group and is quite likely of steric origin. The pK_R of the parent cation is -1.2; the cation has an absorbance maximum at 500 nm and reacts with water with a rate constant of 10^2 s^{-1} at 25 °C.[9b,15] The introduction of the ortho-methylsulfone group decreases the equilibrium constant for reaction with water by a factor of $10^{1.4}$, decreases the excitation energy of the cation by 2.5 kcal/mol, and decreases the rate constant for reaction with water by a factor of 10^3. It is curious, but not unprecedented, that the effect on the rate constant is larger than that on the equilibrium constant for the reaction with water.

Pertinence to Solvolysis Reaction Studies. The results presented here clearly indicate that variations in trapping ratios of solvolysis intermediates by various nucleophiles cannot be attributed to differing selectivities of ion pairs and free ions. There is, however, one point which should be made: our model ion pair is most probably similar to an "intimate" ion-pair intermediate. Although any electrostatic effect of counterion should be greater on the "intimate" than the "solvent-separated" ion pairs, there is evidence[9a,16] that the counterion of a solvent-separated ion pair can provide general-base catalysis for reaction of hydroxylic solvents. Such catalysis is usually weak compared with the "water-catalyzed" reaction[9a] and is not expected to cause large changes in rate, although observable effects on stereochemistry, for example, are expected.

We believe that the majority of the trapping results are explicable on the basis that the reactive anionic trapping nucleophile generally used in such studies reacts at diffusion-controlled rates with the highly reactive cations or ion pairs generated in solvolysis reactions. This suggestion has been implied a number of times, and the idea was cleverly used by Jencks[17] to estimate rate constants for reactions of oxocarbonium ions with water from observed trapping ratios. Rappoport[18] has pointed out that it is almost inconceivable that even the relatively stable benzhydryl cations could react with azide ion at less than diffusion-controlled rates.

Acknowledgment. We are grateful to Dr. Larry Hutchinson for the construction of the rapid-scanning spectrophotometer used in this work and to Dr. Joe Murphy for some early exploratory experiments with Phenol Red Dimethyl Ether. This work was supported by grants from the National Institute of General Medical Sciences (No. 2-R01-GM12832) and National Science Foundation (No. CHE 77-24701).

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Conformational Analysis of Linear Peptides. 2. A Vapor-Pressure Osmometry Study of Self-Association in Chloroform

Neal Sugawara,[1] E. S. Stevens,*[1] G. M. Bonora,[2] and C. Toniolo*[2]

Contribution from the Department of Chemistry, State University of New York, Binghamton, New York 13901, and the Biopolymer Research Centre, C.N.R., Institute of Organic Chemistry, University of Padova, 35100, Padova, Italy. *Received March 17, 1980*

Abstract: Activity coefficients, γ, and practical osmotic coefficients, ϕ, measured by vapor-pressure osmometry, are reported for biphenyl, benzoic acid, and six amino acid derivatives and peptides, all in chloroform solutions at 36 °C. Consecutive molal self-association constants are calculated from the data by attributing the osmotic nonideality to self-association. Values for the dimerization step are as follows: Ac-L-Nva-OMe, K_2 = 1.18 m^{-1}; Ac-L-Val-OMe, K_2 = 1.33 m^{-1}; and *t*-Boc-Gly-L-Val-Gyl-OMe, K_2 = 6.59 m^{-1}. The data for Ac-L-Val-Gly-OMe reveal a significant concentration of trimers and give values of K_2 = 37.7 m^{-1} and K_3 = 1086 m^{-2}. Comparable measurements on benzoic acid yield K_2 = 52.4 m^{-1} and K_3 = 878 m^{-2}. The temperature dependence of self-association in *t*-Boc-Gly-L-Val-Gly-OMe was measured, providing values of ΔH = -27 kJ (mol dimer)$^{-1}$ and ΔS = -71 J K^{-1} (mol dimer)$^{-1}$. Corollary infrared absorption data are presented as independent evidence of molecular association. Solutions of *t*-Boc-L-Val-OMe and *t*-Boc-L-Nva-OMe display osmotic nonidealties too small to attribute to self-association, as do biphenyl solutions.

Considering the importance of self-association to the question of interpreting peptide spectroscopic data, especially in relatively nonpolar solvents, little quantitative work has been reported in the literature.[3,4] We felt the present study was necessary in order to arrive at a proper interpretation of our own high-resolution ^1H NMR measurements on a series of linear oligopeptides in chloroform.[5] We report here the results of a vapor pressure osmometry study of Ac-L-Nva-OMe,[6] *t*-Boc-L-Nva-OMe, Ac-L-Val-OMe, *t*-Boc-L-Val-OMe, Ac-L-Val-Gly-OMe, and *t*-Boc-Gyl-L-Val-Gly-OMe.

The principles and practice of vapor-pressure osmometry have been reviewed.[7-9] A matched pair of thermistors is located in a thermally equilibrated chamber saturated with solvent vapor. A drop of pure solvent is placed on one of the thermistor beads; the other bead holds a drop of solution. Since the vapor pressure of the solvent in the drop of solution is lower than that of pure solvent, solvent vapor will condense on the solution drop at a

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(6) The abbreviations used are as follows: Ac, acetyl; *t*-Boc, *tert*-butyloxycarbonyl; Gly, glycine; Nva, norvaline; Val, valine; OMe, methoxy.
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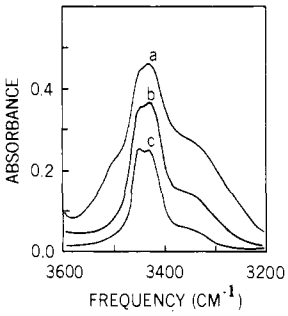

Figure 1. Infrared absorption spectra of *t*-Boc-Gly-L-Val-Gly-OMe in $CDCl_3$ at concentrations of (a) 0.050 M; (b) 0.015 M; (c) 2.6×10^{-4} M.

greater rate than on the drop of pure solvent and the solution is warmed relative to the solvent. In vapor-pressure osmometry studies departures from osmotic ideality can arise from molecular association, as well as from the many other factors contributing to nonideality.[10]

Experimental Section

***t*-Boc-L-Val-OMe.** *t*-Boc-N_3 and HCl·H-L-Val-OMe were reacted in anhydrous ethyl acetate by using *N*-methylmorpholine to deprotonate the ammonium group: yield 60%; oil (from ethyl ether–petroleum ether); $[\alpha]^{21}_D = -21.2°$ (c = 1.1; methanol). Anal. Calcd from $C_{11}H_{21}NO_4$: C, 57.1; H, 9.1; N, 6.1. Found: C, 56.4; H, 9.0; N, 6.2.

Ac-L-Val-OMe.[11] This compound was prepared from acetyl chloride and HCl·H-L-Val-Ome in anhydrous chloroform by using *N*-methylmorpholine to deprotonate the ammonium group: yield 75%; mp 68–69 °C (from ethyl acetate–petroleum ether); $[\alpha]^{21}_D = -30.2°$ (c = 1; methanol); $[\alpha]^{21}_D = -47.3°$ (c = 1; water).

Ac-L-Val-Gly-OMe. HCl·H-L-Val-Gly-OMe[12] and acetyl chloride were reacted in anhydrous chloroform by using *N*-methylmorpholine to deprotonate the ammonium group: yield 51%; mp 200–201 °C (ethyl acetate–petroleum ether); $[\alpha]^{21}_D = -54.2°$ (c = 0.5; methanol). Anal. Calcd for $C_{10}H_{18}N_2O_4$: C, 52.2; H, 7.9; N, 12.2. Found: C, 52.5; H, 7.8; N, 12.0.

***t*-Boc-Gly-L-Val-Gly-OMe.**[12] *t*-Boc-Gly-OH and HCl·H-L-Val-Gly-OMe[12] were reacted in anhydrous methylene chloride by using *N*-methylmorpholine to deprotonate the ammonium group and *N,N'*-dicyclohexylcarbodiimide to activate the carboxyl group: yield 56%; mp 149–150 °C (from ethyl acetate–petroleum ether); $[\alpha]^{21}_D = -29°$ (c = 1.2; methanol).

The synthesis and physical properties of *t*-Boc-L-Nva-OMe and Ac-L-Nva-OMe were described previously.[13] The chemical purity of all amino acid derivatives and peptides was also checked by thin-layer chromatography in a variety of solvent systems. A single spot was obtained in all chromatograms.

Infrared absorption spectra were recorded with a Perkin-Elmer Model 580 spectrophotometer. The positions of the bands are accurate to ±1 cm^{-1}.

Vapor-pressure osmometry data were obtained on a Model 301A apparatus manufactured by Mechrolab, Inc., a division of Hewlett-Packard, Inc., or a Model 232A apparatus manufactured by Wescan Instruments, Santa Clara, CA. Fisher Certified chloroform was used as solvent after passing through an alumina column. Benzil, from Aldrich Chemical Co., was used in the calibration of the osmometer to obtain the difference between thermistor resistances, ΔR, as a function of the stoichiometric molal concentration, m_s. Solutions of all other compounds were prepared at known stoichiometric concentration, and the measured values of ΔR were used to read colligative concentrations, m_c, from the calibration curve. In addition to the compounds described above, biphenyl from Eastman Kodak Chemical Co. and benzoic acid, Fisher Certified grade, were also studied.

Results

Infrared Absorption Measurements. Figure 1 shows the concentration dependence of the infrared absorption of *t*-Boc-Gly-L-Val-Gly-OMe in chloroform, at concentrations of 2.6×10^{-4}, 1.5×10^{-2}, and 5.0×10^{-2} M. Other infrared data are summarized in Table I.

Table I. Infrared Absorption Data (in cm^{-1}) in Deuteriochloroform

	10^{-3} concn, M	NH		CO	
		free	H bonded	ester	amide (urethane)
t-Boc-L-Nva-OMe	0.6	3442		*a*	*a*
	30	3441		1739	1712
Ac-L-Nva-OMe	0.6	3435		*a*	*a*
	30	3435	~3350 w	1738	1676
Ac-L-Val-OMe	50	3435	~3360 w	1738	1677
t-Boc-Gly-L-Val-Gly-OMe	0.26	3450, 3430	~3370 w	*a*	*a*
	15	3450 sh, 3430	~3365	1748	1715 w, 1674
	50	3448 sh, 3430	~3360	1748	1710 w, 1677 sh 1673, 1667

[a] At concentrations less than 10^{-3} M, a 10-cm path length cell was used in which case solvent absorption precludes observation of the C=O bands near 1700 cm^{-1}.

Vapor-Pressure Osmometry Measurements. Self-association studies using vapor-pressure osmometry have been described relatively frequently in recent years, so that only a summary description of the method will be given here.

It is the practical osmotic coefficient,[14,15] ϕ, that is measured experimentally

$$\phi = m_c/m_s \tag{1}$$

where m_c is the colligative molal concentration and m_s the stoichiometric molal concentration.

The activity coefficient, γ, can be related to the osmotic coefficient by application of the Gibbs–Duhem equation[16] to give

$$\ln \gamma = (\phi - 1) + \int_0^{m_s} \frac{(\phi - 1)}{m_s}\, dm_s \tag{2}$$

and

$$\gamma = m_1/m_s \tag{3}$$

where m_1 is the molal concentration of free monomer.[17,18]

Alternatively, the colligative mole fraction of free monomer, X_1, may be computed from the experimental data[19-21]

$$\ln X_1 = \int_0^{m_c} \frac{(\phi - 1)}{m_c}\, dm_c \tag{4}$$

where

$$X_1 = m_1/m_c \tag{5}$$

Numerical integration of osmotic data as a function of concentration thereby gives m_1 either as a function of m_s (eq 2) or

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Table II. Expansion Coefficients Obtained from Fits of Equations 6 and 7[a]

	T, °C	K_2, m^{-1}	K_3, m^{-2}
biphenyl	36	0.75	
t-Boc-L-Val-OMe	36	0.03	
t-Boc-L-Nva-OMe	36	0.54	
Ac-L-Nva-OMe	36	1.18	
Ac-L-Val-OMe	36	1.33	
t-Boc-Gly-L-Val-Gly-Ome	36	6.59	
	(45, 55)	(5.61, 3.61)	
Ac-L-Val-Gly-OMe	36	37.7	1086
benzoic acid	36	52.4	878
	(45, 55)	(58.4, 46)	(363, ...)[32]

[a] For the first three compounds the expansion coefficients, K_2, are not attributable to association but rather reflect other contributions to general osmotic nonideality (see text).

as a function of m_c (eq 4). In either case we can use a series expansion to obtain the association constants:[17-24]

$$(m_s/m_1) - 1 = 2K_2m_1 + 3K_3(m_1)^2 + \ldots \quad (6)$$

or

$$(m_c/m_1) - 1 = K_2m_1 + K_3(m_1)^2 + \ldots \quad (7)$$

where $K_2 = m_2/m_1^2$, $K_3 = m_3/m_1^3$, etc., and m_2 is the molal concentration of dimer, m_3 of the trimer, etc.

The integral in eq 2 was evaluated by constructing a smooth curve through a plot of $(\phi - 1)/m_s$ vs. m_s, followed by analytical integration. Generally, fits higher than quadratic were not justified, given the scatter in the experimental data. That integration gives m_1/m_s, so that the left hand side of eq 6 is known as a function of m_1. Those curves are then fit to extract the expansion coefficients of eq 6.

We also treated the data by using eq 4 and 7 in an entirely analogous manner. In the absence of experimental scatter, the expansion coefficients would be independent of the method of data treatment; we used both methods as an internal check for consistency. Values of K_2 obtained in the two treatments differed by approximately 10% and values of K_3 by about 20%. Averaged values of the expansion coefficients are given in Table II.

Discussion

We have measured the osmotic behavior of our compounds relative to the osmotic behavior of the calibration benzil–chloroform solutions. We do not assume that the calibration benzil–chloroform solutions behave ideally. Two totally monodisperse solutions may display different osmotic behavior by virtue of differences in other contributions to nonideality. Therefore, even if we were to assume that the calibration solutions behave ideally, we could not attribute to associations all of the observed differences in osmotic behavior between our compounds and benzil. On the other hand, it is reasonable to attribute large differences to associations, especially when there is independent evidence, e.g., from infrared absorption measurements, that associations exist.

In the first three compounds in Table II we therefore do not conclude that the expansion coefficients have the significance of association constants. First, their osmotic behavior is very similar to that of the calibration solutions. Second, infrared data show only a single band due to free, solvated NH groups (e.g., *t*-Boc-L-Nva-OMe, Table I), indicating that any association that occurs is below the limits of detection by infrared absorption at the concentrations studied.

On the other hand, two infrared absorption NH stretching bands appear in the cases of Ac-L-Nva-OMe and Ac-L-Val-OMe (Table I). The position of the low-frequency band indicates intermolecular associations. (The only intramolecularly hydrogen-bonded conformation those compounds can adopt is the C_5 structure, in which case the low-frequency NH band is not observed at less than 3400 cm^{-1}.[25]) In the case of the tripeptide, *t*-Boc-Gly-L-Val-Gly-OMe, the low-frequency NH band could be attributable to intramolecular hydrogen bonding, but its concentration dependence (Figure 1, Table I) indicates that at least part of it arises from self-association. We, therefore, take the expansion coefficients in these cases (Table II) to have the significance of association constants.

The accuracy of the expansion coefficients, apart from their significance as association constants, is limited by the precision of the raw experimental data and not in the details of the curve fitting we use.[20,21] The main uncertainty lies in the extrapolation of the $(\phi - 1)/m_s$ values or $(\phi - 1)/m_c$ values back to zero concentration, which is required in evaluating the integrals in eq 2 and 4. Obtaining data of acceptable reproducibility is difficult at concentrations much less than 0.01 M. Two methods of data analysis were used (see above) as a check on internal consistency and were used in order to give a realistic estimate of the accuracy of the expansion coefficients. Average values of the two treatments are given in Table II and should be understood to represent the actual expansion coefficients to within ±10% for K_2 and ±20% for K_3. Compared to these uncertainties other limits on the accuracy are small, e.g., standard deviations of the actual fits and sample purity.

Therefore, the interpretation of the data in Table II in terms of association constants is to be understood in light of the above discussions, i.e., the attribution of osmotic nonideality to association, the model of associations as successive addition of monomers, and the experimental scatter of data points.

Our results (Table II) show a dependence of the association constants on N blocking group and on chain length. In the case of the compounds Ac-L-Nva-OMe and *t*-Boc-L-Nva-OMe, we had earlier interpreted[13] the difference in temperature dependence of NH chemical shift at 0.3 m concentration in $CHCl_3$ in terms of Ac-L-Nva-OMe being more prone towards aggregation than *t*-Boc-L-Nva-OMe, and the present data now justify that interpretation.

Large association constants are observed for the dipeptide, Ac-L-Val-Gly-OMe, and tripeptide, *t*-Boc-Gly-L-Val-Gly-OMe. A chain-length dependence is easily accounted for in terms of an increase in the average number of intermolecular hydrogen bonds formed as the chain increases. (One must speak of the *average* number of hydrogen bonds in the aggregated species, since not all aggregates need be held together with the maximum number of bonds possible). The dipeptide, Ac-L-Val-Gly-OMe, has a greater disposition towards aggregation than the tripeptide *t*-Boc-Gly-L-Val-Gly-OMe presumably because of a dependence on the N-blocking group.

Our treatment of osmotic pressure data is more general than has often been carried out. Typically,[17,18,26-29] two restricted association models have been presumed and then tested to determine which describes the experimental data better. In the first model, only dimerization is considered; i.e., $K_2 = K_d$; $K_n = 0$, $n > 2$. In that model, the dimerization association constant, K_d, is given simply by $K_d = (1 - \phi)/[m_s(2\phi - 1)^2]$. In the second model, multiple associations are presumed to form with all constants, K_n, equal, in which case the single polymerization constant, K_p, is directly obtained from the experimental data as $K_p = (1 - \phi)/(m_s\phi^2)$. When only these two models are used, the one which shows the greater consistency with the data, i.e., independence of either K_d or K_p over a greater range of concentration, is usually selected as describing the system better. For weakly associating compounds the dimerization model would of course give a good representation of the association process. In most of the com-

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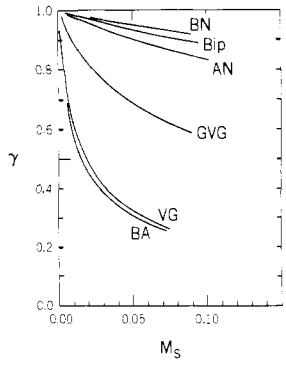

Figure 2. Osmotic activity coefficients as a function of stoichiometric concentration: *t*-Boc-L-Nva-OMe (BN); biphenyl (Bip); Ac-L-Nva-OMe (AN); *t*-Boc-Gly-L-Val-Gly-OMe (GVG); Ac-L-Val-Gly-OMe (VG); and benzoic acid (BA).

pounds we examined, for example, the self-associations which form are simple dimers at concentrations below 0.05 m. For more strongly associating compounds, e.g., Ac-L-Val-Gyl-OMe or benzoic acid in $CHCl_3$, neither the dimerization model nor the multiple association model with equal consecutive association constants would give an accurate description of the associations. The general treatment we use here, similar to that used by Steiner and Magar[19-22] for nucleoside association, is not especially laborious and generates a more complete picture of the association.[30]

In Figure 2 we present our results in terms of the activity coefficient displayed as a function of stoichiometric concentration calculated from eq 3 and 6. Since the activity coefficient is the ratio of free monomer to total stoichiometric concentration, the figure is a clear representation of the relative importance of associated species in the compounds we studied. For example, in a 0.02 m solution of Ac-L-Val-Gly-OMe, half of the peptide molecules are in aggregated forms (at 36 °C). On the other hand, Ac-L-Nva-OMe has only a slight tendency to aggregate, and the osmotic nonidealities of biphenyl and of *t*-Boc-L-Nva-OMe solutions are so small as to not allow the nonidealities to be attributed to aggregation.

The temperature dependence of *t*-Boc-Gly-L-Val-Gly-OMe dimerization gives values of $\Delta H = -27$ kJ (mol dimer)$^{-1}$ and $\Delta S = -71$ J K^{-1} (mol dimer)$^{-1}$. These values are within the range of expectation,[4,29] but there are no data in chloroform with which to make comparisons.

Infrared absorption data provide evidence for the existence of self-associated species when there is a concentration dependence in the bands attributable to free and H-bonded NH groups. If one assumes equal oscillator strengths, then the areas of the H-bonded (A_H) and free (A_F) NH bands can be related to the relative proportions of the two types of NH group, but the validity of that assumption is generally not known. On the other hand, the relative concentrations of H-bonded and free *molecular* species can be calculated from our association constants measured thermodynamically. For *t*-Boc-Gly-L-Val-Gly-OMe our value of K_2 at 36 °C and our calculated ΔH, assumed constant over the relevant temperature range, give $K_2 = 11.7$ m^{-1} at 20 °C, the temperature of the infrared measurements. For m_s values of 0.010 and 0.034 m (corresponding to molar concentrations of 0.015 and 0.050 M used in the infrared absorption measurements), this value of K_2 gives activity coefficients of 0.836 and 0.657, respectively, from which the percentage of *molecules* hydrogen bonded can be calculated from $2m_2/m_s$ to be 16% and 34%, respectively, at the two concentrations. Values of $A_H/(A_H + A_F)$ from the data in Figure 1 are 22% and 46%. These numbers indicate the percentage of *NH groups* hydrogen bonded. The higher figures from the infrared data are understandable on the ground that there may be (and probably are) more than one H-bonded NH group for each associated species.

Some of the compounds examined here have already been the object of conformational studies by ^{1}H NMR in chloroform solutions.[31,32] A discussion of this specific point is fully developed in ref 5 (following paper in this issue).

In conclusion we wish to emphasize the significance and the limitations of our results and the method we applied. (1) Association of peptides in chloroform can be substantial at concentrations as low as 10 mM. (2) Independent evidence for association, e.g., by infrared absorption spectroscopy, is necessary before osmotic nonidealities can be attributed to association. (3) Quantitative measurements of association in chloroform can be obtained by osmometry; other contributions to nonideality can be minimized by working at as low concentrations as possible, compatible with acceptable reproducibility. (Higher concentrations would decrease uncertainty in K values, but also increase the potential role of other contributions to nonideality; we consider our concentrations to be optimal.) (4) Additional measurements on peptides will be necessary before general trends and conclusions can be established concerning the dependence of association on chemical structure.

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(30) We are aware that reference to trimeric benzoic acid is usually not made. According to our data this species is not negligible, even though it is in small concentrations. For example, at a stoichiometric concentration of 0.02 m, 95% of the colligative species are either the monomer (63%) or dimer (32%), and the remaining 5% are trimers. Any higher molecular weight species which might have been present at even smaller concentrations (e.g., less than 1%) would not have been detected in this experiment. We also point out that the presence of trimers or higher molecular weight species would not be demonstrated in any simple spectroscopic experiment, since, whatever the molecular weight, there would by only two types of carboxyl groups, hydrogen bonded and free; thus, IR might well display only two bands, and NMR display only two chemical shifts. Furthermore, the formation of a trimer is easily pictured as being formed when an oper dimer (i.e., a noncyclic dimer) hydrogen bonds with a third monomer.

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