Interaction of the Peptide Bond with Solvent Water: A Vapor Phase Analysis†

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ABSTRACT: A dynamic technique, using radioactivity as a means of detection, makes it possible to measure the partial pressures of highly polar compounds in dilute aqueous solution. The results can be expressed in terms of the dimensionless distribution coefficient for transfer of a compound from dilute aqueous solution to the vapor phase. For acetic acid this coefficient is 1.1×10^{-5}, for acetamide 7.6×10^{-8}, for *N*-methylacetamide 4.1×10^{-8}, and for *N,N*-dimethylacetamide 5.4×10^{-7}. Thus acetamide is much more strongly solvated than the uncharged acetic acid molecule. The results suggest: (1) that the peptide bond represents an extreme among uncharged functional groups in the degree to which it is stabilized by solvent water; (2) that the very great hydrophilic character of the peptide bond may be associated mainly with hydrogen bonding of the solvent to the carbonyl oxygen atom (rather than the N—H group); and (3) that the observed equilibria of biosynthesis and hydrolysis of peptide bonds in aqueous solution are largely determined by differences between reactants and products in their free energies of solvation. It is anticipated that where "bound" water is found in proteins, it will often be found to be associated with peptide bonds, and will tend to be associated with the C=O group rather than with the N—H group.

The interaction of biological compounds with solvent water affects their reactivity with other molecules, and there is little doubt that solvation effects have exerted a profound influence on the evolution of self-assembling systems and biological catalysts. The action of enzymes, for example, can be said to require the preferential extraction from solvent water of activated intermediates in substrate transformation, as compared with substrates themselves (Wolfenden, 1972, 1976a). In attempting to understand the observed affinities of active sites and other biological receptors for ligands such as substrates and intermediates, it would be useful to have some idea of the absolute tendencies of these ligands to leave water and enter a featureless cavity of unit dielectric constant. It might then be possible to infer the presence of specific attractive or repulsive interactions between these ligands and the sites at which they are bound. With such information it should also be possible to calculate equilibria in the vapor phase from solution measurements and compare the results with quantum mechanical calculations for gas phase reactions.

The hydrophilic character of a molecule can be determined, in an absolute sense, by evaluating the dimensionless equilibrium constant for its distribution from the dilute vapor phase (in which intermolecular forces are virtually absent) to an aqueous solution so dilute that solute-solute interactions are negligible (Butler, 1937, and references cited therein). Many compounds have been examined in this way and the results, collected recently by Hine and Mookerjee (1975), tend to confirm Butler's impression that the hydrophilic character of complex molecules can be estimated with reasonable accuracy as an additive function of their constituent groups. One manifestation of this additivity is the unvarying effect of adding methylene increments (+0.15 kcal/methylene group) in a homologous series of compounds on their hydrophilic character; nor does this effect vary significantly with the class of compound considered (Wolfenden and Lewis, 1976).

Because it is difficult to analyze solutes at very low concentration in the vapor phase, such measurements have mainly been confined to relatively volatile solutes exhibiting substantial vapor pressures in aqueous solution. Most classes of compounds of interest to biochemists (peptides, phosphate esters, purines, and so on) have never been studied even as simple examples, and it seemed desirable to try to extend these measurements to include compounds of the more polar types encountered in biological systems. As was shown in a preliminary communication (Wolfenden, 1976b), a substantial increase in sensitivity can be achieved by means of a dynamic technique, using radioactivity to detect the solute in the vapor phase. This paper describes the technique in detail, and the results obtained with acetamide, *N*-methylacetamide, and *N,N*-dimethylacetamide.

The hydrogen-bonding propensities of peptides are too well known to require extensive comment. In solubility studies, it was shown that peptides have a strong preference for water as compared with nonaqueous solvents (Cohn and Edsall, 1943; Nozaki and Tanford, 1971). Their preference for hydrogen bonding to water, rather than to other peptides, was discussed by Schellman (1955) and Kauzmann (1959), and placed on a firm experimental basis by the infrared studies of Klotz and Franzen (1962) on the self-association of peptides in various solvents. An elegant method for determining the amount of bound water, in solutions containing peptides or proteins, has been developed by Kuntz (Kuntz et al., 1969; Kuntz, 1971), and molecular orbital calculations on hydrogen bonding of amides are in progress (see Johansson et al., 1974, and references cited therein). The present findings provide further information concerning the strength and probable geometry of hydration of peptides in dilute aqueous solution.

Experimental Section

[1-^{14}C]Acetamide, purchased from Calbiochem Corp., and [1-^{14}C]acetic acid, purchased from ICN Co., were used without further purification. *N*-Methylacetamide was prepared by treating [^{14}C]methylamine hydrochloride (1 μmol, 0.1 mCi, obtained from ICN Co.) in aqueous potassium acetate (1 mL, 0.25 M) with acetic anhydride (20 μL) added in four portions over a period of 2 h, adding KOH (1 M) in such a way

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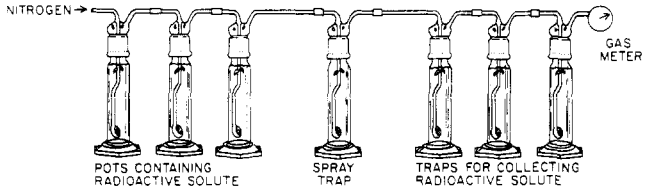


FIGURE 1: Apparatus for determining the partial pressure of polar solutes.

as to maintain pH in excess of 9. After standing overnight at room temperature, the product moved as a single spot on paper chromatography (in 1-butanol:acetic acid:water, 40:10:50, product R_f 0.92; methylamine R_f 0.45). A similar procedure was followed with dimethylamine (1 μmol, 0.05 μCi, obtained from ICN Co.) with a similar result, *N,N*-dimethylacetamide appearing as the sole radioactive component of the product mixture.

The apparatus used for determining the vapor pressure of radioactive solutes is shown in Figure 1. The glass train used in these experiments consisted of a series of seven gas washing bottles (150 mL capacity) equipped with sintered glass discs. Each of the first three bottles contained radioactive solute at a concentration of approximately 0.01 M in 100 mL of water. The concentration of radioactivity in these "pots" was determined accurately at the beginning and end of the transfer measurements and did not change appreciably in any of these experiments. Each of the last three bottles contained water (100 mL), and the concentration of radioactivity in these traps was determined at intervals during the course of the experiment. The center bottle, containing no liquid, was used as a spray trap to ensure that no radioactivity was conducted mechanically from the pots to the traps. No mechanical transfer of radioactivity was observed with this, or even with much simpler arrangements involving, for example, a U tube 15 cm in length. This also served as an effective spray trap as was demonstrated with radioactive acetate in alkaline solution. In experiments in which the number of pots and traps was varied, it was found that equilibration of the carrier gas with acetamide was over 90% complete using a single pot, and that of the radioactivity transferred to the traps, over 90% appeared in the first trap.

To conduct a typical experiment, the pots and spray trap were immersed in a constant temperature bath at 25.0 ± 0.1 °C, and water-pumped nitrogen was passed through the train at a rate of 100 mL/min or less. The volume of carrier gas that had passed through the train was monitored continuously with the aid of a Precision Scientific Co. Wet-Test Flowmeter exposed to atmospheric pressure at the end of the train. With this configuration, a pressure of 1.175 atm was required at the beginning of the train to produce a flow rate of 100 mL/min. Since the flowmeter was operating at atmospheric pressure, this resulted in a slight overestimate (in the neighborhood of 10% in the third pot) of the actual volume of carrier gas to which the equilibrating pots had been exposed; no correction was made for this small deviation from atmospheric pressure.

The chemical identity of the material transferred to the traps was determined in each case by concentration under vacuum, followed by measurement of the distribution coefficient of radiolabeled material between water and 1-octanol in the presence of acid (0.1 M HCl) and base (0.1 M KOH) and comparison with distribution equilibria observed for the starting material and recorded in the literature. Observed equilibria are given in Table I.

TABLE I: Octanol/Water Distribution Coefficients, 25 °C.

	$(X)_{octanol}/(X)_{0.1\ N\ HCl}$	$(X)_{octanol}/(X)_{0.1\ N\ KOH}$
Acetic acid	0.51[a]	<0.001
Acetamide	0.070	0.070
N-Methylacetamide	0.079[a]	0.079
Methylamine	<0.001	0.27[a]
N,N-Dimethylacetamide	0.17[a]	0.17
Dimethylamine	<0.001	0.42[a]

[a] Similar value reported by Leo et al. (1971).

It required between 1 and 10 days (depending on the amide and the concentration of radioactivity in the pots) to accumulate sufficient (3000 cpm or more) radioactivity in the traps to characterize this material by distribution experiments. In each experiment the rate of transfer of radioactivity was found to be linear with time, and proportional to the rate of gas flow, when the latter was caused to vary by a factor of 3 (from 30 mL to 100 mL per min). At the end of each experiment, the material remaining in the pots had not changed detectably in the concentration of radioactivity (much less than 1% was transferred even in the longest experiments), and no change had occurred in distribution properties of the solute between octanol and water (either acidic or basic). It was found necessary, in experiments involving radioactive acetamide, to maintain the pots at pH 9 (by adjustment with KOH using phenolphthalein) at the outset of the experiment, in order to trap slight traces of acetic acid that were generated during the course of the experiment, since this compound as the free acid is much more volatile from aqueous solution than any of the amides. When this precaution was observed, material accumulating in the traps was identical in its distribution properties between water and octanol, in either acid or base, to the authentic amide. In experiments with amides containing a radioactive label in the amino portion of the molecule, pots were maintained at pH 3 (by adjustment with HCl using bromcresol green) in order to trap traces of relatively volatile amine that were generated during the course of the experiment.

Results

With the apparatus shown in Figure 1, it proved possible to obtain reproducible measurements of the partial pressure of acetic acid, acetamide, *N*-methylacetamide, and *N,N*-dimethylacetamide. In each case the rate of transfer of radioactive solute from the pots to the traps was proportional to the rate of flow of the carrier gas, and to the concentration of radioactive solute in the pots. The rate of transfer was unaffected by increasing the number of pots containing radioactive solute (indicating that equilibration was complete under these conditions) or by varying the concentration of nonradioactive solute in the pots from 0.01 M to 0.001 M (consistent with previous demonstrations that acetamide in dilute aqueous solution does not undergo detectable self-association (Davies and Hallam, 1951)).

Measurements on [1-^{14}C]acetic acid were performed with 0.18 M H_2SO_4 present in the pots (to maintain the acid in its fully protonated state), and 0.1 M KOH present in the traps. Measurements on [1-^{14}C]acetamide were performed with 0.01 M KOH in the pots in order to trap traces of radioactive acetic acid generated during long-term experiments. Measurements on *N*-[^{14}C]methylacetamide and *N,N*-[^{14}C]dimethylacetamide were performed with the pots adjusted to maintain an acid reaction to bromcresol green, to trap traces of radioactive methylamine and dimethylamine generated during long-term

TABLE II: Vapor/Water Distribution Coefficients of Amines, 25 °C.

	$(X)_{vapor}/(X)_{water}$	Ref
NH_3	7.7×10^{-4}	*a*
CH_3NH_2	2.9×10^{-4}	*a*
$(CH_3)_2NH$	7.2×10^{-4}	*b*
$(CH_3)_3N$	4.0×10^{-2}	*b*

[a] Calculated from Landolt-Bornstein (1936). [b] Hine and Mookerjee (1975).

TABLE III: Carbonyl Stretching Frequencies in Water and Vapor (cm^{-1}).

	Aqueous (D_2O)	Vapor	(Vap) − (Aq) shift
Acetamide	1633[a] (1627)[b]	1753, 1728[c]	107
N-Methylacetamide	1622[a]	1728, 1711[c]	97
N,N-Dimethylacetamide	1608[a]	1683[c]	75
Acetic acid	1710[b]	1785[d]	75
Ethyl acetate	1710[b]	1765[d]	55

[a] This investigation. [b] Jencks et al. (1960). [c] Cutmore and Hallam (1969). [d] Hartwell et al. (1948).

experiments. When these precautions were observed, the radioactive material transferred to the traps was found to be identical, in its distribution properties, with the authentic solutes present in the pots (Table I). Examination of material in the pots showed that breakdown was negligible in all cases and that no detectable loss of radioactive material occurred during the course of the experiment.

Distribution coefficients of acetic acid and various amides (moles per liter in the vapor phase divided by moles per liter in water) were obtained by dividing the concentration of radioactivity in the carrier gas transferred to the traps by the concentration of radioactivity in the pot solutions (which did not change detectably during the course of the experiment), as measured at 25 °C. Each value was determined in at least five independent experiments. The value, 1.1×10^{-5}, observed for acetic acid was comparable with a value, 1.23×10^{-5}, determined titrimetrically by Friedenhager and Liebster (1932). For the amides, observed values were $7.6 (\pm 2.5) \times 10^{-8}$ for acetamide, $4.1 (\pm 1.3) \times 10^{-8}$ for *N*-methylacetamide, and $5.4 (\pm 2.5) \times 10^{-7}$ for *N,N*-dimethylacetamide.

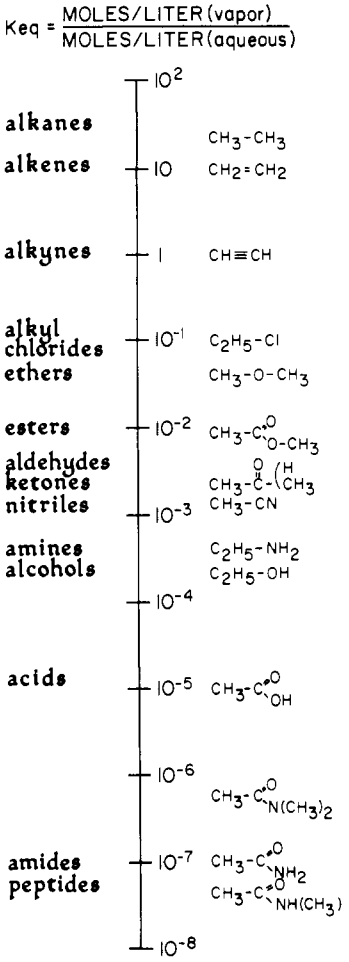
Carbonyl stretching frequencies of amides were recorded in D_2O solution as described by Jencks et al. (1960), and the results are shown in Table III.

Discussion

It might be supposed that the greater hydrophilic character of acetamide, as compared with uncharged acetic acid, is partly due to its ability to form a larger number of hydrogen bonds to water. The present findings show, however, that the hydrophilic character of *N*-methylacetamide is even greater than that of acetamide. When the remaining hydrogen atom is replaced, in *N,N*-dimethylacetamide, there is a modest decrease in hydrophilic character, but this compound is still much more hydrophilic than acetic acid. Of all uncharged bonds to carbon that have been examined, the peptide bond (as represented in *N*-methylacetamide) thus appears to be unusual and perhaps unique in its degree of stabilization by solvation (Scheme I).

It is difficult to account for the order of free energies of

SCHEME I: Vapor-Water Distribution Coefficients of Organic Compounds of Various Classes.[a]



[a] Many of these entries are from the tabulation of Hine and Mookerjee (1975).

hydration of acetamide and its N-methylated derivatives in terms of any single effect: inductive, steric, or otherwise. It is worth noting, however, that the corresponding amines exhibit similar behavior, with methylamine exhibiting greater hydrophilic character than either ammonia or dimethylamine (Table II). The fact that N-methylation of acetamide has relatively little effect on its hydrophilic character suggests that properties of the carbonyl oxygen, rather than the N–hydrogen atoms, may be largely responsible for the hydrophilic character of amides. Consistent with this possibility is the finding that the C=O stretching frequencies of acetamide, *N*-methylacetamide, and *N,N*-dimethylacetamide undergo large and comparable shifts when these compounds are transferred from the vapor phase to dilute aqueous solution (Table III). In the series ethyl acetate, acetic acid, *N,N*-dimethylacetamide, acetamide, *N*-methylacetamide, the magnitude of the shift in stretching frequency is correlated to some extent with increasing hydrophilic character.

As models for peptide bonds in proteins, it would be desirable to examine compounds more complicated than acetamide and its N-methylated analogues; however, the introduction of even a single additional polar substituent would result in a vapor pressure too low to measure except perhaps by using with very short-lived isotopes. Previous work has shown that the hydrophilic character of complex molecules can usually be estimated with reasonable accuracy from their constituent groups (Butler, 1937; Hine and Mookerjee, 1975; Wolfenden and

Lewis, 1976), providing some assurance that these simple models may be generally applicable. The present findings suggest several influences that can be tested qualitatively against the observed structures of real proteins.

First, hydrophilic character reaches a maximum in *N*-methylacetamide, the closest simple model of the peptide bond. It might thus be expected that water should often be found associated with peptide bonds in proteins especially at exposed positions, such as the β turn, where there is little possibility of forming intramolecular hydrogen bonds in the protein. Second, the vapor pressure of aqueous acetamide is found to be so little affected by substitution of methyl groups for protons on nitrogen, as to suggest that interaction of water with the carbonyl group (rather than with the N–H protons) is mainly responsible for the hydrophilic character of acetamide. It might then be predicted that, in the structures of real proteins, localized water would tend to be found closely associated with carbonyl oxygen atoms of peptide bonds. Hydrogen bonding between solvent water and peptide N–H groups would be expected to be found less frequently, and this prediction is in agreement with the results of the molecular orbital calculations of Alagona et al. (1973) and of Johansson et al. (1974). Finally, bond water might be expected to be found in association with carboxylate and carboxamido side chains.

These postulates were tested by examining the structure of rubredoxin from *C. pasteurianum* (53 amino acids), as determined at 1.5 Å resolution by Watenpaugh et al. (1973). These investigators were able to obtain coordinates not only for the heavier atoms of the protein molecule, but also for the oxygen atoms of 100 bound water molecules. A computer search of this structure was performed in collaboration with Dr. J. Hermans, assuming a maximum distance of 3.4 Å between electronegative atoms of a hydrogen bond. Forty-four water molecules were found to be within hydrogen-bonding distance of the carbonyl oxygen atoms of peptide bonds, and 29 of the 52 peptide bonds were so involved. Many fewer water molecules, 18, were found to be within hydrogen-bonding distance of the N–H groups of peptide bonds. Much of the remaining localized water, not bound near peptide bonds, appeared to be associated with carboxylate and carboxamido functions, in keeping with expectations from the present experiments. Further refinements of structure will doubtless alter these coordinates in detail, but seem unlikely to alter the general tendencies observed. A similar distribution of bound water molecules, as between peptide C=O an ' N—H groups, has been observed very recently in the structure of a snake venom neurotoxin at 1.3 Å resolution (Dr. Gregory Petsko, personal communication).

Whereas the free energy of hydrolysis of ethyl acetate, expressed in terms of uncharged reactants and products, is −23.8 kJ at 25 °C, the free-energy change for this reaction in the vapor phase is +5.8 kJ (Wolfenden, 1976b, and references cited therein). The exergonic character of the aminolysis of amino acid esters of transfer RNA, in protein biosynthesis, can thus be explained entirely in terms of the stronger solvation of products as compared with reactants. Conversely, equilibrium favors hydrolysis of amides and peptides very much more strongly in the vapor phase than in dilute aqueous solution, provided the concentration of reactant water is included in the equilibrium expression in both cases.

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