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Accelerated Publications

Lack of Water Transport by Amino Acid Side Chains or Peptides Entering a Nonpolar Environment[†]

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ABSTRACT: Water has been reported to enter cyclohexane in association with 3-methylindole, a model compound representing the side chain of tryptophan. Entrainment of water would cloud the interpretation of measured partition coefficients as a simple index of hydrophobicity. NMR and isotope-exchange experiments indicate that the reported entrainment of water resulted from unrecognized exchange of ^{3}H from water into the –NH– group of the indole ring. A more detailed analysis shows that no significant amounts of excess water (less than 0.1 molecule/molecule of solute) enter cyclohexane with molecules representing the side chains of tryptophan, phenylalanine, threonine, lysine, or the peptide bond itself.

Interactions between biological molecules in aqueous solution usually appear to involve the stripping away of solvent water, at least in part, from the groups that make contact. Apparent affinities between these groups often seem to reflect the cost of removing them from water, in addition to electrostatic or steric factors that may be at work. For example, those amino acid side chains which are most hydrophobic, as judged by their partitioning from water to the vapor phase or to nonpolar solvents, show the most pronounced tendencies to be found in the interiors of globular proteins (Radzicka & Wolfenden, 1988).

Most interpretations of hydrophobic character have been based on the premise that solute molecules, themselves present at extremely low concentrations, tend to be completely surrounded by representative molecules of the solvent mixture in each phase. Although difficult to test directly, that assumption seemed reasonable. Wet cyclohexane, for example, contains approximately 1 water molecule for every 10^4 molecules of cyclohexane (Staudhammer & Seyer, 1957), and cyclohexane is similarly rare in cyclohexane-saturated water. The presence of organic solutes also has not been found to enhance the solubility of water in organic solvents, in the few cases that have been examined. For example, we were unable to detect coextraction of excess water into cyclohexane in the presence of organic solutes representing the side chains of common amino acids, above the water content that had been present in the absence of solutes, using proton NMR as a method of detection (Radzicka & Wolfenden, 1988).

This conventional interpretation has been opened to question by a recent report (Wimley & White, 1992) that tritiated water enters cyclohexane along with a compound representing the side chain of tryptophan (3-methylindole), when this compound partitions between water and cyclohexane. Observing an apparent 2:1 ratio of 3-methylindole to entrained water at equilibrium in the cyclohexane phase, the authors infer that 3-methylindole and its monohydrate are equally abundant in wet cyclohexane, over the temperature range from 8 to 55 °C. Scheme I shows that if **I** (uncomplexed 3-methylindole) and **II** (a 1:1 complex of water and 3-methylindole) are both present in wet cyclohexane, then the total concentration of 3-methylindole in the cyclohexane phase is greater than it would be if only **I** were present. If only **I** is present in cyclohexane, then the distribution coefficient $K_{W\to C}$ is accurately represented by the concentration of 3-methylindole in each phase at infinite dilution. If, instead, **I** and **II** are equally abundant in wet cyclohexane, then $K_{W\to C}$ will be overestimated by a factor of 2 on the basis of a simple comparison of total concentrations of 3-methylindole in each

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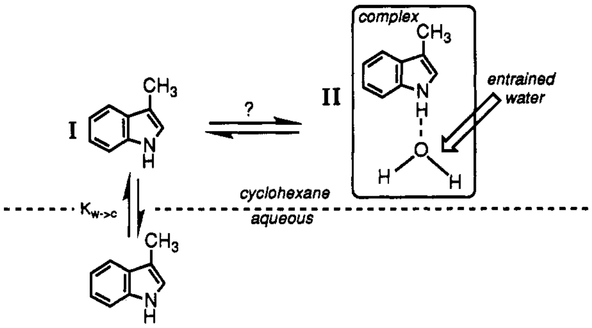
Scheme I



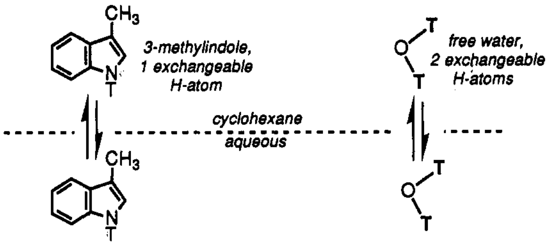
phase, giving rise to the misleading impression that **I** is more hydrophobic than is actually the case.

Aside from their important bearing on the relative hydrophobicity of tryptophan, these observations would lead to other interesting conclusions. First, if 3-methylindole and its monohydrate were equally abundant in wet cyclohexane, then water could be extracted into nonaqueous surroundings by the simple expedient of forming a single H-bond to indole in the nonpolar phase. Such bonds could furnish potential water binding sites in the nonpolar interiors of globular proteins and furnish a potential mechanism for water transport across membranes. Second, earlier work has shown that hydrophobic character tends to bear a simple relationship to the number of polar groups that are present in a solute molecule (Hansch & Leo, 1979). Owing to the presence of additional polar atoms and a greater tendency toward H-bond formation, hydrated complexes such as **II** might be expected to be substantially more hydrophilic than anhydrous solutes such as **I**. Instead, the anhydrous and monohydrated forms of 3-methylindole appear to be equivalent in hydrophobic character, since they are reported to be equally abundant in cyclohexane. In addition, the putative hydrate of 3-methylindole appears to possess unusual thermal properties, in that most hydrates tend to dissociate with increasing temperature. In contrast, a 1:1 molar ratio of hydrated to anhydrous 3-methylindole is reported to persist over a temperature range of 40 °C.

In view of their potential importance, it seemed desirable to consider whether these results might be open to a simpler interpretation. Wimley and White chose tritium to follow the distribution of water between phases, assuming that ^3H entered cyclohexane only in the form of water. Proton exchange is known to occur at the NH group of tryptophan under acidic conditions (Wüthrich, 1986), and it seemed possible that proton exchange could have occurred in these experiments. With an expected fractionation factor of approximately unity (Schowen, 1982), the enrichment of tritium at the -NH- group of 3-methylindole might eventually be expected to approach that of the water, which was present in large molar excess over 3-methylindole. Scheme II shows that if tritium (T) had equilibrated between water and the -NH- group of 3-methylindole (one exchangeable H atom) but were considered to represent water (two exchangeable H atoms), then one extra water molecule would appear to have entered cyclohexane with every two molecules of 3-methylindole. This ratio would remain unchanged with varying temperature, and this would explain the otherwise puzzling observation (Wimley & White, 1992) that the hydrated and unhydrated species of 3-methylindole in cyclohexane appeared to remain in a 1:1 ratio at all temperatures.

To test this possibility under controlled conditions, we performed two sets of experiments. In both sets of experiments, an aqueous layer, containing HCl to promote exchange, was shaken with a layer of cyclohexane in the presence and absence of added 3-methylindole. In the first set of experiments, the aqueous layer consisted of D_2O, and exchange of hydrogen atoms between water and the ring nitrogen atom of 3-methylindole in cyclohexane was monitored by deuterium NMR. In the second set of experiments, ^3H_2O was present in the aqueous layer, and the appearance of ^3H in the cyclohexane layer was measured to determine whether excess water was transported into cyclohexane in the presence of 3-methylindole.

Scheme II



MATERIALS AND METHODS

3-Methylindole, toluene, *n*-butylamine, *N*-butylacetamide, cyclohexane, and D_2O (99.8 atom % excess) were obtained from Aldrich Chemical Co. 3-Methylindole was purified by sublimation, and other compounds were used without further purification.

Distribution experiments were performed by shaking an aqueous phase (1 mL) with a cyclohexane phase (50 mL) for various time intervals in 100-mL round-bottomed flasks, using a Burrell wrist-action shaker at 20 °C. When the phases had separated, aliquots were removed for analysis by NMR (using a Bruker AMX-500 spectrometer equipped with a deuterium probe) or by scintillation counting. In deuterium NMR experiments, concentrations were determined from the integrated intensities of deuterons, using deuterium at natural abundance in cyclohexane as a reference. In experiments with tritium, concentrations of exchangeable hydrogen atoms in the cyclohexane phase were calculated from the specific activity of tritium in the aqueous phase, typically 5.0×10^8 cpm/mol of water.

RESULTS AND DISCUSSION

Distribution experiments were performed by shaking an aqueous layer (1 mL, containing 10^{-2} M HCl to promote exchange) with a layer of cyclohexane (50 mL) in the presence and absence of added 3-methylindole at 20 °C. Under these conditions, the distribution coefficient of 3-methylindole from water to cyclohexane is 51.3 (Radzicka & Wolfenden, 1988), and the solubility of water in cyclohexane is approximately 0.9×10^{-3} M (see below). With 3-methylindole initially present in the cyclohexane phase at a concentration of 2×10^{-3} M or more, no significant amount of solute is lost to the aqueous phase, and transport of 3-methylindole-associated water into the cyclohexane phase should be easily detected.

Under these conditions, Figure 1 shows that, using ^3H_2O, tritium partitioning between phases occurred rapidly in the absence (●) or presence (◆) of 3-methylindole in the cyclohexane phase, equilibrium being achieved with a half-time of less than 15 min. Subsequent experiments were routinely performed after shaking for a period of 4 h, to ensure complete equilibration. Table I shows that when the same experiment was performed using D_2O as the aqueous phase, the integrated intensity of the N–D peak of 3-methylindole,

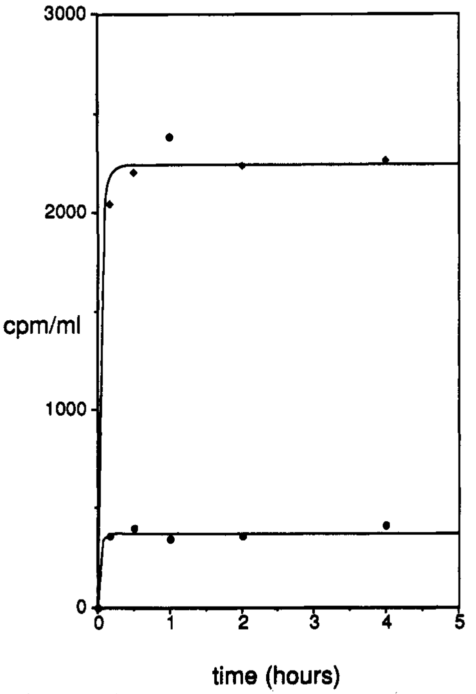


FIGURE 1: ^{3}H (cpm/mL) appearing in the cyclohexane phase as a function of time elapsed since the commencement of shaking, in the absence (●) and in the presence (◆) of 3-methylindole (8×10^{-3} M).

Table I: Water-Exchangeable ^{2}H Atoms Appearing in the ^{2}H NMR Spectrum in Cyclohexane in the Presence of Various Concentrations of 3-Methylindole at 20 °C[a]

concn of 3-methylindole in cyclohexane (mM)	integrated intensity of N–D resonance of 3-methylindole	integrated intensity of D_2O resonance
0.0	0.0	0.1
2.0	0.2	0.1
4.0	0.5	0.1
8.0	1.0	0.1
16.0	2.0	0.1

[a] $CDCl_3$, used as a reference in cyclohexane, was assigned a resonance at 7.24 ppm downfield from TMS, by analogy to its position in proton spectra. On the basis of this reference, the resonance of ^{2}H of natural abundance in C_6H_{12} was at 1.63 ppm, the N–D resonance of 3-methylindole was at 7.59 ppm, and D_2O was assigned a resonance at 1.05 ppm. Integrated intensities were normalized with respect to ^{2}H at natural abundance in C_6H_{12}.

at 7.59 ppm, increased in proportion to solute concentration. However, the integrated intensity of deuterons of water in cyclohexane, at 1.05 ppm, did not vary in the presence of varying concentrations of 3-methylindole and corresponded to the concentration of water in water-saturated cyclohexane in the absence of solutes at 20 °C determined separately (see below). However, it remained possible that some additional water might have been present that escaped detection by NMR. It was therefore desirable to perform an experiment that would allow detection of all the exchangeable hydrogen atoms in the cyclohexane phase.

$^{3}H_2O$ was used in place of $^{2}H_2O$, to determine whether more tritium entered cyclohexane than could be accounted for as free water and as the –NH– group of 3-methylindole. Table II shows that tritium entered the cyclohexane phase in concentrations numerically matching the concentration of 3-methylindole, 0–32 mM, that was present in the cyclohexane phase, after subtracting the unvarying concentration (0.9 ± 0.1 mM) of water that was always observed in cyclohexane in the absence or presence of added 3-methyindole.

Table II: Concentration of Water-Exchangeable ^{3}H Atoms Appearing in Cyclohexane in the Presence of Various Concentrations of 3-Methylindole

concn of 3-methylindole in cyclohexane (mM)	total ^{3}H in cyclohexane (cpm/mL)	concn of exchangeable H atoms in cyclohexane (mM)
0.0	434 ± 75	0.0 (1.8)[a]
3.2	1255 ± 95	3.4
8.0	2374 ± 239	8.0
16.0	4408 ± 207	16.4
32.0	7732 ± 73	30.1

[a] The concentration in parentheses represents the concentration of tritium due to the concentration of water (0.9 mM) in water-saturated cyclohexane.

In summary, the possibility that an additional half-molecule of water is entrained by 3-methylindole, when it enters cyclohexane, appears to be excluded (1) by the NMR observation that no excess water enters cyclohexane with 3-methylindole and (2) by the findings with tritium, indicating that the total concentration of exchangeable hydrogen atoms matches the concentration of 3-methylindole entering the cyclohexane phase, plus the small but constant concentration of "free" water that is required to saturate cyclohexane at any given temperature. From the results obtained using tritium, at the highest concentrations of 3-methylindole, we estimate that less than 0.1 molecule of water could be entrained by each molecule of 3-methylindole that enters cyclohexane.

It remained of interest to consider the possibility that such entrainment might occur in the presence of molecules that are more or less polar than 3-methylindole. To test that possibility, we performed similar experiments with toluene (the side chain of phenylalanine), ethanol (the side chain of threonine), or uncharged *n*-butylamine (the side chain of lysine). In addition, we examined the behavior of *N*-butylacetamide, as a model of the peptide bond itself. In every case, the amount of tritium transferred to the cyclohexane phase did not exceed expectations based on the number of exchangeable hydrogen atoms in the solute that partitioned into the cyclohexane phase.

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