can be trapped by continuous illumination at 140 K at the expense of generation of the S_2 state EPR signal. The moderately large zero-field splitting parameter D_2 required to fit our data (3.0 cm^{-1}) can be explained by a strong axially symmetric crystal field around the $s_2 = 1$ spin.[15]

If the $s_1 = {}^1/_2$ spin is indeed the ground state of an antiferromagnetic Mn dimer, then there must be excited states above the state giving rise to the EPR signals. As discussed earlier, the presence of an excited state provides a relaxation pathway for the spin system via an Orbach mechanism. Therefore, the values of Δ obtained from our power saturation data may reflect the extent of exchange coupling within the dimer. As an example, we considered a trimer consisting of $s_1 = 2$, $s_2 = {}^3/_2$, and $s_3 = 1$, which could arise from a site consisting of Mn(III) ($s_1 = 2$), Mn(IV) ($s_2 = {}^3/_2$), and low-spin Fe(IV) ($s_3 = 1$), where $J_{12} > 0$, $J_{13} < 0$, and $J_{23} < 0$. Provided that $|J_{12}| > |J_{23}|$ and $|J_{12}| > |J_{13}|$, the ground and lowest excited states of this trimer will have $s = {}^3/_2$ and $s = {}^1/_2$, respectively. The exchange Hamiltonian for three interacting spins can be written as $\hat{\mathbf{H}}_{ex} = \sum_{i<k} J_{ik}\hat{\mathbf{s}}_i\hat{\mathbf{s}}_k$ and the spin–spin interaction is given by $\hat{\mathbf{H}}_{ss} = \sum_i \hat{\mathbf{s}}_i \mathbf{D}_i \hat{\mathbf{s}}_i$.

The energy levels were obtained by diagonalizing $\hat{\mathbf{H}}_{trimer}$ in the uncoupled representation $|s_1 s_2 s_3 m_1 m_2 m_3\rangle$, where

$$\hat{\mathbf{H}}_{ex} = \sum_{i<k} J_{ik}[{}^1/_2(\hat{\mathbf{s}}_{i+}\hat{\mathbf{s}}_{k-} + \hat{\mathbf{s}}_{i-}\hat{\mathbf{s}}_{k+}) + \hat{\mathbf{s}}_{iz}\hat{\mathbf{s}}_{kz}] \quad (8)$$

and

$$\hat{\mathbf{H}}_{ss} = \sum_i D_i[\hat{\mathbf{s}}_{iz}^2 - {}^1/_3 s_i(s_i + 1)] + {}^1/_2 E_i(\hat{\mathbf{s}}_{i+}^2 + \hat{\mathbf{s}}_{i-}^2) \quad (9)$$

It was assumed that $E_i = 0.0$ cm^{-1} and that $J_{13} = J_{23} = J_2$ and $J_{12} = J_1$. In the case of the active S_2 state EPR signal generated by 170 K illumination, it was assumed that the value of Δ is the same as that for the active state obtained by 160 K illumination. The temperature-dependence data could be fitted by using J_2 values which were identical with the J values obtained from the previous model and $D_3 = 3.0$ cm^{-1}. The values of J_1 which fitted the power-saturation data were 34.0 (resting state) and 12.7 cm^{-1} (active state).

Conclusions

The data presented in this paper show that the S_2 state EPR signals from the OEC arise from the interaction of more than one paramagnetic site. However, our temperature dependence data cannot be accounted for by a simple Mn dimer model, unless such a dimer arises from a ferromagnetic exchange coupling of low-spin forms of Mn. As noted above, low-spin Mn complexes are rare and not likely to be present in the OEC. A more reasonable model for the species giving rise to the S_2 state EPR signal is one in which an antiferromagnetically exchange coupled Mn(III)–Mn(IV) dimer is ferromagnetically exchange coupled to another site with $s = 1$. This second site could possibly be low-spin Mn(III), low-spin Fe(IV), or even a second Mn dimer.

We assign the S_2 state EPR signal to a thermally populated $s = {}^1/_2$ state. Therefore, if our interpretation of the data is correct, an EPR signal from the $s = {}^3/_2$ ground state should be observable at low temperature. This signal is expected to be anisotropic and, therefore, difficult to detect. Current efforts in this laboratory are directed at using the ferromagnetic exchange coupling scheme to accurately simulate the three S_2 state EPR spectra and attempting to observe EPR signals from the $s = {}^3/_2$ ground state of the S_2 state of the OEC.

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A New Method for Obtaining Isotopic Fractionation Data at Multiple Sites in Rapidly Exchanging Systems

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Abstract: A new method for rapidly and conveniently obtaining isotopic fractionation factors in dilute aqueous solutions of compounds containing rapidly exchanging OH, NH, and SH groups is described. Shifts in the positions of NMR peaks for spectroscopically observable nuclei induced by isotopic substitution are the basis of this procedure which has the unique capability of separately measuring the isotopic exchange constants simultaneously for several different groups in the same molecule. The results for a series of alcohols, amines, thiols, phenols, acids, and amides with use of ^{13}C NMR spectroscopy are reported. Atypically low values of K_{frac} are observed in several cases, indicating that there are strong internal hydrogen bonds in competition with those to water, yielding conformational information.

For an isotopic exchange reaction

$$HA + DB \overset{K_{frac}}{\rightleftharpoons} DA + HB \quad (1)$$

the equilibrium constant K_{frac} is known as the fractionation factor. Many molecules containing OH, NH, and SH groups exchange hydrogen and deuterium rapidly with hydroxylic solvents, setting up such equilibria. These fractionation factors are closely related to details of molecular structure and are sensitive to changes in factors like hydrogen bonding. Strong internal hydrogen bonds (in competition with those to the solvent) should be indicators of the conformation of flexible molecules.

Existing and established experimental methods cannot conveniently measure fractionation factors for hydrogen–deuterium exchange in dilute aqueous solution,[1] nor can previously reported techniques obtain information about individual exchangeable sites

(1) For a review of isotope effects see: (a) M. Wolfsberg, *Acc Chem. Res.*, *5*, 225 (1972). (b) M. Wolfsberg, *Annu. Rev. Phys. Chem.*, **20**, 449 (1969).

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in a molecule which may contain several distinguishable sites. We report here a new, generally applicable procedure for measuring multiple exchange fractionation factors for rapidly exchanging molecules in dilute aqueous solution.

Previously reported methods[1] designed to obtain proton–deuteron fractionation factors between water and other exchangeable species all have serious limitations. The first experimental measurements of isotopic equilibrium constants date back to the late 1930's.[2] Compounds with readily exchangeable protons were allowed to achieve equilibrium with water containing a known small fraction of deuterium.[3,4,5] After the solute and solvent were separated and purified, the deuterium content of each was determined. The aqueous solutions obtained from careful combustion of the solute (as well as the solvent itself) were subjected to density measurements, refractometric measurements, or thermal conductivity measurements,[6] and from calibration curves, the deuterium concentrations were obtained. The entire process could take over 2 weeks![7] The separation must be done in a manner which does not perturb the equilibrium, and great care must be taken to prevent isotopic exchange with atmospheric water.

In principle, experimental measurement of infrared (IR) and raman spectra of molecules,[8] followed by normal-mode analysis and the use of formulas from the theory of isotope effects,[9] can be used to calculate equilibrium constants for isotopic exchange reactions. However, the frequencies are affected by solvation (particularly in aqueous solution), and it often is difficult or impossible to measure, assign, and analyze the peaks due to all the species present because of strong solvent absorption.

Mass spectroscopy is one of the most extensively used techniques for measuring isotopic fractionation constants. Gas-phase equilibria such as

$$H_2 + D_2 \rightleftharpoons 2HD$$

or NH_3/ND_3 exchange,[10,11] or gases in equilibrium with liquids as in[12]

$$^{10}BF_3(g) + {}^{11}BF_3\text{–anisole} \rightleftharpoons {}^{11}BF_3(g) + {}^{10}BF_3\text{–anisole}$$

can be examined. Aqueous solutions such as 1,3,5-trimethoxybenzene in water (exchange catalyzed by perchloric acid)[13] have also been studied. The solute must either be recovered from the solution in pure form and subjected to mass spectral analysis[13] or the water vapor over a solution can be examined for changes in deuterium content. The latter method generally requires quite concentrated solutions for accurate results. Although mass spectroscopy is an extremely sensitive and potentially precise probe, it is not without complications. The technique is best suited to systems where the vapor can be directly examined. When attempting to measure the self-exchange equilibrium constant of water (see eq 5) by mass spectroscopy, assumptions and corrections must be made for differences in total ionization cross section for H_2O, D_2O, and HOD. Technical problems such "the memory effect" and background noise must be overcome.[14,15]

More recently, Fenby has used isotopic vapor pressures to determine values of K_{frac} for compounds in aqueous solution.[16] As pointed out by Jancso,[17] accuracy is often poor when examining certain types of compounds (such as amines) and there is considerable discrepancy in the values reported by different authors for isotopic vapor pressures of the same compounds.

Another important tool for obtaining isotopic fractionation data is nuclear magnetic resonance (NMR) spectroscopy. In the relatively few cases where proton exchange between solute and solvent occurs and happens to be slow on the NMR time scale, integration of the proton or deuterium peaks for the different species can yield the fractionation factors.[18] It is often difficult to measure these integrals well enough to obtain accurate values for the fractionation factors. In the more common cases where exchange is rapid on the NMR time scale, techniques based on measurement of the averaged proton or deuterium frequencies[19] can be used; however, these NMR methods (as well as all previously mentioned methods) are poorly suited for molecules containing several distinguishable exchangeable sites. The single measured NMR frequency for the averaged protons or deuterons must be used to obtain several different equilibrium constants. In addition, the accuracy becomes poor in cases where one substance (water or another hydroxylic solvent) is in large excess, since shifts in the NMR frequencies of the averaged peaks resulting from differences in the fractionation factors are very small.

We report here a new, general method for rapidly and conveniently measuring isotopic fractionation factors under conditions of rapid proton exchange between solvent water and molecules containing OH, NH, and SH functional groups. Changes in the NMR chemical shifts of any spectroscopically observable nuclei near the sites of isotopic incorporation in molecules are the basis of this procedure. This paper focuses on the use of ^{13}C NMR spectroscopy to demonstrate the method's generality and applicability to a wide variety of organic molecules, but other nuclei can also serve as reporters for this purpose. Since it is not the averaged peak of the exchanging protons or deuterons that is observed spectroscopically, the fractionation factor can be accurately measured in dilute aqueous solution, and at high and low H_2O/D_2O ratios. This new procedure also has the unique capability of simultaneously determing the fractionation factors between water and *each* exchangeable site in a molecule containing many such sites.

Method Employed

Introduction of an isotope into a molecule induces chemical shifts in the NMR peaks of nuclei near the substituted atom. These shifts, which have been called intrinsic isotope shifts, have been much studied.[20] Deuterium substitution is particularly easy in molecules containing functional groups with readily exchangeable protons such as OH, NH, and SH. Shifts in the ^{13}C resonance on deuteration of COH, CNH, and CSH groups can be readily measured and have been helpful in making spectral assignments.[21] However, if one uses known mixtures of H_2O and D_2O (and HOD), instead of pure H_2O and pure D_2O, as solvents for the molecules to be studied, an isotopic exchange situation (as in eq 1) is created. The fraction of D in the exchangeable group will not necessarily be the same as that in the water. If the proton/deuteron exchange between solute and solvent is rapid, as it is in most OH, NH, and SH groups, the observed CMR frequency will be the average of the frequencies of the H and D substituted molecules weighted by their amounts. Since the relative amounts are functions of the fractionation factor, in principle, accurate measurement of the average frequency could yield a value for the fractionation factor. It was our intention to discover

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Table I. CMR at 125.76 MHz and 308 K; Average δ (in Hz)[a] for Approximate D_2O Fraction

case	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
A1	2.35	4.80	7.25	10.15	12.72	15.00	17.75	19.75	22.35	25.06
A2	1.80	3.45	5.30	7.60	9.50	11.25	13.20	17.70	16.65	18.73
A3	1.72	3.45	5.40	7.58	9.42	11.18	13.12	14.78	16.42	18.60
A4	1.65	3.65	5.45	7.50	9.68	11.05	13.40	14.80	16.45	18.47
A5	0.90	1.72	2.70	3.60	4.22	4.95	5.92	6.60	7.50	8.57
A6	0.70	1.15	2.00	2.50	2.75	3.00	3.95	4.40	5.20	6.07
A7	0.70	1.30	1.65	1.85	1.93	2.00	2.25	2.65	3.40	4.03
B	5.49	10.06	15.13	20.23	25.09	31.08	35.31	40.33	45.87	51.64
C	5.32	10.77	14.98	21.54	25.98	32.04	37.44	44.18	49.59	55.83
D	2.95[b]	7.82	10.16	15.23	18.16	23.13	27.24	32.04	37.77	43.04
E	1.64	2.94	4.46	6.00	7.23	8.43	9.84	11.04	11.84	12.94
F	1.64	3.72	5.34	7.15	8.78	10.36	11.84	13.22	14.54	15.97
G	1.62	3.12	4.60	5.66	7.07	7.91	9.18	10.13	10.95	11.98
H1	1.62[b]	3.73	5.60	7.36	8.92	10.61	12.04	13.50	14.92	16.63
H2	0.00	2.10[b]	3.84	5.20	6.50	7.55	8.48	9.40	10.29	11.39
I1	0.0	2.68[b]	4.30	5.81	7.16	8.16	9.60	10.62	11.87	13.17
I2	0.0	2.25[b]	4.03	5.36	6.65	7.48	8.67	9.34	10.34	11.19
J1	2.97	6.01	8.94	11.68	14.55	17.02	19.86	22.33	24.93	28.50
J2	3.31	6.24	9.33	12.44	15.80	19.10	22.90	26.60	30.67	36.60
K	1.26[b]	3.06	4.86	6.70	8.69	10.80	13.09	15.66	18.62	22.28

case		concn (M)	K_{frac}
A1	methyl-α-D-glucopyranoside (C-3)	0.50	1.05 +/- 0.06
A2	methyl-α-D-glucopyranoside (C-4)	0.50	1.04 +/- 0.06
A3	methyl-α-D-glucopyranoside (C-2)	0.50	1.05 +/- 0.06
A4	methyl-α-D-glucopyranoside (C-6)	0.50	1.08 +/- 0.09
A5	methyl-α-D-glucopyranoside (C-5)	0.50	1.04 +/- 0.07
A6	methyl-α-D-glucopyranoside (CH_3)	0.50	0.88 +/- 0.19
A7	methyl-α-D-glucopyranoside (C-1)	0.50	0.96 +/- 0.58
B	sodium salicylate	1.00	0.95 +/- 0.05
C	potassium phthalate	0.30	0.95 +/- 0.04
D	sodium maleate	0.30	0.77 +/- 0.05
E	diethylamine	1.00	1.31 +/- 0.09
F	morpholine	1.00	1.25 +/- 0.07
G	pyrrolidine	1.00	1.43 +/- 0.05
H1	2,6-dimethylpiperazine (3° carbon)	1.00	1.24 +/- 0.02
H2	2,6-dimethylpiperazine (2° carbon)	1.00	1.35 +/- 0.06
I1	2-methylpiperidine CH	1.00	1.17 +/- 0.05
I2	2-methylpiperidine CH_2	1.00	1.44 +/- 0.08
J1	1-cysteine (C-NH_2)	0.80	1.09 +/- 0.03
J2	1-cysteine (C-SH)	0.80	0.83 +/- 0.05
K	2-mercaptoethanesulfonic acid (Na salt)	1.00	0.65 +/- 0.01

[a] All values reported relative to signal in H_2O, taken as zero. [b] Indicate values measured with large uncertainty due to severe overlap between peaks.

how reproducibly and accurately we could obtain fractionation factors using this procedure.

Experimental Section

A 5 or 10 mm dual cell with equal volume concentric compartments, similar to that of Pfeffer,[21] was used. A 1 M solution of the solute in distilled H_2O was placed in one compartment and a 1 M solution of the solute in a known H_2O/D_2O mixture was placed in the other compartment. The deuterium oxide (99.8% D) was obtained and used as purchased from MSD company. All compounds examined are commercially available and were used as purchased or purified by standard methods.[22] The H_2O/D_2O mixtures were prepared by combining appropriate convenient volumes (with pipet) of 1 M stock solutions of the compound in H_2O and in D_2O. The mass of each aliquot of stock solution was also determined to provide verification for the D_2O fraction of each mixture. For consistent comparison, the solute in H_2O was placed in the inner compartment, serving as the reference for all examined systems. The fraction of D_2O in the outer compartment was varied from 0% to 100%. Frequency differences between corresponding proton-decoupled CMR signals originating from each compartment were then measured. Up to five separate ^{13}C NMR spectra were recorded for each of the D_2O concentrations at 308 K.

Acquiring high-resolution spectra of solutions in concentric NMR tubes requires considerable attention to magnetic field homogeneity. The double cell arrangement produces somewhat broader peaks than the single cell for the same solution. Generally, it is found that the NMR signal from one compartment can be sharpened by shimming but often at the expense of the other; usually the peak from the inner compartment can be made sharpest. Interchange of the solutions between the two compartments for selected representative systems generally resulted in small differences in the data. For most compounds, interchange of H_2O and D_2O solutions produced a change in data of no more than 0.5 Hz (and had a modest effect on the value for K_{frac}), but our experiment on potassium phthalate yielded a significantly larger chemical shift difference (3.73 Hz, 7% of total splitting) when the H_2O solution was in the inner compartment than when H_2O was in the outer compartment. A suspiciously large trend in the derived value of K_{frac} (a sharp decrease with increasing D_2O concentration) was also noticeable. To evaluate the effect of the error on K_{frac}, the data were adjusted to compensate for the difference due to interchange of the two solutions (attributed, at least in part, to magnetic field inhomogeneity since the difference can be varied somewhat by substantial changes in shim settings). It was found that the average K_{frac} was essentially unchanged, but the computed uncertainty associated with the fractionation constant for potassium phthalate decreased considerably.

Data for all other systems listed in Tables I and II are uncorrected for this source of error. The uncertainty given with each K_{frac} is considered conservative. Examintation of the same solutions (either H_2O, D_2O, or an H_2O/D_2O mixture) in both inner and outer compartments for a variety of compounds (amines, alcohols, acids, and thiols) revealed a single discernible peak for each carbon resonance at 125.76 MHz. Singe point data acquisition was carried out at either 62.89 and/or 67.90 MHz (Table II) while more extensive data was obtained at 125.76 MHz (Table 1A).

In addition to control experiments based on solution position in the dual cell, factors such as pH, concentration, field strength, and temperature were also investigated. In general, no attempts were made to adjust pH/pD in solutions of the amines, since generated NMR titration curves (chemical shift. vs. pH) indicate that the pH/pD of experimentation, the chemical shift differences for C-NH and C-ND are virtually constant over a range greater than 0.4 pH unit. In various test cases where

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Table II. Single Point NMR Data CMR at 62.89 MHz and 308 K Unless Otherwise Stated; Average δ (in Hz)[a] for Approximate D_2O Fraction

case	concn (M)	50%	100%	K_{frac}
methanol	1.00	6.05	11.91	1.06
ethanol (α carbon)	1.00	5.32	10.41	1.10
ethanol (β carbon)	1.00	4.31	8.46	1.08
isopropyl alcohol (α carbon)	1.00	4.57	9.37	0.98
isopropyl alcohol (β carbon)	1.00	3.80	7.57	1.04
tert-butyl alcohol (α carbon)	1.00	4.30	8.73	1.00
tert-butyl alcohol (β carbon)	1.00	3.36	6.60	1.03
phenol (C-α)[c,d]	0.50	5.42	10.36	1.13
phenol (C-o)[c,d]	0.50	4.72	8.91	1.15
phenol (C-m)[c,d]	0.50	1.42	2.73	1.11
1,3-dihydroxyacetone	1.00	4.11	8.20	1.05
2-nitropropanol	1.00	4.41	8.90	1.01
2-aminoethanol (C—OH)	0.50	4.90	9.27	1.16
salicylaldehyde/*p*-dioxane	0.50	6.19	12.53	0.99
acetic acid (CH_3)[d]	6.00	4.66	9.25	1.02
acetic acid (CH_3)	2.00	4.22	8.84	0.92
acetic acid (C=O)	2.00	4.68	9.96	0.89
maleic acid (C=O)	1.00	8.65	18.89	0.87
maleic acid (=CH)	1.00	10.86	22.78	0.94
oxalic acid	1.00	22.89	48.57	0.89
malonic acid (C=O)	1.00	8.18	18.28	0.81
succinic acid (C=O)	0.50	4.95	10.79	0.86
succinic acid (C—C)	0.50	4.56	9.69	0.91
glutaric acid (α CH2)	1.00	3.84	7.95	0.98
glutaric acid (β CH2)	1.00	2.98	6.08	1.01
glutathione (glu-COOH)	0.25	6.22	13.70	0.86
glutathione (gly-COOH)	0.25	10.17	23.14	0.81
N-methylacetamide (N—CH_3)[e]	1.00	6.56	12.58	1.13
N-methylacetamide (CO—CH_3)[e]	1.00	3.72	7.20	1.11
methylamine	0.50	8.88	17.03	1.15
n-butylamine	1.00	7.88	14.97	1.17
isopropylamine	1.00	9.16	17.55	1.17
sec-butylamine	1.00	8.24	16.09	1.10
tert-butylamine	1.00	9.30	18.61	1.04
dimethylamine	0.50	3.81	6.54	1.49
diisopropylamine	1.00	4.35	8.51	1.08
piperidine[b]	1.00	6.33	11.49	1.30
piperazine[b]	0.50	8.81	15.28	1.40
2,6-dimethylpiperidine[b]	0.50	9.18	18.46	1.01
2-aminoethanol (C—NH_2)	0.50	9.22	17.90	1.12
2,5-dimethylpiperazine CH	1.00	4.41	8.46	1.14
2,5-dimethylpiperazine CH_2	1.00	3.97	7.18	1.27
2-methylpiperidine CH[b]	0.50	9.03	16.84	1.18
2-methylpiperidine CH_2[b]	0.50	7.60	12.57	1.56
2-methylpiperidine/ 0.1 M NaOH CH[b]	0.50	8.66	15.81	1.24
2-methylpiperidine/ 0.1 M NaOH CH_2[b]	0.50	7.71	13.25	1.43
2-methylpiperidine/ 0.5 M NaOH CH[b]	0.50	8.32	15.11	1.28
2-methylpiperidine/ 0.5 M NaOH CH_2[b]	0.50	7.94	13.95	1.38
2-methylpiperidine/ 0.75 M NaOH CH[b]	0.50	9.09	17.13	1.19
2-methylpiperidine/ 0.75 M NaOH CH_2[b]	0.50	8.54	15.64	1.27
2-methylpiperidine/ 1.0 M NaOH CH[b]	0.50	8.90	16.97	1.15
2-methylpiperidine/ 1.0 M NaOH CH_2[b]	0.50	8.49	15.55	1.28

[a] All values reported relative to signal in H_2O, taken as zero. [b] ^{13}C NMR (125.76 MHz). [c] ^{13}C NMR (67.90 MHz). [d] 303 K. [e] pH 13.

dissociation can occur in neutral aqueous solution, sulfuric acid or sodium hydroxide was added to suppress possible ionization and for the most part showed no significant effect on the measured value of K_{frac} for the reported systems. This indicates that for the majority of classes and concentrations of compounds reported, the extent of ionization is non-interfering in the measurement of K_{frac}. The effect of modest changes in concentration was explored by examination of selected samples at 0.5 and 1.5M, in addition to 1.0 M, which did not significantly affect the measured fractionation factors, in most cases. Since most compounds were first screened at 62.89 MHz and then more complete data were obtained at 125.76 MHz, data were available on the relation between K_{frac} and field strength. Although the uncertainty associated with the measurement of K_{frac} decreases as field strength is increased (due to an increase in the splitting to line width ratio), the values are identical within experimental error.

Working with water as the solvent, temperature studies for many compounds are restricted to a practical range of about 60°. In addition to data acquisition at 308 K, a one point K_{frac} determination was also carried out, for certain cases, at lower and higher temperatures (about 280 and 335 K), so as to establish K_{frac}'s sensitivity to temperature. The value of K_{frac} for tested systems appears quite constant (within experimental error) over this temperature range; however, the chemical shifts of certain compounds (such as 2,6-dimethylpiperazine) are considerably temperature sensitive, indicating that proper temperature regulation is essential to achieve an acceptable level of precision. To ensure that using a proton decoupling power of 1.5 W was not causing an undesired and undetected heating of the solution, the calibration method developed by Led and Peterson[23] was utilized. Proton decoupled and non-decoupled ^{13}C NMR spectra of an equal volume mixture of acetone-d_6 and carbon tetrachloride in the inner cell and an aqueous solution of various solutes in the outer compartment of the coaxial duel cell were taken. Comparison of the sets of spectra indicated that there was essentially no unaccounted heating caused by proton decoupling for most systems (alcohols, amines, carboxylic acids, etc.) and that the actual sample temperature was within three degrees of the indicated temperature.

Discussion

In this experiment, each carbon atom bonded to (or near) an exchangeable site appears as a doublet in the ^{13}C NMR spectrum.[21] Carbon atoms α, β, and γ to the site of exchange experience (with the magnitude decreasing as a function of distance) an upfield shift due to deuterium incorporated in ND, OD, and SD groups.[24] Generally, the largest splitting detected (upon isotopic substitution at a nearby site) in the spectrum is the one used to evaluate K_{frac} for that site.[25] Close examination of the cyclic amine piperidine revealed that the β and γ ring carbon atoms display no information about the extent of deuterium incorporation at nitrogen. Thus, no splitting is detected for ring carbon atoms not directly adjacent to the nitrogen atom; the intrinsic isotope chemical shift differences at these ring positions are small relative to peak width. The NMR signal for the methyl groups of 2,6-dimethylpiperidine does appear as a doublet.

In the CMR spectrum, the downfield peak of each pair—corresponding to the solute in H_2O—serves as the reference; the upfield peak is the averaged frequency for the carbon of the solute in the D_2O/H_2O mixture (see Figure 1). This chemical shift difference (δ) is a function of the shift difference between the resonances for the fully protonated and fully deuterated species and the fractionation factor. Assuming that concentrations are proportional to activities

$$K_{frac} = \frac{[\text{solute-D}][\text{water-H}]}{[\text{solute-H}][\text{water-D}]} \quad (2)$$

$$K_{frac} = \frac{F_{av}[\text{water-H}]}{(F_{D20} - F_{av})[\text{water-D}]} \quad (3)$$

$$\frac{F_{av}}{F_{D_2O}} = \frac{K_{frac}}{(\%\ \text{water-H}) + (K_{frac}\ (\%\ \text{water-D}))}(\%\ \text{water-D}) \quad (4)$$

With respect to the carbon frequency in H_2O, taken to be zero, F_{av} is the averaged carbon frequency in the D_2O/H_2O mixture, and F_{D_2O} is the carbon frequency (corrected to complete deuteration of solute) in 100% D_2O, which corresponds to the intrinsic isotope chemical shift difference.

Since up to five separate spectra are recorded for each D_2O fraction, a total of 25 values ($5F_{av} \times 5F_{D_2O}$) for K_{frac} are determined for each of the 9 D_2O concentrations (10–90%). When the generated 225 points were used, the average for K_{frac} along with the uncertainty association with one standard deviation is reported (see Table 1B). K_{frac} obtained from a nonlinear least-squares fit of the data is totally consistent with the average value

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(25) For many compounds, K_{frac} can be accurately determined from the resonances in the CMR due to the carbon β (as well as α) to the exchangeable site, providing an internal check on the measurement of K_{frac}.

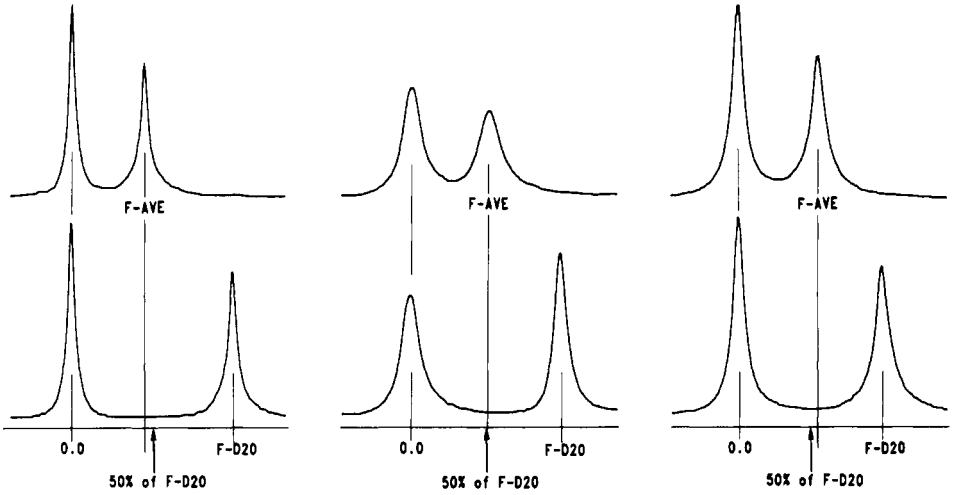

Figure 1. Lower row: solute in H_2O vs. solute in D_2O. Upper row: solute in H_2O vs. solute in equal molar H_2O/D_2O. First column: C–SH of cysteine (K_{frac} less than unity). Middle column: C–OH of methyl-α-D-glucopyranoside C-3 (K_{frac} of about unity). Last column: C–NH of morpholine (K_{frac} greater than unity).

calculated for each case. The χ^2 value associated with the best fit for each system was calculated and typically found to be in the range of 0.1 to 1×10^{-4}. The values for the fractionation constants are calculated by assuming that the self-exchange equilibrium constant of each system does not differ significantly from the statistical value and that multiple exchangeable sites in a molecule exchange independently from each other.

To obtain sufficient resolution, parameters are typically set such that the spectra are acquired with an interval of around 0.2 Hz/point. This corresponds to a spectral width of about 3000 Hz for a 32 000 point acquisition (the exact settings depend on natural peak widths, separation distance between pairs of peaks, and the number of pairs examined in the specific molecule). A 45° pulse in generally used and 100–200 scans accumulated for most solutions examined. With a typical peak width at half-height of about 1 Hz and a separation distance on the order of several hertz, chemical shift differences are found to be reproducible to within +/−0.1 Hz or better. The scatter of these values is the major source of error in determining K_{frac}. Due to the nature of the calculation, K_{frac} is determined most accurately from frequency measurements in the range of 40% to 60% D_2O when K_{frac} is close to unity. As values for fractionation factors become greater than one, the optimal range for its determination shifts to lower D_2O concentrations; for K_{frac} less than unity, higher D_2O concentrations are optimal. Data at very high and low deuterium concentrations are used to verify the intrinsic isotope chemical shift difference and establish the equilibrium constants for the exchange with D_2O and with HOD, respectively. The curve generated from the data points spanning 0% to 100% D_2O concentration also helps to point out systematic errors that may skew the data. With proper care in sample preparation and temperature control, K_{frac} can be determined to an accuracy of better than 5% (depending on the magnitude of F_{D_2O} and the peak width). A plot of F_{av}/F_{D_2O} vs. D_2O fraction clearly illustrates how the fractionation constant deviates from unity when an isotope effect exists (see Figures 2 and 3).

At low deuterium concentration, the fraction of D_2O is small and K_{frac} essentially measures the exchange equilibrium between solute and HOD. At high deuterium concentration, little H_2O is present and K_{frac} reflects the exchange equilibrium between solute and D_2O. In principle, the self-exchange equilibrium constant for water (K of eq 5) may be determined from the change in apparent K_{frac} with deuterium fraction.

$$H_2O + D_2O \overset{K}{\rightleftharpoons} 2HOD \qquad (5)$$

In the cases we have studied so far, the experimentally derived values for K_{frac} do not appear to be a function of the D_2O fraction beyond experimental error. Therefore, a meaningful experimental value of K cannot be extracted from our present data. A case with an extremely large intrinsic isotope shift and very narrow lines would be required for this purpose.

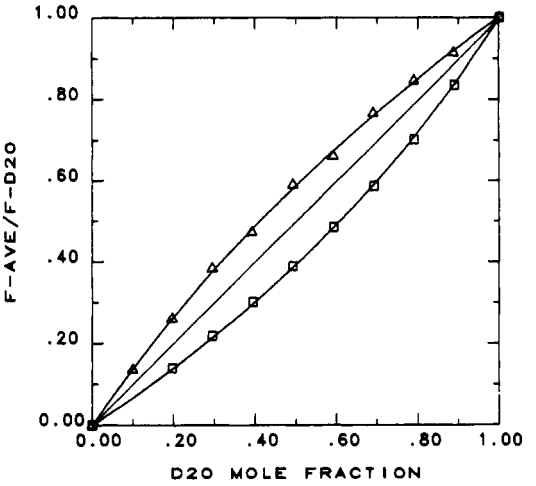

Figure 2. Triangle: pyrrolidine (K_{frac} greater than unity). Square: 2-mercaptoethanesulfonic acid sodium salt (K_{frac} less than unity). Straight line: theoretical K_{frac} of exactly unity.

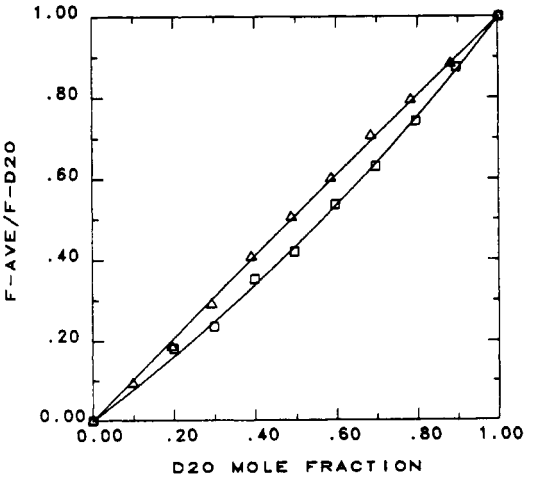

Figure 3. Triangle: methyl-α-D-glucopyranoside C-2 (K_{frac} of approximate unity). Square: sodium maleate (K_{frac} less than unity).

Results

In exploring the utility and generality of this new method to organic molecules, data were obtained for the wide variety of

systems—alcohols, amines, thiols, phenols, acids, and amides—described in Tables I and II. Strong internal hydrogen bonds (in competition to those in water) are implicated for certain cases, where abnormally low values are measured for K_{frac}, and are indicative of the preferred conformations in dilute aqueous solution.

In the cases examined thus far, certain secondary amines show the largest fractionation factors (in the range of 1.2 to 1.4) with deuterium favored on nitrogen. In Gold's study on the acid–base behavior of cationic indicators in H_2O–D_2O mixtures,[26] he evaluated the fractionation factors for the exchangeable hydrogen nuclei to be about 1.3 for *p*-nitroaniline and *p*-nitro-*N*-methylaniline. The fractionation factor reported here for diethylamine differs substantially from the value of unity reported by Fenby.[27] It should also be pointed out that Fenby's calculated equilibrium constant[16] (from enthalpies of reaction) for the deuterium exchange reaction with diethylamine at 298 K is 1.4—which supports our value nicely. Other secondary amines (diisopropylamine and 2,6-dimethylpiperidine) yielded values for K_{frac} only slightly greater than unity. The presence of methyl groups flanking the exchaning site appears to lower the value of K_{frac}, indicating a decrease in the preference for deuterium about the nitrogen atom of the amine. Asymmetric secondary amines (such as 2-methylpiperidine) provide two different α carbons that can each be used to monitor the fraction of H/D exchange. Although the value of K_{frac} measured with each α carbon can be significantly different, these systems display unusual sensitivity to pH. As the pH is increased, the measured values of K_{frac} for each α carbon approach each other (the value from the tertiary carbon increases while the value from the secondary carbon decreases) to within experimental uncertainty.

The lowest fractionation factors were observed for hydrogen attached to sulfur (as in 2-mercaptoethanesulfonic acid sodium salt) and for hydrogen involved in strong internal hydrogen bonding (as found in sodium maleate), where deuterium is in higher concentration in the surrounding solvent water. Preference for deuterium on oxygen rather than sulfur is in accord with the early findings of Small.[28] A fractionation constant considerably less than unity for an OH group is consistent with the presence of strong internal hydrogen bonding, as is expected for sodium maleate.[29] Our value of 0.77 for the K_{frac} of sodium maleate agrees well with the value of 0.84 reported by Kreevoy.[30]

Primary amines and alcohols are generally found to have fractionation factors of about 1.1, which are comparable to the values compiled by Miklukhin.[2] Direct comparison between K_{frac} values reported here and those existing in the literature for specific compounds is restricted to a few simple systems, mostly alcohols. Methanol has been reported[31] to have a fractionation factor of 1.09 which is good agreement with the value reported here of 1.06; the value of K_{frac} for ethanol has been cited as 1.11[5] and 1.05[32] which is consistent with the average value of 1.09 reported here; *tert*-butyl alcohol has been assigned[18] a fractionation factor of 1.1, and we have measured an average value that is slightly greater than unity; a fractionation factor of 1.08 has been reported for phenol, and our values of 1.13 is in good agreement; our average reported value of K_{frac} for acetic acid is 0.94 which is in good agreement with the value of 0.96 reported by Gold.[33] Succinic acid has been found[34] not to differ drastically from unity which is consistent with our results.

With regard to the limitations of this technique, one point seems clear within the confines of the measurements made in executing this experiment in its simplest form (as presented in the paper), K_{frac} can only be evaluated with confidence under conditions where the solute exists predominantly as a single species. That is, if in addition to the exchange reaction, a separate process (such as dissociation) is occuring to a large extent and can be perturbed by isotopic incorporation, the measured K_{frac} may not accurately represent the exchange reaction. It is in instances such as these when changes in pH, temperature, and concentration can result in substantial changes in the measured value of K_{frac}.

Conclusions

The major feature of this technique is that with a single experiment, the fractionation factors for *several* distinguishable exchangeable sites in a molecule can be simultaneously measured, directly in dilute aqueous solution. That is, to a first approximation, the average NMR frequency of each carbon (relative to its frequency when the attached exchangeable group is fully protonated or deuterated) allows measurement of the isotopic fractionation factor for the exchangeable site directly bonded to it (such as in 2,6-dimethylpiperazine, l-cysteine, and methyl-α-D-glucypyranoside).[35]

This new technique is not limited to ^{13}C NMR spectroscopy nor to the use of water as an exchanging solvent. What is required is a molecule with a rapidly exchanging proton near an observable nucleus. One can then set up an equilibrium with a pool of available protons/deuterons (such as found in water, water/Me_2SO, methanol, etc.). If the solute is sufficiently soluble in the solvent and the solvent has a carbon atom near the site of exchange, K_{frac} of the solvent can be determined (which is inversely proportional to K_{frac} of the solute). If the solvent and solute each have a carbon atom near their respectively site of exchange, the dual cell need not be used. In principle, measuring the CMR chemical shift differences between solute and solvent as a function of deuterium fraction can provide K_{frac}.

Depending on the system studied, this method can be employed with use of a wide variety of nuclei: ^{13}C, ^{31}P, ^{14}N, ^{17}O, ^{1}H, etc. Thus far ^{13}C NMR spectroscopy has proven to be most useful, but work on the use of other nuclei as probes for measuring fractionation factors is in progress.

Since hydrogen bonding produces substantial changes in the IR frequencies for stretching and bonding, it should also affect fractionation factors significantly as those are related to zero-point energies. The effect of hydrogen bonding on K_{frac} is seen (see Figure 3) when comparing the fractionation factors for phenol (K_{frac} greater than 1.1) and sodium salicylate (K_{frac} less than unity) or when comparing sodium maleate (K_{frac} of 0.77) with a typical alcohol or carboxylic acid, such as acetic acid or glutaric acid (which have an average K_{frac} of approximately unity). As hydrogen bond strength in compounds increases, relative to water, the fractionation factor for that particular site of exchange decreases.[36] Evidence for internal hydrogen bonding can then provide insight into the preferred three-dimensional conformation of the solute in aqueous solution. Although the difference in K_{frac} measured for the series of diacids under identical NMR conditions is small, it is interesting to note that malonic acid has the smallest value of K_{frac}, indicating the strongest hydrogen bonding (forming a six membered ring); oxalic acid and succinic acid are intermediate in their value of K_{frac} and therefore intermediate in hydrogen-bonding strength (formation of a five-membered ring and a seven-membered ring, respectively); glutaric acid shows the larges K_{frac}, that of a typical carboxylic acid, revealing that no detectable hydrogen bonding (formation of eight-membered ring) occurs in aqueous solution at these concentrations.

Therefore, obtaining measured fractionation factors for molecules containing several rapidly exchanging groups, using this new technique, may lead to the detection and evaluation of the

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importance of strong internal hydrogen bonds (if they occur) in aqueous solution. These should in turn provide direct experimental information closely connected to the preferred conformations of these molecules in solution.

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Registry No. H_2, 1333-74-0; methyl α-D-glucopyranoside, 97-30-3; sodium salicylate, 54-21-7; potassium phthalate, 29801-94-3; sodium maleate, 18016-19-8; diethylamine, 109-89-7; morpholine, 110-91-8; pyrrolidine, 123-75-1; 2,6-dimethylpiperazine, 108-49-6; 2-methylpiperidine, 109-05-7; L-cysteine, 52-90-4; sodium 2-mercaptoethanesulfonate, 19767-45-4; methanol, 67-56-1; ethanol, 64-17-5; isopropyl alcohol, 67-63-0; *tert*-butyl alcohol, 75-65-0; phenol, 108-95-2; 1,3-dihydroxyacetone, 96-26-4; 2-nitropropanol, 2902-96-7; 2-aminoethanol, 141-43-5; salicylaldehyde, 90-02-8; acetic acid, 64-19-7; maleic acid, 110-16-7; oxalic acid, 144-62-7; malonic acid, 141-82-2; succinic acid, 110-15-6; glutaric acid, 110-94-1; glutathione, 70-18-8; *N*-methylacetamide, 79-16-3; methylamine, 74-89-5; *n*-butylamine, 109-73-9; isopropylamine, 75-31-0; *sec*-butylamine, 13952-84-6; *tert*-butylamine, 75-64-9; dimethylamine, 124-40-3; diisopropylamine, 108-18-9; piperidine, 110-89-4; piperazine, 110-85-0; 2,6-dimethylpiperidine, 504-03-0; 2,5-dimethylpiperazine, 106-55-8.

A Study of L-Proline, Sarcosine, and the Cis/Trans Isomers of *N*-Acetyl-L-proline and *N*-Acetylsarcosine in Aqueous and Organic Solution by ^{17}O NMR[1]

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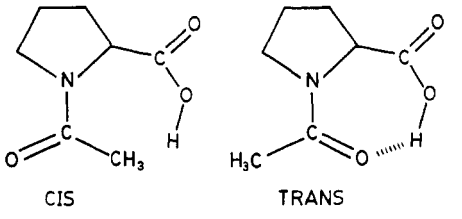
Contribution from the Institut de chimie organique de l'Universite, CH-1005 Lausanne, Switzerland. *Received August 10, 1984*

Abstract: ^{17}O NMR at 48.8 MHz has been used to study the cis/trans isomerism of *N*-acetyl-L-proline and *N*-acetylsarcosine in both aqueous and organic solution, and the results have been discussed in terms of possible hydrogen-bonded structures in the solvents examined. L-Proline, sarcosine, and their *N*-acetylated derivatives have been selectively enriched in ^{17}O either at the carboxyl or at the amide group and their spectra measured in aqueous solution throughout the whole pH range. Two resonances were observed for the carboxyl group of *N*-acetyl-L-proline, and these could be assigned to the cis and trans isomers due to differences in their signal intensity at low pH. The chemical shift difference of the two isomers was independent of the protonation state of the carboxyl group. This difference, as well as the difference in pK_a, is explained by an electric field effect of the amide group. The amide resonances of *cis*- and *trans*-*N*-acetyl-L-proline were poorly resolved at 8.4 T, and those of *N*-acetylsarcosine were inseparable. A low-frequency shift was observed for the amide resonances on deprotonation of the remote carboxyl group. The appearance of the carboxyl and amide resonances of the two isomers of *N*-acetyl-L-proline in methanol was similar to that of water. In contrast, using the solvents acetone and chloroform a large separation of the amide resonances was observed accompanied by a coalescence of the carboxyl resonances. The chemical shift of the cis amide resonance of *N*-acetyl-L-proline was found to be concentration dependent in acetone. This along with the chemical shift difference of the cis and trans amide resonances at low concentration (17.5 ppm) is discussed in terms of a γ-turn structure in the trans isomer. For *N*-acetylsarcosine the chemical shift difference was only 6.4 ppm, indicating a lower γ-turn probability. In chloroform, the chemical shifts and line widths of both the amide and carboxyl oxygens of *N*-acetyl-L-proline and the cis/trans isomer ratio are concentration dependent, indicating an increase in aggregation of the system with concentration. The chemical shift difference of the cis and trans amide resonances at dilute concentrations (22 ppm) proves the high tendency of formation of the γ-turn structure in this solvent.

Numerous studies have been reported on the hindered internal rotation of amide and peptide bonds, and a variety of spectroscopic techniques have been applied.[2] The presence of a cyclic side chain and/or alkyl substitution of the peptide bond as in proline and sarcosine is of particular interest because of the resulting increase in population of the cis isomer about the X-Pro bond and the restriction in conformational freedom.[3-5]

The compounds *N*-acetyl-L-proline (AcProOH) and *N*-acetyl-L-proline *N'*-methylamide (AcProNHMe) have been extensively studied as models for secondary structure in proline-containing peptides. The assignment of their cis and trans isomers was originally done by ^1H NMR,[6] and it was indicated that the trans isomer may exist as an intramolecularly hydrogen bonded

Scheme I



C_7 conformer (γ-turn structure, Scheme I) in nonpolar solvents, this model being subsequently extended to the situation in aqueous solution at low pH.[7]

The complexity of the ^1H NMR spectra of proline derivatives led to an early application of ^{13}C NMR;[8] however, the possible formation of an intramolecular hydrogen bond was not considered. The pH titration curves for the ^{13}C NMR resonances of AcProOH were interpreted[9] as supporting a γ-turn structure for the trans

(1) Presented in part at the 6th International Meeting on NMR Spectroscopy, Edinburgh, Scotland, July 1983.
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