Heteronuclear Two-Dimensional NMR as a Conformational Probe of Cellular Phosphates

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Abstract: Heteronuclear two-dimensional NMR has been used for the correlation of the proton and phosphorus chemical shifts of cellular phosphates. Experimental spectra are in good agreement with theoretical predictions based on a novel approach using subspectral analysis. The spectra are sensitive to the homonuclear scalar couplings between the protons as well as the heteronuclear proton–phosphorus coupling. Thus, the two-dimensional spectra provide information about the conformation of cellular phosphates. Since proton signals are only observed from those protons located in the vicinity of the phosphate groups, the signals are referred to as "local" proton spectra.

Cellular phosphates have been extensively studied by both proton and phosphorus NMR to investigate the conformations, concentrations, and metal binding properties of these molecules. High-resolution ^1H NMR has been utilized to study the scalar (spin–spin) couplings of the protons which have provided valuable conformational information.[1] These studies have usually been limited to pure solutions of cellular phosphates in deuterated solvents since resonances from other molecules, such as an enzyme or a solvent, obscure the proton resonances of interest. The concentrations of cellular phosphates present in mixtures, for example, in acid extracts of cells, can be measured by phosphorus NMR, since the resonances are sufficiently resolved.[2] Thus, it has been possible to study enzyme reaction kinetics in vitro as well as metabolic processes in vivo. The assignment of the phosphorus resonances of complicated mixtures is not always straightforward because of the environmental dependence of the phosphorus chemical shifts.[3] A method of labeling the phosphorus resonances by some characteristic feature in addition to their chemical shift would facilitate the assignments.

An elegant novel technique for the correlation of the chemical shifts of different nuclei has recently been proposed by Ernst and co-workers.[4,5] This technique, which is known as heteronuclear two-dimensional NMR, has been applied to the correlation of proton and carbon-13 spectra.[4-8] The chemical shifts of the different nuclei are depicted in a two-dimensional "map" in which each signal has two independent frequency coordinates. One dimension reflects the chemical shifts of the protons, while the other is reserved for the spectrum of the "other" nucleus.

The investigation of cellular phosphates by this method is particularly attractive. Since phosphorus is a relatively rare nucleus in biological systems, the two-dimensional map will not normally be obscured by extensively overlapping signals. In the proton dimension, only those protons which are coupled to phosphorus will give rise to signals. These resonances offer some insight into the conformation, since they are sensitive not only to heteronuclear but also to proton–proton couplings. The two-dimensional method thus provides simultaneous information about both concentrations and conformations, while the correlation between the proton and phosphorus shifts is useful in assigning both signals.

It is the purpose of this paper to review some of the basic principles of heteronuclear two-dimensional NMR spectroscopy and to show how this method can be applied to cellular phosphates. The analysis is extended to strongly coupled spin systems which commonly occur in these compounds.

Basic Principles

The experiment consists of two rf pulses applied at the proton frequency, separated by an evolution period t_1, and followed by a normal phosphorus observation pulse with subsequent acquisition of the free induction decay, as shown schematically in Figure 1a. This signal is subjected to a Fourier transformation and yields a phosphorus spectrum with the usual frequencies. The amplitudes, however, depend in a periodic manner on the duration of the evolution period. The mechanism which gives rise to the modulation of the amplitudes may be discussed by considering a simple AX system, consisting of a single proton coupled to a single phosphorus nucleus. The energy levels and transitions of this system are depicted in Figure 1b. There are two proton lines which belong to the polarizations α and β of the phosphorus nucleus. In the language of spectral analysis, each line may thus be considered as an elementary "subspectrum". This concept is also applicable to complex spin systems and is helpful in that the two proton pulses act independently on the two subspectra. The first proton pulse generates two transverse magnetization vectors which precess at ν_{12} and ν_{34}, the difference of the frequencies being equal to J_{PH}. As the evolution period increases, the magnetization vectors acquire different phase angles. Some typical situations are represented in Figure 2a. The second proton pulse causes all magnetization vectors to rotate through an angle of 90°. Depending on the free precession angle, the magnetization may be rotated back to its equilibrium position (for example, M_{12} at t_1 = 25 ms or M_{34} or 50 ms in Figure 2b) or experience an inversion with respect to the equilibrium position (M_{12} at t_1 = 50 ms) which is reminiscent of an inversion–recovery experiment. The resulting z components of the magnetization vectors correspond to populations that differ from those found at equilibrium. The populations of the energy levels for different durations of the t_1 evolution period are shown in Figure 2c. A more detailed analysis of the effect of the proton pulses shows that the modulation of the populations exhibits a sinusoidal dependence on t_1. This modulation is referred to as "population stirring" by Freeman.[6] The amplitudes of the phosphorus lines which are observed experimentally are proportional to the population differences, as indicated by Figure 2d.[9]

Close inspection of the mechanism reveals an important feature. Consider the phosphorus transition between levels 1 and 3. The intensity of this line is enhanced when the population in level 1 is decreased, or when the population in level 3 is increased. Therefore, the inversion of the proton magnetization M_{34} enhances the (1,3) line, whereas the inversion of the M_{12} vector has the opposite effect. This behavior is re-

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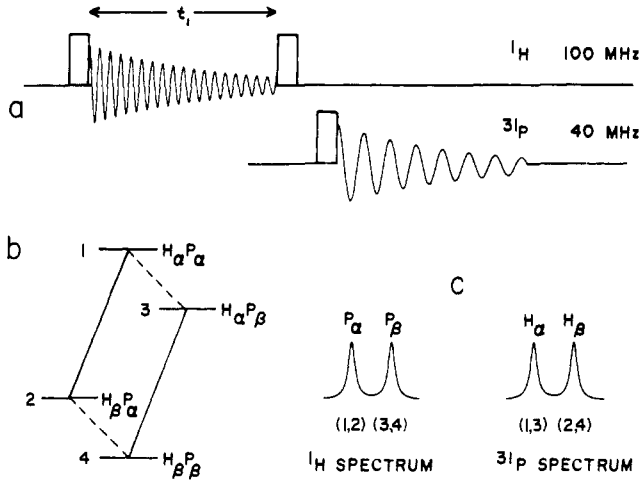

Figure 1. (a) The pulse sequence used for heteronuclear two-dimensional spectroscopy: two 90° proton pulses, separated by the evolution period t_1, are followed by a normal 90° phosphorus pulse. The energy levels (b) and spectra (c) for the AX system, consisting of one proton coupled to one phosphorus nucleus, are schematically depicted. The proton subspectra for P_α and P_β are affected independently by the proton pulses.

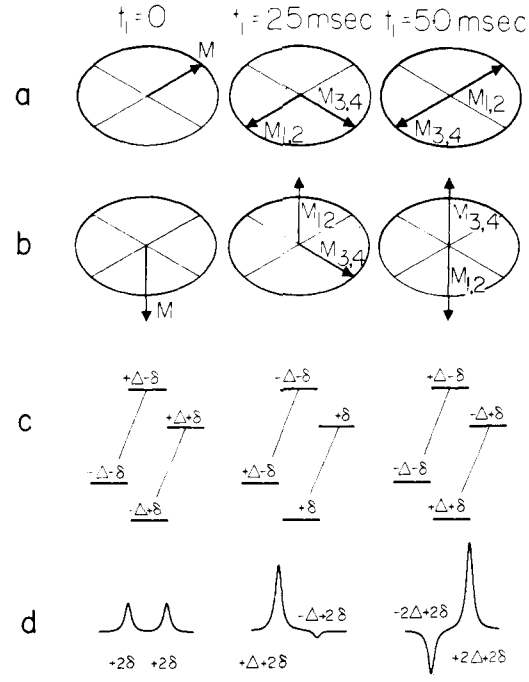

Figure 2. (a) The transverse proton magnetization, assumed here to consist of two vectors precessing at 10 and 20 Hz with respect to the frequency of the proton transmitter, evolves freely until the second proton pulse, which generates nonequilibrium longitudinal magnetization (b) and corresponding nonequilibrium populations of the energy levels (c) that affect the amplitudes of the phosphorus transitions (d). By convention the population difference is 2Δ across the proton transitions and 2δ across the phosphorus transitions in thermal equilibrium.

flected in the two-dimensional spectrum by the appearance of lines at ν_{34} and ν_{12} with opposite algebraic signs. In general, the modulations originating from the proton subspectra corresponding to the α and β polarizations of the phosphorus nucleus are 180° out of phase with each other.

The pulse sequence and the first Fourier transformation are repeated for some 300 regular increments of the evolution period. The modulation of the phosphorus signal is then analyzed by a second Fourier transformation with respect to t_1. The new frequency dimension F_1 reflects the precession of the proton magnetization during the evolution period. The F_1 frequencies are identical with those of an ordinary proton free induction decay.

Thus, the principle is to alter the populations of the energy levels in order to transfer information about the proton resonances to the phosphorus spectrum. We note that the proton and phosphorus transitions must share a common energy level, a condition which is only fulfilled by a small number of protons.

Pyridoxal Phosphate: A Simple Example

Pyridoxal phosphate, also known as codecarboxylase, is a precursor of vitamin B_6 and is involved in several cellular processes. The phosphorus nucleus is coupled to two equivalent methylene protons, which are not significantly coupled to any other nuclei. Many features of the AX system can be found in this A_2X spin system. The phosphorus spectrum of pyridoxal phosphate is shown in Figure 3 for regular increments of the evolution period t_1. The outer lines of the phosphorus triplet show modulation patterns with opposite phases, as expected from the discussion of the AX system. The two degenerate central components of the triplet do not exhibit any modulation. This feature can be readily explained by extending the "population stirring" analysis to the energy levels of the A_2X system.

The data in Figure 3 may be thought of as a matrix $S(t_1,F_2)$ which can be transposed and then subjected to a second Fourier transformation, which substitutes the new frequency dimension F_1 for the time variable t_1.[11] The result is the two-dimensional spectrum shown in Figure 4c in absolute value display, which obscures the phase properties for the benefit of clarity. The two traces with the largest signals in the F_1 dimension are shown separately in phase-sensitive mode. Both feature a proton doublet with one peak pointing up and the other down, forming

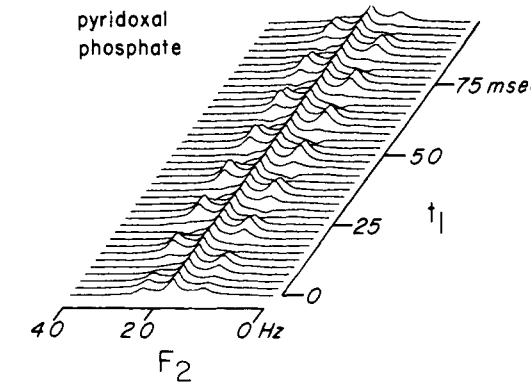

Figure 3. Phosphorus spectra of the A_2X group of pyridoxal phosphate, obtained at regular increments of the evolution period t_1. The central component remains unaffected by the proton pulses while the outer lines experience modulations with opposite phases. The actual experiment extends over 300 t_1 values.

a pattern reminiscent of a dispersion signal. However, the phase correction applied in the F_1 dimension is known to produce absorption mode lines from earlier experiments on a well-resolved doublet. The signals in Figure 4 are centered about the chemical shift of the methylene protons. We note that all traces show a signal at the frequency origin of the F_1 dimension, which corresponds to the fraction of the population that is not affected by the population stirring of the proton pulses.

Strongly Coupled Spin Systems

The two-dimensional spectra of nucleotides are complicated by extensive scalar coupling between the protons. Successful interpretation of the spectra requires the ability to calculate both line positions and intensities from trial values of the chemical shifts and coupling constants. Ultimately, an iterative

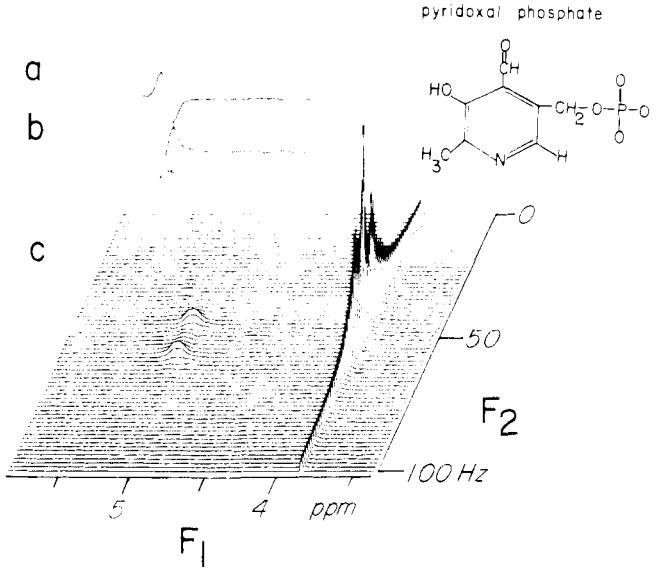

Figure 4. Two-dimensional spectrum of pyridoxal phosphate obtained by transposition and Fourier transformation of the data shown in part in Figure 3. The signals in (a) and (b) appear in phase-sensitive mode while (c) is shown in absolute-value display.

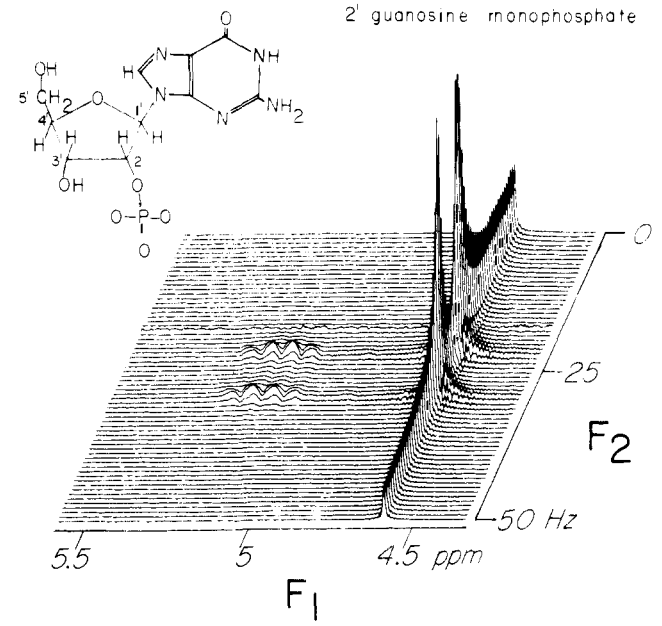

Figure 5. Two-dimensional spectrum of 2′-guanosine monophosphate (2′GMP). The absolute-value display obscures the fact that the two groups of proton signals around 5.1 ppm are in opposite phase.

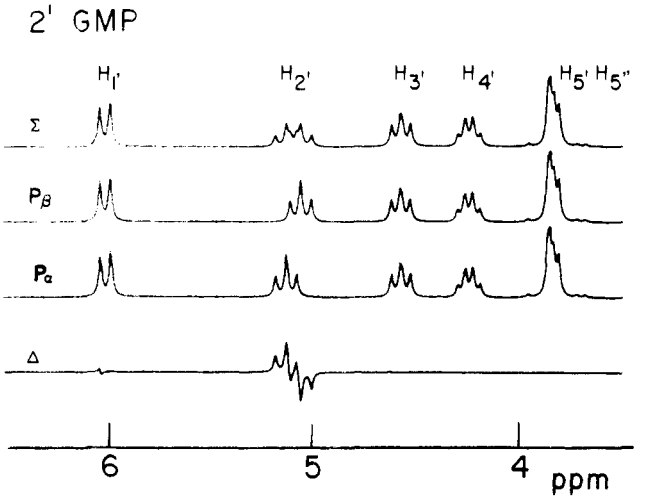

Figure 6. Simulated proton spectra of 2′GMP at 100 MHz, with line widths of 2.5 Hz. The complete proton spectrum (top) may be decomposed into two subspectra (middle); the local spectrum (bottom) is obtained by subtracting P_β from P_α.

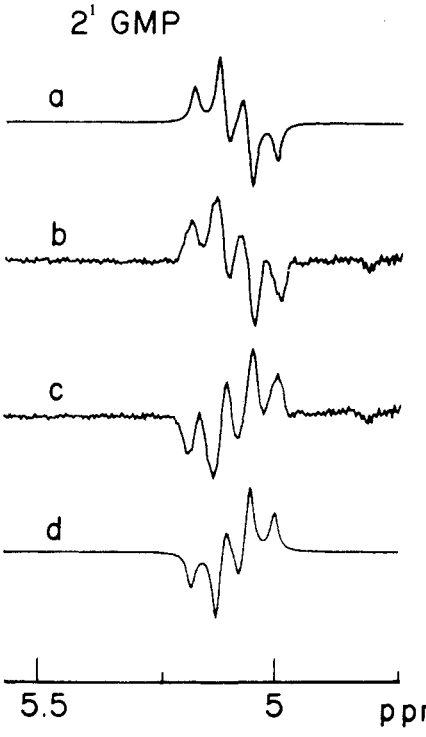

Figure 7. The experimental local spectra of 2′GMP (b and c) extracted from the two-dimensional spectrum presented in Figure 5. The theoretical spectra are reproduced from Figure 6 for comparison. The signal to noise ratio may be improved by using the difference of (b) and (c).

analysis in the manner of the LAOCOON program[13] should be feasible.

For noncyclic nucleotide monophosphates, the P-O-C-H coupling is normally the only heteronuclear coupling observed.[14] Strong coupling between the protons broadens the lines of the phosphorus doublet, though the fine structure is not resolved.

The proton subspectra of the α and β polarizations of the phosphorus differ only in the effective chemical shift of the proton coupled to the phosphorus: $\delta_{eff} = \delta_0 \pm {}^1/_2 J_{PH}$. Each subspectrum is calculated separately with a six-spin spectral simulation program. The ordinary proton spectrum is the sum of the two subspectra, whereas the lines observed in the F_1 dimension of the two-dimensional spectrum correspond to the *difference* of the subspectra.

The two-dimensional spectrum of 2′-guanosine monophosphate (2′-GMP) provides a test of this theory. The full spectrum is shown in absolute value mode in Figure 5. The phosphorus doublet appears along the F_2 axis, while the proton signals extend into the F_1 dimension.

The simulated subspectra shown in Figure 6 differ in the effective chemical shifts of the 2′ proton (5.054 and 5.126 ppm, since J_{PH} = 7.2 Hz or 0.072 ppm at 100 MHz, and the chemical shift of the 2′ proton is 5.09 ppm[15]). The sum of the subspectra is equivalent to the ordinary proton spectrum. The difference spectrum in Figure 6 clearly shows that only the 2′ proton gives rise to a signal. This leads us to refer to the difference spectrum as a *local* spectrum since it only reflects the resonance of the proton nearest to the phosphate group. The simulated local spectra are shown in Figure 7 for comparison

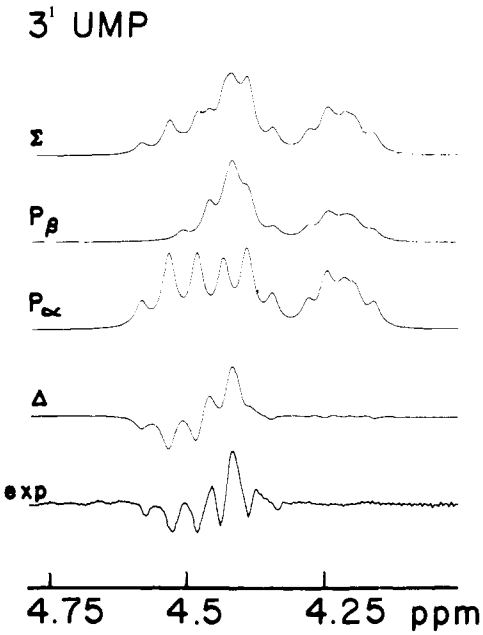

Figure 8. Theoretical proton spectra of 3'UMP at 100 MHz with 2.5-Hz line widths. The normal spectrum Σ is equivalent to the sum of the subspectra P_β and P_α whereas the difference Δ may be compared with the experimental local spectrum (bottom).

with the experimental spectra. It is noted that neither the coupling constants of the theoretical spectrum nor the phase of the experimental spectrum was adjusted to obtain agreement.[16]

The two-dimensional spectrum of 3'-uridine monophosphate (3'UMP) provides a more stringent test for the subspectral analysis, because the coupling among the protons is significantly stronger. The experimental, phase-sensitive local spectrum of 3'UMP is shown in Figure 8 (bottom). The simulated spectrum Δ is in good agreement, although the shape of the multiplet is rather sensitive to small variations of the line width.

The local spectra of 2'GMP feature a remarkable inversion symmetry which is an indication of the relatively weak coupling between the protons. The two subspectra in Figure 6 show responses of the 2' protons that are merely displaced in frequency but retain the same multiplet pattern. In the case of 3'UMP, however, the subspectra P_β and P_α exhibit typical second-order intensity effects.[17] The multiplets of the 3' proton are quite different in the two subspectra; as a result, the difference spectrum Δ has an asymmetric pattern (Figure 8).

Application of Local Proton NMR to Conformational Studies

Two-dimensional spectroscopy may prove to be a powerful method for the study of the conformations of nucleotides and other cellular phosphates. In order to assess the potential of this approach we have simulated local spectra for some typical nucleotide conformations which are shown in Figure 9. The simulations are based on theoretical values for the coupling constants of $J_{1',2'}$, $J_{2',3'}$, and $J_{3',4'}$ given by Cheng and Sarma[18] and chemical shifts from the work of Davies and Danyluk.[15] The local spectra of both 2'- and 3'-phosphates show a pronounced dependence on the conformation. It should be possible to determine the coupling constants from experimental local spectra by iterative analysis.[19] This approach appears promising because of the inherent simplicity of the two-dimensional spectra, which are not encumbered by signals from the solvent or from the majority of biomolecules, as evidenced by the phophorus spectra of acid extracts of cells.

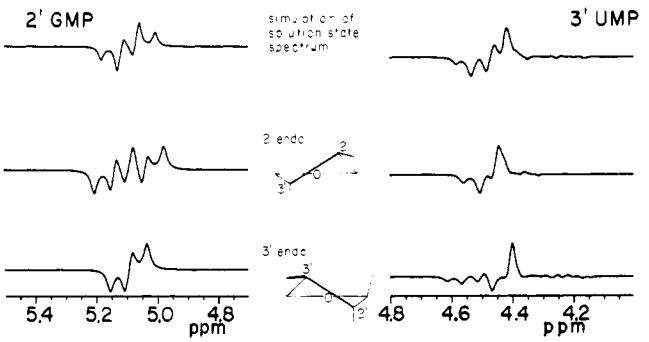

Figure 9. Simulated local spectra of 2'GMP and 3'UMP derived from published data for three typical conformations.[15,18] In going from the 2'-endo to the 3'-endo conformation, $J_{1',2'}$ changes from 10.1 to 0.1 Hz, $J_{2',3'}$ from 5.5 to 4.9 Hz, and $J_{3',4'}$ from 0.2 to 10.3 Hz. The solution-state spectra (top) have been observed experimentally (see Figures 7 and 8).

Experimental Section

All samples were obtained from P-L Biochemicals and passed through a Bio-Rad Chelex 100 column to remove traces of paramagnetic metals. The samples were then lyophilized and dissolved in 15 mM ethylenediaminetetraacetic acid, lyophilized again, and finally dissolved in approximately 1:1 H_2O-^2H_2O. The concentrations were typically 1-2 M with a pH between 7 and 8 as determined by direct reading of a Beckman 3500 pH meter with a Markson 668 electrode. The spectra were obtained using a 2.34 T JEOL spectrometer with a 10-mm ^{31}P probe operating at 40 MHz. The proton pulses were generated by gating the 100-MHz coherent decoupler for intervals of 45 μs, corresponding to 90° flip angles for the protons. All pulses were controlled by a Nicolet 293A pulse programmer. The data were processed with a Nicolet 1180 computer with associated software for two-dimensional Fourier transformation.

The proton decoupled phosphorus line widths were typically 1 Hz, limited by the imperfect homogeneity of the magnetic field. This corresponds to 2.5 Hz for the proton line widths in the local spectra since $\gamma_{1H}/\gamma_{31P} \simeq 2.5$. The two-dimensional spectrum of pyridoxal phosphate was obtained in 30 min and the 2'GMP spectrum in 6 h.

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(12) Homonuclear coupling between the phosphorus nuclei in di- and triphosphates is not considered here.
(13) A. A. Bothner-By and S. M. Castellano in "Computer Programs for Chemistry", Vol. I, D. F. DeTar, Ed., W. A. Benjamin, New York, N.Y., 1968.
(14) For cyclic phosphates the phosphorus spectrum consists of a doublet of doublets. The analysis presented here is applicable to the proton modulation of the outer lines, but the algebraic signs associated with the inner lines obey more complex rules.
(15) D. B. Davies and S. S. Danyluk, *Biochemistry*, **14**, 543 (1975).
(16) The phase correction in the F_1 dimension has been calibrated to obtain absorption mode lines for trimethyl phosphate, which features a well-resolved doublet. This phase correction can be applied to all spectra provided that the initial value of the evolution period is not changed.
(17) In the presence of strong coupling, the intensities are proportional to the square of the F_x matrix elements of the proton transitions, as in continuous

wave or ordinary Fourier transform spectroscopy. This can be shown by analyzing the effect of the two proton pulses on the ABX system in terms of the Ernst formalism.[4,5]

(18) See Table V in D. M. Cheng and R. H. Sarma, *J.Am. Chem. Soc.*, **99**, 7333 (1977).

(19) Some modifications to the existing spin simulation programs would be required. For example, the nucleotides could be treated as seven-spin systems (one phosphorus and six protons) with the convention that all states with P_α are even numbered and those with P_β odd. All proton transitions between odd-numbered states could then be made negative. The iterative analysis should allow for the variation of only those coupling constants to which the local spectrum is sensitive.

Heat of Formation of the 2-Norbornyl Cation in the Gas Phase

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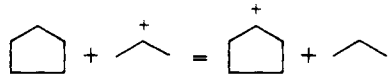
Abstract: Proton transfer equilibria BH^+ + 2-norbornene = B + 2-norbornyl$^+$ and their temperature dependence were measured with a high-pressure mass spectrometer. The entropy change obtained from a van't Hoff plot of the equilibrium constant indicates that there is no rearrangement on protonation, i.e., that the product from the reaction is the 2-norbornyl cation. The enthalpy change, when connected to a proton affinity ladder, leads to a heat of formation of the norbornyl cation which is in very good agreement with the earlier results by Field and by Beauchamp. Compared to other secondary cyclic ions like cyclopentyl, norbornyl is unusually stable.

Introduction

Recently Solomon and Field[1] published several measurements of hydride ion transfer equilibria 1 measured with a pulsed high-pressure mass spectrometer. van't Hoff plots of K_1 lead to ΔH_1 and ΔS_1. In particular these authors deduced the energy changes for reactions 2, 3, and 4 (see Table I) which

$$R_1H + R_2^+ = R_1^+ + R_2H \quad (1)$$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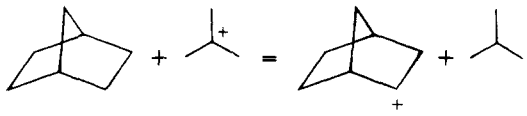
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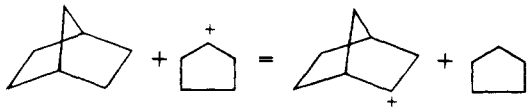
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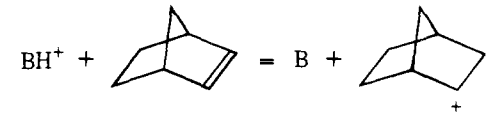
relate to the 2-norbornyl cation. The enthalpies for reactions 2 and 3 were obtained from van't Hoff plots of K_2 and K_3 while ΔH_4 was deduced from ΔH_2 and ΔH_3 and available thermochemical data. Since in mass spectrometric measurements only the mass, but not the structure, of the ion is identified, the assumption was made that the ionic product in reaction 3 is the most stable ion resulting from hydride abstraction without skeletal rearrangement, i.e., the 2-norbornyl cation. Reaction 4 shows that the norbornyl cation, which is nominally a secondary ion, is 11.4 kcal/mol more stable than the cyclopentyl cation.

Because of the importance of Solomon and Field's[1] results it is clearly desirable to obtain additional experimental data for the norbornyl cation. A second gas-phase route for production of the ion is the proton transfer reaction 5. This method was used in an early ion cyclotron resonance study by Kaplan,[2] which preceded Field's[1] work, and in a recent brief report by Beauchamp.[3] Kaplan's results[2] were not based on ion equilibria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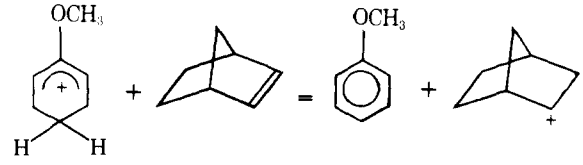
measurements but on the inferior[4] kinetic bracketing technique, which examines the direction in which a proton transfer reaction like (5) proceeds with different standard bases B. The proton affinities of B used by Kaplan were early values which were subsequently revised.

Beauchamp's[3] brief study gives results for equilibrium 5 with B = diethyl ether at room temperature. The known proton affinity of the ether with assumption of a small $\Delta S°_5$ leads[3] to a heat of formation of the 2-norbornyl cation which is in close agreement with Field's result.

The present work describes one more study of proton transfer equilibria 5. However, since the temperature dependence of K_5 was determined $\Delta S°_5$ could be evaluated. Knowledge of the magnitude of ΔS_5 is useful since a small entropy change can be taken as evidence that no extensive skeletal rearrangement has occurred, i.e., that the likely product of (5) is the 2-norbornyl cation.

Results and Discussion

The temperature dependence of the equilibrium constant K_6 for proton transfer reaction 6 (see Table I) was determined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in a number of runs with a pulsed electron beam high-pressure mass spectrometer (see Experimental Section). A van't Hoff plot of K_6 is shown in Figure 1. The invariance of K_6 with changing ratio of anisole and norbornene and total ion source pressure was tested at every temperature. Some of these results are also shown in Figure 1. The energy and entropy changes for reaction 6 are given in Table I. Anisole was selected as the

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