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Selective Observation of Phosphate Ester Protons by $^1H\{^{31}P\}$ Spin-Echo Difference Spectroscopy

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Several pulse sequences have been described which enable the selective observation of signals of protons attached to ^{13}C and ^{15}N nuclei (*1–4*). In earlier work, we have applied one such sequence, namely $^1H\{^{13}C\}$ spin-echo difference (SED) spectroscopy, to the observation of ^1H resonances attached to enriched ^{13}C atoms (*5*). In each case the main motivation for the application of such sequences is the limited NMR sensitivity of ^{13}C (3.1×10^{-2} compared to ^1H as unity) and ^{15}N (3.2×10^{-3}). Thus, taking advantage of the large ^{13}C–^1H and ^{15}N–^1H scalar couplings provides spectral information about the compound containing the heteronucleus, but at ^1H sensitivity.

We now wish to describe the analogous $^1H\{^{31}P\}$ SED method. This is not as strongly justified from the point of view of sensitivity as the heteronuclei mentioned above because ^{31}P NMR is much more sensitive (0.10) than ^{13}C and ^{15}N compared to ^1H. Also, while both these nuclei may be enriched far above their natural-abundance levels (^{13}C, 1.1%; ^{15}N, 0.37%), ^{31}P is present as 100% natural abundance. However, there are additional reasons beyond that of sensitivity, to justify testing the $^1H\{^{31}P\}$ method. Phosphates are important in biological systems and ^{31}P NMR is now widely used to investigate the metabolic states of cells and organisms (6–8). But, while ATP and inorganic phosphate (P_i) give well resolved ^{31}P signals, the sugar phosphate monoesters, such as glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), or glucose 1,6-diphosphate (G16P) all absorb in the same region of the ^{31}P spectrum, and are not adequately resolved. Hence it has not been generally possible to follow these important metabolites independently. In several cases the proton resonances of these metabolites are well resolved. Many metabolic processes occur rapidly, so that any means that can be found to gain sensitivity in this area should be exploited. Thus, for both reasons of resolution and sensitivity the $^1H\{^{31}P\}$ SED method deserves consideration.

A simple phosphate on which the $^1H\{^{31}P\}$ SED pulse sequence can be demonstrated is trimethyl phosphate (Fig. 1). The pulse sequence used was

Proton: $\pi/2(x)$–τ–d–$\pi(y)$–d–τ–AQ

Phosphorus: $\pi/2$ $\pi/2$ (on/off)

which is analogous to the previous ^{13}C SED sequence (*5*), but with several changes. The sequence in the present work was altered by applying the ^{31}P π pulse as two $\pi/2$ pulses during symmetrical delays (d) before and after the ^1H π pulse, to maintain

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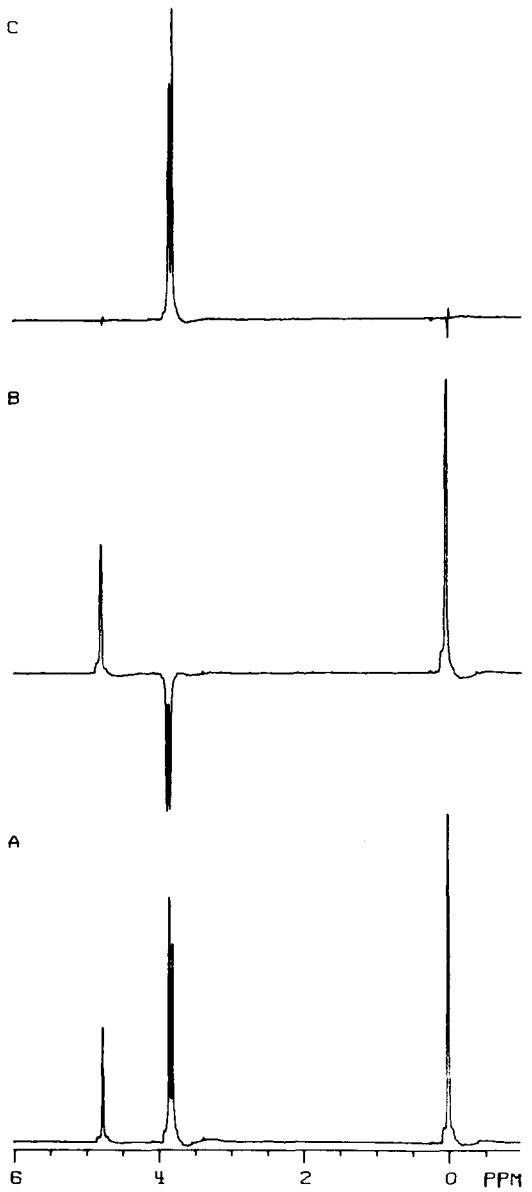

FIG. 1. ^1H NMR spectra (270 MHz) of trimethyl phosphate (5%) in HDO containing 0.075% TSP. These experiments were carried out using the ^1H decoupler coil for ^1H observation, and the ^{31}P pulse supplied through the decoupler channel into the tuned ^{31}P coil. Five millimeter sample tubes were used and the ^1H $\pi/2$ pulse length was 43 μs. Thirty-two scans were usually collected. (A) One-pulse spectrum. (B) Spin-echo spectrum in which the ^{31}P frequency (109.3 MHz) was subjected to a simultaneous π pulse (38 μs; d = 19 μs). (C) $^1H\{^{31}P\}$ SED spectrum, showing the suppression of the HDO and TSP signals. The delay τ was set to 47 ms for optimum cancellation, the theoretical value from the ^{31}P–^1H coupling constant of 11.1 Hz is 45 ms.

correct phasing. The interval τ between the ^1H pulses was much longer than that for the comparable ^{13}C experiment, because the 3-bond coupling constant $^3J_{POCH}$ is much smaller than the direct ^{13}C–^1H bond coupling, and this interval for maximal inversion is equivalent to $1/2J$ (Fig. 1B). In the $^1H\{^{31}P\}$ SED difference spectrum, it is clear that the POCH signal is greatly increased relative to those of HDO and TSP in this sample. The measured signal-to-noise enhancement for observation of the trimethyl phosphate protons by the $^1H\{^{31}P\}$ method versus direct $^{31}P\{^1H\}$ observation was a factor of 17. In principle, similar results could be obtained by ^{31}P decoupling difference spectroscopy, but since the net overall intensity in such a difference spectrum is always zero, this method is unsatisfactory for the poorly resolved spectra often encountered in biological systems.

Two difficulties arise in adapting the $^1H\{X\}$ SED method to ^{31}P, namely the values of $^3J_{POCH}$ are small and they are quite variable, in the range of 3–12 Hz. While in principle, the value of J could be measured directly both from the ^1H and ^{31}P spectra, in practice this was not always readily possible. An extra complication, not found with the ^{13}C and ^{15}N cases, occurs because the magnitude of $^3J_{PH}$ is comparable to the $^3J_{HH}$ of the homonuclear proton couplings. This introduces phase modulation (*9–11*) of the proton resonances during the time 2τ, since the π proton pulse does not refocus the dephasing due to homonuclear coupling. This effect will be briefly analyzed for the simple case of an AMX spin system, where A and M are mutually coupled protons and X is the ^{31}P nucleus, which is coupled to proton A.

The positions of the A-spin magnetization vectors in the transverse plane at the beginning of acquisition are sketched in Fig. 2A for the case of no π ^{31}P pulse, and in Fig. 2B for the case where a π ^{31}P pulse was applied at time τ after the initial proton pulse. For reasons of clarity in the diagram the homonuclear coupling constant, J_{AM}, is assumed to be much larger than J_{AX}. The difference in transverse magnetization, for example, for the lowest field A-spin multiplet component in the two experiments is then:

$$M_{\alpha A} - M'_{\alpha A} = M_0[\exp(2\pi i J_{AM}\tau) - \exp\{2\pi i(J_{AM} + J_{AX})\tau\}]. \quad [1]$$

For $\tau = 1/2J_{AX}$ the signals obtained in the two experiments are in antiphase relative to each other, and the difference spectra will yield only resonances from protons

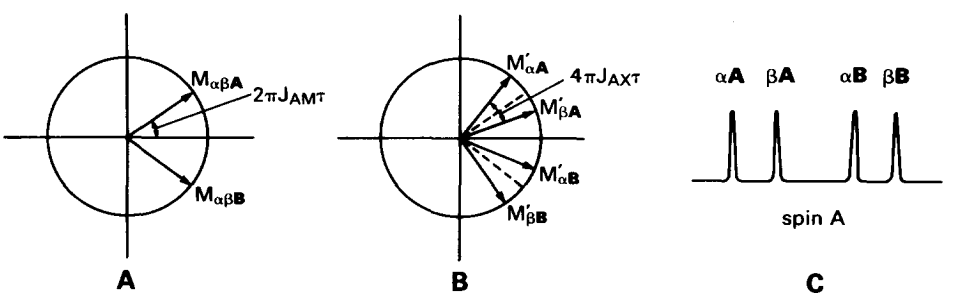

FIG. 2. Positions of magnetization vectors of spin A in the transverse plane at the center of the spin echo (time 2τ after the $\pi/2$ ^1H pulse). (A) With no ^{31}P pulse applied. (B) With a ^{31}P π pulse applied at time τ. The magnetization vector, **M**, of proton A, has indices corresponding to the spin state of the coupled ^{31}P (α and β) and of the coupled proton (**A** and **B**, for the $m = 1/2$ and $m = -1/2$ states, respectively). The four corresponding A-spin resonance lines are shown schematically in (C).

coupled to ^{31}P. However, it can be seen from Eq. [1] that the phase of the magnetization in the difference spectrum equals $2\pi J_{AM}\tau$. In general, all proton multiplet components will have different phases and cannot be phased to the pure absorption mode. This is illustrated in the next three figures. In Fig. 3, the pulse sequence has been applied

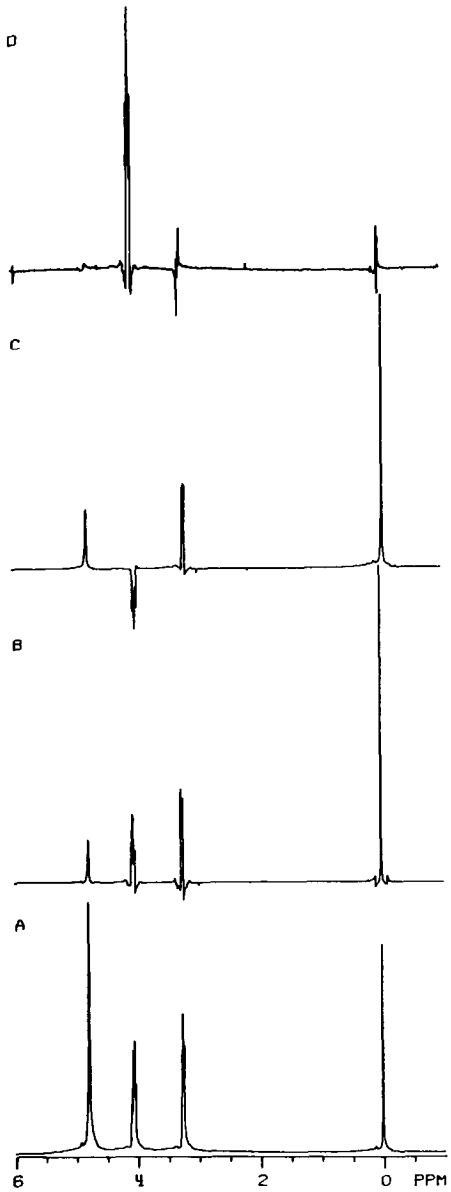

FIG. 3. ^1H NMR spectra of phosphoethanolamine (0.1 *M*) in HDO containing 0.075% TSP. (A) One-pulse spectrum. (B) ^1H spin-echo spectrum with conditions as in Fig. 1, except the value of τ was chosen to be optimal (86 ms), while the theoretical value from the coupling contant (6.1 Hz) is 83 ms. The value of $^3J_{HH}$ is 4.8 Hz. (C) Same as in (B) except that the ^{31}P frequency was pulsed. (D) $^1H\{^{31}P\}$ SED spectrum.

to phosphoethanolamine, containing two methylenes. In this case the spectrum derived from the application of the pulse sequence without the ^{31}P pulse is given (Fig. 3B), as well as that showing the inverted proton signal derived from the methylene adjacent to the phosphorus atom. These were the best results that could be obtained in varying τ, and it is clear that there is some phase distortion of the final difference spectrum (Fig. 3D) which derives from the homonuclear coupling effect. There is also some residual signal from the other methylene protons, presumably resulting from small four-bond POCCH coupling.

Further complications are revealed in the case of fructose 6-phosphate (Fig. 4). While the $^1H\{^{31}P\}$ SED spectrum (Fig. 4B) clearly shows the enhancement of the ester protons adjacent to phosphate at the expense of all the other proton signals, nevertheless this is a complex pattern consisting of the overlap of two doublets of the nonequivalent methylene protons, each modulated by the spin echo sequence

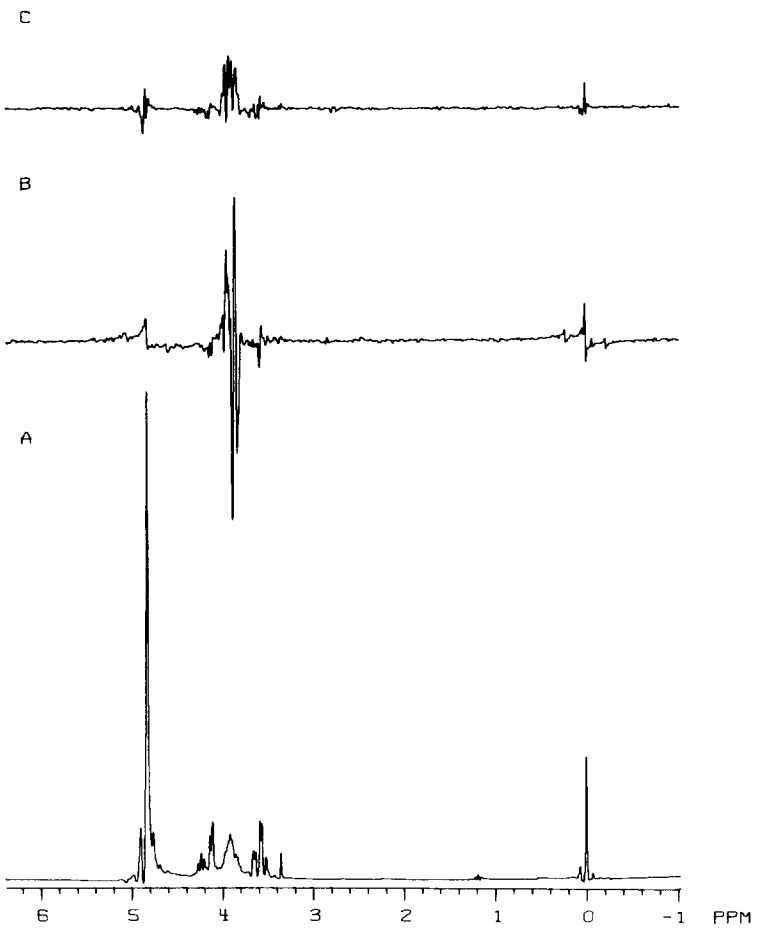

FIG. 4. ^1H NMR spectra of fructose 6-phosphate (0.4 *M*) in HDO with 0.075% TSP. (A) One-pulse spectrum. (B) $^1H\{^{31}P\}$ SED spectrum with τ set to the optimal value of 55 ms (this is less than the theoretical value from the expected coupling constant of ca. 3 Hz). (C) as in (B), but with τ set to 10 ms.

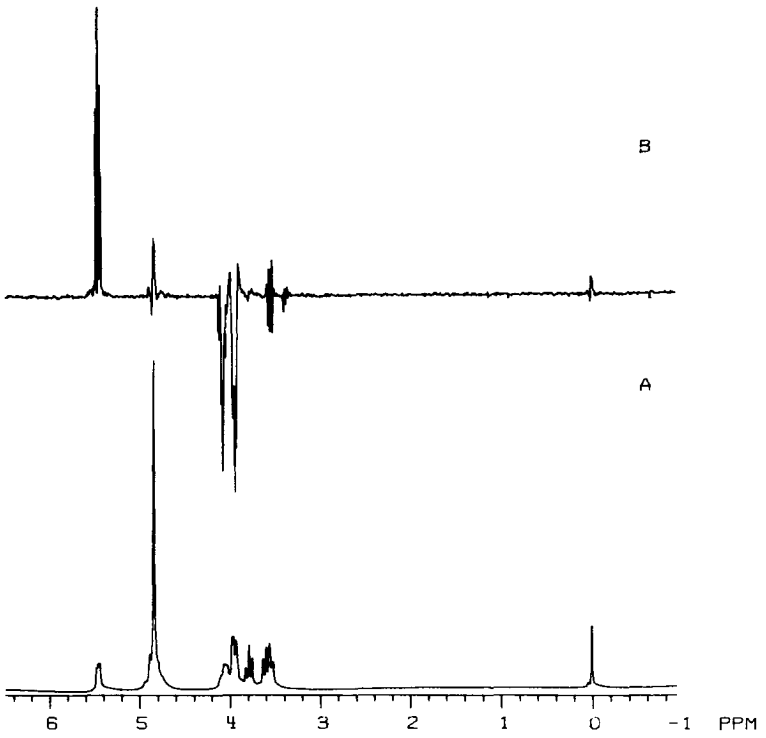

FIG. 5. ^{1}H NMR spectra of glucose 1,6-diphosphate (0.28 *M*) in HDO with 0.075% TSP. (A) One-pulse spectrum. (B) $^{1}H\{^{31}P\}$ SED spectrum with τ set to 40 ms.

with one positive and one negative component. This pattern of two doublets is more readily seen (Fig. 4C) when a very short τ value is used, which has the effect of allowing subtraction of the other proton spins while preventing phase modulation due to the homonuclear couplings. However, one unfortunate consequence of adopting this approach to simplify the spectrum is the loss of sensitivity in the phosphate ester proton signals. A final illustrative example is the case of glucose 1,6-diphosphate (Fig. 5). The selective enhancement of the resolved phosphate ester protons is seen. The 1-proton is the downfield signal, and the 6-protons show an inverted doublet due to coupling to the 5-proton (Fig. 5B).

Modifications of the pulse sequence described above with extra phosphorus pulses are being tested, to reduce the effect of homonuclear coupling. It remains to be seen how useful this general method will be when applied to metabolic studies, due to the possible summation of positive and negative components from different phosphate esters. However, these problems should be surmountable in view of the small number of important phosphate esters present in high concentration at various times during a process such as glycolysis. In addition, this approach might be useful for imaging studies, where a signal intensity correlated with the degree of glycolytic activity is desired, such as in the case of ^{11}C- or ^{18}F-labeled glucose in positron emission tomography.

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