High-Resolution NMR Spectroscopy of Solids and Surface-Adsorbed Species in Colloidal Suspension: ^{31}P NMR Spectra of Hydroxyapatite and Diphosphonates

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An increasingly widely used method for obtaining high-resolution NMR spectra of solids is magic-angle sample spinning.[1] In this technique, a rapid coherent mechanical motion of the solid sample is used to eliminate interactions due to both dipolar couplings and chemical shift anisotropy. A conceptually simpler approach would be to suspend sufficiently small particles of the solid in a fluid medium (gas or liquid), allowing the incoherent isotropic tumbling motions of the particles to eliminate these broadening interactions.[1] Since our first experimental demonstration of this idea,[2a] Siedle and Newmark[2b] have applied it to the study of surface species in a different system. This note reports in detail the successful application of the method to the measurement of the ^{31}P chemical shift of solid calcium hydroxyapatite, $Ca_{10}(OH)_2(PO_4)_6$, in colloidal suspension. In addition, the high-resolution ^{31}P NMR spectrum of a surface-adsorbed diphosphonate species has also been observed.

There are two requirements for this method to work: (1) the particles must be small enough so that the product of their rotational correlation time τ_c with the "local field" of the broadening interaction $(\Delta\omega_0^2)^{1/2}$ satisfies the criterion for motional narrowing,[3] $[\Delta\omega_0^2]^{1/2}\tau_c \ll 1$; (2) the particles must form a stable colloidal suspension. The former requirement can often be met by controlling the conditions under which the solid is formed. The latter condition will generally require the addition of a peptizing agent and/or mechanical dispersion to disaggregate the solid, unless a colloidal suspension can be formed directly by chemical reaction. In the present study, the surface-adsorbed species of interest functioned as a peptizing agent, as is quite common for charged adsorbates.

Calcium hydroxyapatite (HAP) was synthesized by an adaptation of literature methods.[4] A 0.25 M Na_2HPO_4 solution was added to 0.75 M $CaCl_2$ at pH 10.5 and 35 °C. The specific surface area (BET measurement) of the poorly crystalline solid was 87 m^2/g, and the typical particle size was 125 Å wide by 500 Å long (transmission electron microscopy). Infrared spectroscopy revealed the presence of some CO_3^{2-}.

The ^{31}P NMR spectrum of the HAP powder obtained at 36.4 MHz on a Bruker HX-90 spectrometer is shown in Figure 1a. The resonance is approximately Gaussian, with a half-width at half-intensity of 0.925 kHz. This corresponds to a second moment of $(\Delta\omega_0/2\pi)^2 = 0.61$ kHz^2, which is used in the calculation below.

No stable colloidal suspension of HAP could be formed in the absence of a peptizing agent. However, the addition of diphosphonates (near pH 7) such as disodium 1-hydroxyethane-1,1-diphosphonate [Na_2EHDP, Na_2 $CH_3(OH)C(PO_3H)_2$] or disodium dichloromethanediphosphonate together with ultrasonication for several hours using a microtip sonifier resulted in the formation of a stable colloidal suspension.

The ^{31}P NMR spectrum of the colloidal suspension resulting from the addition of EHDP is shown in Figure 1b. The peak at 19.1 ppm (downfield from 85% H_3PO_4) arises from EHDP (discussed below). There are two peaks in the phosphate region of the spectrum. The sharp peak at 0.9 ppm arises from the orthophosphate ions of HAP liberated into solution when EHDP is added to solid HAP.[5] The broader peak at 2.8 ppm is assigned

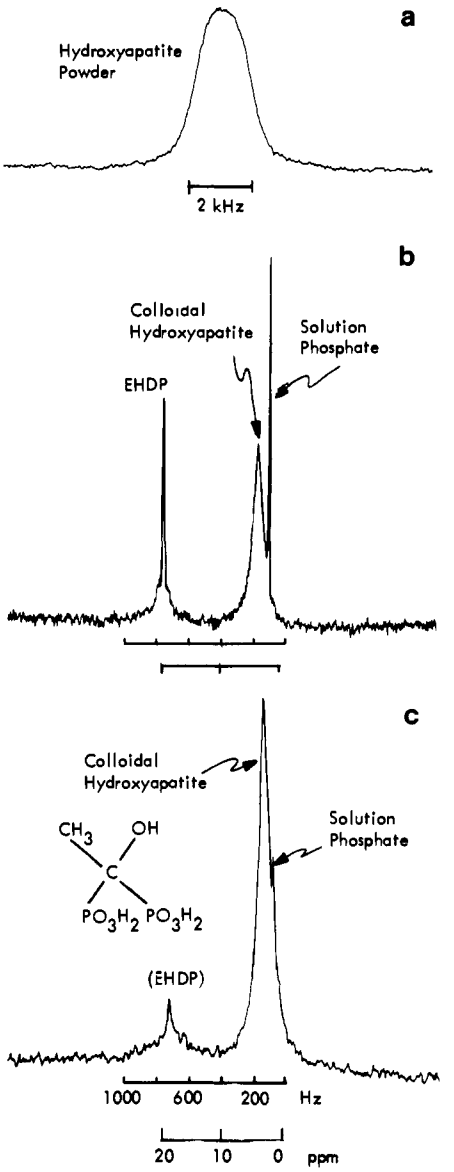

Figure 1. (a) Undecoupled ^{31}P NMR spectrum at 36.4 MHz of solid hydroxyapatite (HAP) powder, (b) ^{31}P NMR spectrum (proton-decoupled) of colloidal suspension of ≲0.6 g of HAP in 2 mL of H_2O + 0.075 g of Na_2EHDP, 1.4-h data accumulation, scale same as (c), and (c) ^{31}P NMR spectrum (proton-decoupled) of colloidal suspension formed by ultracentrifuging sample in (b) and resuspending pellet in 2 mL of H_2O.

to solid HAP; it was fitted to a Lorentzian line shape with a half-intensity half-width of 36 Hz. These assignments were confirmed by two experiments: (1) raising the pH to 8.0 caused the solution phosphate peak to shift downfield to 2.4 ppm, as expected,[6] while the HAP peak position remained constant; (2) ultracentrifugation of the colloidal HAP suspension and resuspension of the solid pellet of HAP in pure H_2O resulted in the nearly complete loss of the solution phosphate signal but not the HAP resonance (Figure 1c). Magnetic field inhomogeneity is not solely responsible for the breadth of the EHDP and HAP resonances, since the solution phosphate peak is considerably sharper.

Figure 1a,b demonstrates that the formation of a colloidal suspension has resulted in an approximately 30-fold reduction in the width of the ^{31}P resonance of HAP. This reduction in line width can be calculated theoretically for comparison. For the case of "motional narrowing", which applies to the colloidal suspension

(1) E. R. Andrew *Int. Rev. Sci., Phys. Chem., Ser. 2*, **4**, 173-207 (1975).
(2) (a) J. P. Yesinowski, abstract published from the 20th Experimental NMR Conference in Asilomar, CA, Feb 1979. (b) A. R. Siedle and R. A. Newmark, *J. Am. Chem. Soc.*, **103**, 1240 (1981).
(3) A. Abragam, "The Principles of Nuclear Magnetism", Oxford University Press, London, 1970, Chapter X.
(4) E. D. Eanes and A. S. Posner, *Trans. N.Y. Acad. Sci. II*, **28**, 223 (1965).
(5) W. G. Robertson, D. B. Morgan, H. Fleisch, and M. D. Francis, *Biochim. Biophys. Acta*, **261**, 517 (1972).
(6) M. M. Crutchfield, C. F. Callis, R. R. Irani, and G. C. Roth, *Inorg. Chem.*, **1**, 313 (1962).

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in Figure 1b (see below), a Gaussian resonance in the absence of motion and with a second moment $\Delta\omega_0^2$ is narrowed by any motions which modulate the anisotropic broadening interactions. The result is a Lorentzian line with a half-intensity half-width δ given by

$$\delta = \Delta\omega_0^2\tau_c/2\pi \text{ (in Hz)} \quad (1)$$

where τ_c is the correlation time of the motion.

For a rigid solid such as HAP, the relevant correlation time τ_c is that for rotational reorientation of the solid particle in a colloidal suspension.

The Stokes–Einstein equation for a sphere of radius a immersed in a continuous medium of viscosity η can be used to estimate this rotational correlation time:

$$\tau_c = 4\pi\eta a^3/3kT \quad (2)$$

Assuming values of a = 250 Å (for a 500-Å-long particle) and η = 1 cP (for water at 20 °C)[7] yields a value for τ_c at 20 °C of 1.6×10^{-5} s. Since this estimate results in $(\Delta\omega_0^2)^{1/2}\tau_c = 0.08 \ll 1$, the criterion for motional narrowing is met,[3] and eq 1 is applicable.

Substituting the estimated τ_c and the measured $\Delta\omega_0^2$ into eq 1 yields δ = 61 Hz, compared to the observed δ = 36 Hz. The calculated line width would become smaller if the shorter correlation time for motion around the long (500 Å) axis were taken into account, but the quantitative expression for δ would become more complicated. In view of the possibility of incomplete disaggregation and particle size dispersion, we consider the observed agreement in line widths satisfactory. Further sharpening should be achievable by lowering η, either by raising the temperature or by using a lower viscosity liquid.

The measured isotropic chemical shift of HAP, 2.8 ppm, agrees with the value measured for other hydroxyapatite samples by ^{31}P magic-angle sample spinning methods;[8] it differs somewhat from the value of ca. 6 ppm observed[6] for aqueous PO_4^{3-}.

The use of colloidal suspensions serves to sharpen the NMR signals of surface-adsorbed species as well as of the colloidal solid itself. The peak at 19 ppm in Figure 1c arises from EHDP molecules chemisorbed on the HAP surface. The ^{31}P NMR spectrum of this sample *before* ultracentrifugation and resuspension (Figure 1b) exhibits a sharp component at 19.1 ppm superimposed upon a broad component. The sharp component arises from free EHDP molecules in solution and the broader component from chemisorbed EHDP that is not in rapid exchange with free molecues in solution. Apparently chemisorption on the HAP surface does not result in a detectable change in the ^{31}P chemical shift of EHDP.

The assignment of the broader component at 19 ppm to chemisorbed EHDP is supported by the ^{31}P NMR spectrum of another sample of colloidal HAP in which the EHDP resonance showed only the broader component. The amount of EHDP added to this sample and the measured surface area of the HAP implied that about 36 Å^2 of HAP surface was available for each EHDP molecule (incomplete suspension of all the HAP introduces some error). This is a plausible value for a monolayer coverage and suggests that the broader component arises from all of the chemisorbed EHDP.

We have obtained spectra with better signal/noise at 121.5 MHz on a Bruker CXP-300 in which the bound EHDP has a half-height width of about 660 Hz. This line width is plausible in view of the expected larger second moment of solid EHDP compared to that of HAP. The colloidal HAP line width is also greater than that observed at lower field because of a measurable phosphorus chemical shift anisotropy.[8]

As shown here, the use of colloidal suspensions offers a means of obtaining high-resolution NMR spectra of selected solids and surface-adsorbed species using conventional high-resolution NMR equipment.

(7) "Handbook of Chemistry and Physics", 57th ed., R. C. Weast, Ed., CRC Press, Cleveland, OH, 1976.

(8) W. P. Rothwell, J. S. Waugh, and J. P. Yesinowski, *J. Am. Chem. Soc.*, **102**, 2637 (1980).

Effect of DNA Molecular Weight, Temperature, and Magnetic Field Strength on the ^{31}P NMR Results of DNA Complexed with Ethidium

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Because of its sensitivity to phosphate bond and tortional angles,[1] ^{31}P NMR spectroscopy should be widely applicable in conformational studies of nucleic acids. Analysis of smaller molecules such as tRNA and synthetic complementary deoxynucleotide segments has yielded information on the structure of these molecules and how their conformation changes with factors such as temperature, pH, and bound ligands.[1a,2] Recently ^{31}P NMR spectroscopy has been applied to higher molecular weight DNA samples such as synthetic double helical deoxypolynucleotides,[3] nucleosomes,[4] viruses,[5] and low[6] and high molecular weight[7] DNA fragments. Application of ^{31}P NMR spectroscopy to the analysis of the interaction of DNA with intercalating ligands has produced somewhat conflicting results.[8,9] Hogan and Jardetsky found that the ^{31}P chemical shift of DNA did not change, and the area was totally lost as the DNA was titrated to saturation with ethidium.[9] Jones and Wilson studied ethidium and several other intercalating ligands and found downfield shifts for the ^{31}P resonance of DNA in the presence of these compounds and no significant area loss.[8] The downfield shifts were best correlated with the unwinding angle produced by the intercalating ligand.[8]

In an effort to explain the apparently different results with the important intercalating drug, ethidium, we sonicated DNA for different time periods and fractionated the samples by gel exclusion chromatography.[10] We have obtained for the first time ^{31}P spectral results for a high molecular weight (1400 base pairs) DNA complex with an intercalating ligand. These results are reported here, and are compared to a low molecular weight DNA sample (190 base pairs), as a function of ionic strength, temperature, magnetic field strength, and ethidium to DNA ratio.[11] The chemical shifts, T_1 values, and line widths at 24.15 MHz for the low and high molecular weight fractionated DNA samples as a function of added ethidium are collected in Table I. As can

(1) (a) Gorenstein, D. G. *Annu. Rev. Biophys. Bioeng.* **1981**, *10*, 355–386. (b) Gorenstein, D. G. In "Nuclear Magnetic Resonance Spectroscopy in Molecular Biology"; D. Reidel: Dordrecht, Holland, 1978; pp 1–15. (c) Gorenstein, D. G.; Kar, D. *Biochim. Biophys. Res. Comm.* **1975**, *65*, 1073.

(2) (a) Kearns, D. R. In *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 477–523. (b) Krugh, T. R.; Nuss, M. E. In "Biological Applications of Magnetic Resonance"; Academic Press: New York, 1979; pp 113–175. (c) Patel, D. J. *Acc. Chem. Res.* **1979**, *12*, 18. (d) Schweizer, M. In "Magnetic Resonance in Biology"; Wiley Interscience: New York, 1980; pp 259–302.

(3) (a) Patel, D. J.; Canuel, L. L. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 674. (b) *Ibid.* **1979**, *76*, 24. (c) Shindo, H.; Simpson, R. T.; Cohen, J. S. *J. Biol. Chem.* **1979**, *254*, 8125. (d) Simpson, R. T.; Shindo, H. *Nucl. Acids Res.* **1980**, *8*, 2093. (e) Klysik, J.; Studivant, S. M.; Larson, J. E.; Hart, P. A.; Wells, R. D. *Nature (London)* **1981**, *290*, 672.

(4) (a) Cotter, R. I.; Lilley, D. M. J. *FEBS Lett.* **1977**, *82*, 63. (b) Kallenbach, N. R.; Appleby, D. W.; Bradley, C. H. *Nature (London)* **1978**, *272*, 134. (c) Klevan, L.; Armitage, I. M.; Crothers, D. M. *Nucleic Acids Res.* **1979**, *6*, 1607. (d) Shindo, H.; McGhee, J. D.; Cohen, J. S. *Biopolymers* **1980**, *19*, 523.

(5) diVerdi, J. A.; Opella, S. J. *Biochemistry* **1981** *20*, 280.

(6) (a) Mariam, Y. H.; Wilson, W. D. *Biochim. Biophys. Res. Commun.* **1979**, *88*, 861. (b) Bolton, P. H.; James, T. L. *J. Am. Chem. Soc.* **1980**, *102*, 25. (c) Hogan, M. E.; Jardetzky, O. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 6341. (d) Shindo, H. *Biopolymers* **1980**, *19*, 509.

(7) (a) Opella, S. J.; Wise, W. B.; diVerdi, J. A. *Biochemistry* **1981**, *20*, 284. (b) Hanlon, S.; Glonek, T.; Chan, A. *Ibid.* **1976**, *15*, 3869.

(8) Jones, R. L.; Wilson, W. D. *J. Am. Chem. Soc.* **1980**, *102*, 7776.

(9) Hogan, M.; Jardetzky, O. *Biochemistry* **1980**, *19*, 2079.

0002-7863/81/1503-6267$01.25/0