Solid-State Phosphorus-31 Nuclear Magnetic Resonance Studies of Synthetic Solid Phases of Calcium Phosphate: Potential Models of Bone Mineral†

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ABSTRACT: Phosphorus-31 NMR spectra have been obtained from a variety of synthetic, solid calcium phosphate mineral phases by magic angle sample spinning. The samples include crystalline hydroxyapatite, two type B carbonatoapatites containing 3.2 and 14.5% CO_3^{2-}, respectively, a hydroxyapatite in which approximately 12% of the phosphate groups are present as HPO_4^{2-}, an amorphous calcium phosphate, monetite, brushite, and octacalcium phosphate. Spectra were observed by the standard Bloch decay and cross-polarization techniques, as well as by a dipolar suppression sequence, in order to distinguish between protonated and unprotonated phosphate moieties. The spectra of the synthetic calcium phosphates provide basic information that is essential for interpreting similar spectra obtained from bone and other calcified tissues.

Calcium and inorganic orthophosphate form a variety of crystalline and noncrystalline solid phases that have been extensively used to help identify and characterize the solid inorganic mineral phases in bone and tooth. However, none of the synthetic calcium phosphate solid phases of known structure has the identical chemical, compositional, and structural characteristics of the mineral phase(s) found in bone and tooth. For example, the failure to uniquely identify the mineral phase(s) of bone by X-ray diffraction stems in part from the fact that the mineral crystallites are very small and the resulting X-ray diffraction patterns are too poorly defined to permit a unique solution to the structural analysis (Glimcher et al., 1981). In order to overcome this problem, we have examined the short-range atomic order around the phosphate groups of the solid mineral phases of biologically calcified tissues by ^{31}P MASS[1] NMR (Roufosse et al., 1984). However, in order to interpret the spectra obtained from bone and other calcified tissues, it is first necessary to examine and understand the ^{31}P MASS NMR spectra obtained from well-defined and characterized synthetic solid phases of $CaPO_4$. A number of the synthetic $CaPO_4$ solid phases studied have been postulated to be present in bone tissue.

The synthetic solid phases of $CaPO_4$ studied in the experiments reported in this paper may be divided into three categories: (1) crystalline and poorly crystalline hydroxyapatites (HA and PCHA, respectively); (2) amorphous calcium phosphates (ACP); (3) crystalline compounds containing HPO_4^{2-} groups. The first category includes the crystalline compounds hydroxyapatite (HA), two type B carbonatohydroxyapatites (CA) containing 3.2 and 14.5% structurally substituted CO_3^{2-} ions, a type A carbonatohydroxyapatite (CA) in which all of the hydroxyl groups are replaced by CO_3^{2-} groups, a hydroxyapatite with approximately 12% of its phosphate groups present as HPO_4^{2-}, and a "poorly crystalline" hydroxyapatite (HA) obtained by the hydrolysis of ACP. The third group contains the well-defined mineral phases brushite (BRU), monetite (MON), and octacalcium phosphate (OCP).

Standard Bloch decay and cross-polarization spectra were obtained on all samples, as were dipolar suppression traces to help distinguish resonances arising from protonated and unprotonated phosphate moieties. A computer study of the ^{31}P MASS NMR spectra obtained from these well-defined synthetic $CaPO_4$ solid phases forms the basis for the interpretation of similar spectra observed from bone and other calcified tissues (Roufosse et al., 1984).

Materials and Methods

Preparation and Description of the Synthetic $CaPO_4$ Solids. Most of the synthetic $CaPO_4$ solids analyzed in this study were gifts of the Laboratory of Physical Chemistry of Solids at High Temperature, The Université Paul Sabatier and Institut National Polytechnique, Toulouse, France. These include ACP, a poorly crystalline HA obtained from the hydrolytic conversion of ACP under strictly controlled conditions (Heughebaert & Montel, 1982), HA with approximately 12% of the phosphate groups present as HPO_4^{2-} (Trombe, 1973), a type A carbonatohydroxyapatite (Trombe et al., 1972a, b) in which all of the OH^- groups are replaced by CO_3^{2-}, and two type B carbonatohydroxyapatites (CA) containing 3.2 and 14.5% CO_3^{2-}, respectively (Labarthe et al., 1975; Elliott et al., 1980). Monetite (MON), brushite (BRU), and crystalline HA were prepared as previously reported (Roufosse et al., 1979). Octacalcium phosphate (OCP) was kindly donated by M. S. Tung of the National Bureau of Standards, Washington, D.C. All samples were characterized by powder X-ray diffraction (Roufosse et al., 1979).

^{31}P Magic Angle Sample Spinning (MASS) NMR Spectra. All of the NMR spectra were obtained on a home-built pulse spectrometer operating at 119 and 294 MHz for ^{31}P and ^{1}H, respectively. The typical radio-frequency (RF) field strengths were 13 G for protons and 24 G for phosphorus. The sample size was typically 80 mg. Powders were tightly packed into

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[1] Abbreviations: MASS, magic angle sample spinning; NMR, nuclear magnetic resonance; HA, hydroxyapatite; CA, carbonatohydroxyapatite; PCHA, poorly crystalline hydroxyapatite; ACP, amorphous calcium phosphate; OCP, octacalcium phosphate; BRU, brushite; MON, monetite; $CaPO_4$, calcium phosphate in solid phase.

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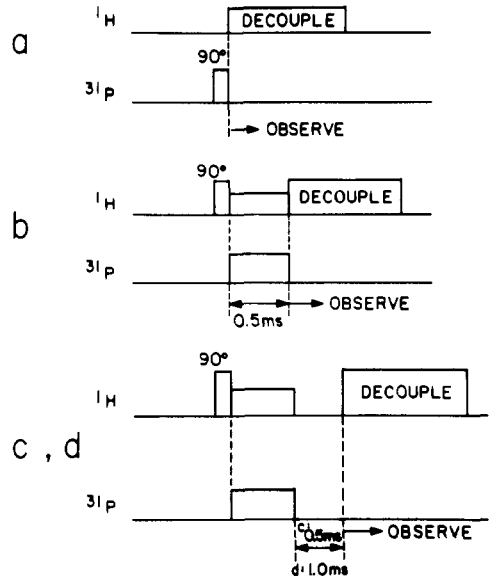

FIGURE 1: Schematic representation of the sequences of pulses used in the ^{31}P MASS NMR experiments. See description in text.

Andrew–Beams type rotors machined from Delrin (Andrew, 1971), and all spectra were taken at 2-kHz spinning rates to facilitate comparison of sideband intensities. Chemical shifts are referenced to external 85% H_3PO_4.

In Figure 1 are shown the pulse sequences employed to obtain ^{31}P MASS NMR spectra of the $CaPO_4$ samples. Figure 1a illustrates a proton-decoupled Bloch decay experiment, the sequence commonly used in liquid-state Fourier-transform NMR spectroscopy. For the samples studied here, it was necessary to employ a 60-s recycle delay to provide adequate time for repolarization of the ^{31}P nuclei. Figure 1b is the normal cross-polarization experiment (Pines et al., 1973) employed for studying dilute spin systems in solids. In contrast to the sequence shown in Figure 1a, this experiment produces spectra that have enhanced contributions for phosphate groups with nearby protons—e.g., HPO_4^{2-} groups. The recycle delay was 10 s for the cross-polarization measurements. Figure 1c illustrates a variation of the cross-polarization experiment, namely, the dipolar suppression technique (Opella & Frey, 1979; Munowitz et al., 1981), which we have also found useful in comparing the various samples. In this sequence, we insert a period τ following the cross-polarization in which ^{1}H decoupling is not present. During this period, any ^{31}P nucleus that is adjacent to a proton experiences a dipolar coupling, and its signal is suppressed. The number and proximity of the protons determine the amount of suppression. Typically, τ was either one or two rotational periods of the rotor (0.5 or 1 ms); the recycle delay used in this experiment was also 10 s.

Results

Hydroxyapatite (HA). In part 1 of Figure 2 is shown ^{31}P MASS NMR spectra of crystalline HA obtained with the four pulse sequences discussed above. The powder spectrum of this material is about 2 kHz wide at our operating field. At a spinning rate of 2 kHz, this spectrum collapses to a sharp centerband flanked by weak sidebands containing about 10% of the total intensity of the spectrum. Although the monoclinic crystal structure of HA has three nonequivalent PO_4^{3-} groups, they apparently have the same isotropic shift. The observed isotropic shift for HA is given in Table I and is in agreement with a previously reported value (Rothwell et al., 1980). However, we find the chemical shift anisotropy is considerably smaller than that reported by Rothwell et al. (1980). Although this observation might arise from samples with varying water content, it probably results from the inaccuracies of the moment analysis previously employed when it is applied to chemical shift tensors with a small anisotropy such as HA.

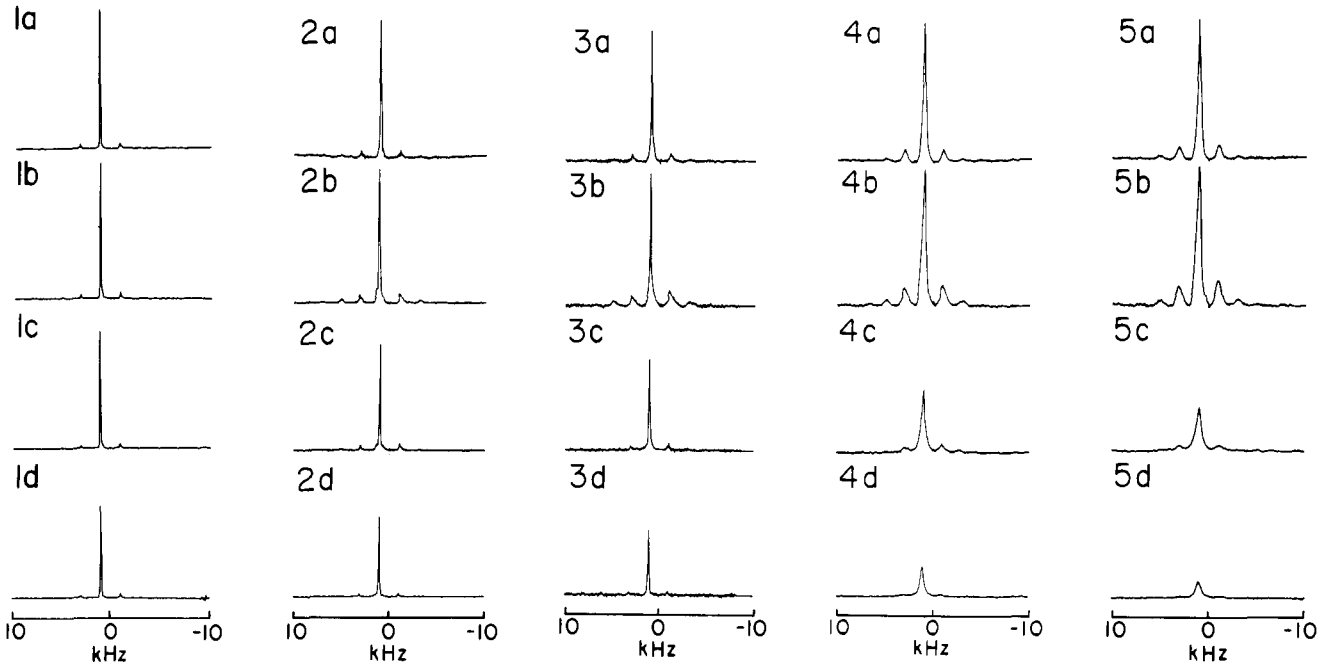

FIGURE 2: Proton-decoupled ^{31}P MASS NMR spectra of several hydroxyapatite minerals. Each column presents data obtained with the four experiments explained in the text and illustrated in Figure 1: (a) Bloch decay, RD = 60; (b) cross-polarization, RD = 10 s, 0.5-ms mixing; (c and d) cross-polarization with 0.5 and 1.0 ms of proton dipolar coupling before data acquisition. For comparison purposes, all figures present (a) and (b) on the same vertical scale, while (b)–(d) are presented on an absolute intensity basis. (1) Crystalline hydroxyapatite; (2) hydroxyapatite containing 12% of its phosphate in the form HPO_4^{2-}; (3) poorly crystalline hydroxyapatite; (4) type B carbonatohydroxyapatite containing 3.2% CO_3^{2-} and type B carbonatohydroxyapatite containing 14.5% CO_3^{2-}. The two main features to note are the significant increases in sideband intensities for (b) over (a) as protons are introduced to the lattice and the significant broadening of the lines caused by carbonate ion.

Table I: Isotropic ^{31}P Chemical Shifts (ppm) Measured for the $CaPO_4$ Solid Phases in MASS NMR Experiments[a]

compd	chemical shift (ppm)
crystalline HA	2.8
HA–12% HPO_4^{2-}	2.8
type B CA	
3.2% CO_3^{2-}	2.8
14.5% CO_3^{2-}	3.0
type A CA	5.5, 4.6, 3.8, 2.5
ACP	3.0
OCP	2.8, −0.5
monetite	−0.3, −1.7
brushite	1.4

[a] Shifts are relative to 85% H_3PO_4, and a positive shift is downfield.

The spectral line shapes of HA obtained with a Bloch decay (Figure 2 part 1a) and cross-polarization (Figure 2, part 1b) are identical within experimental error. However, the proton density in this material is low, and there is actually a 20% suppression of the signal intensity of the cross-polarization signal compared to that obtained by a Bloch decay with a 60-s recycle delay. A second consequence of the low proton density is that the removal of the ^1H decoupling field for 0.5 or 1 ms does not result in an appreciable suppression of the ^{31}P MASS NMR signal. For example, after 1 ms of ^1H coupling, 70% of the signal is still present (Figure 2, part 1d).

Hydroxyapatite Containing Approximately 12% HPO_4^{2-} (HA–12% HPO_4^{2-}). Part 2 of Figure 2 shows the ^{31}P MASS NMR spectra of a sample of HA that contains approximately 12% of its PO_4^{3-} groups substituted by HPO_4^{2-} groups (Trombe, 1973). In the Bloch decay experiment (Figure 2, part 2a), the spectrum is very similar to the one obtained from crystalline HA. The spectrum, therefore, presumably arises principally from the PO_4^{3-} groups in the mineral. However, with cross polarization (Figure 2, part 2b), three small differences between HA and HA–12% HPO_4^{2-} appear in the spectrum: (1) an additional contribution to the centerband, (2) the appearance of a second set of rotational sidebands, and, very importantly, (3) an asymmetry of the first set of sidebands. A small shift (−0.5 ppm) in the position of the central line is also evident. HPO_4^{-2} groups generally exhibit large shift anisotropies (~130 ppm) and isotropic shifts slightly downfield from apatites (vide infra). The spectra in part 2 of Figure 2 are consistent with all of these observations. Furthermore, the higher proton density results in only 56% of the signal remaining after 1 ms of dipolar coupling. The isotropic chemical shift of this sample is given in Table I.

"Poorly Crystalline" Hydroxyapatite (PCHA). ^{31}P MASS NMR spectra from PCHA are shown in part 3 of Figure 2. The sidebands are more intense than those seen from HA and from HA–12% HPO_4^{2-}. In addition, the shapes of the lines differ from those observed in the HA samples. For instance, the centerband in the cross-polarization spectrum appears to be a superposition of a sharp line on a broader background. These same sort of features appear in the sidebands and in the proton-suppression experiment. The broad features appear to be suppressed, leaving only the sharp component. Integration of the spectra indicates that approximately 40% of the intensity remains after 1 ms of dipolar coupling.

Carbonatohydroxyapatite (CA). Parts 4 and 5 of Figure 2 are spectra from two CA's in which CO_3^{2-} ions partially replace the OH^- groups in the apatite lattice. The spectra shown in part 4 of Figure 2 were obtained from a sample containing 3.2% CO_3^{2-}, while the spectra shown in part 5 of Figure 2 were obtained from a sample containing 14.5% CO_3^{2-} (Labarthe et al., 1973). The spectra in parts 4 and 5 of Figure 2 demonstrate a number of important points. Not only are there easily discernible differences between the Bloch decay and cross-polarization spectra, but also the introduction of CO_3^{2-} groups clearly has a pronounced effect on the ^{31}P MASS NMR line widths. In pure HA, PCHA, and HA–12% HPO_4^{2-}, line widths are 0.5–1 ppm. In the type B CA samples, however, the line widths are approximately 5 ppm. Considering the relatively small amount of CO_3^{2-} substitution in these compounds, the effect is rather large and in the range studied (3.2–14.5% CO_3^{2-}) shows only a small concentration dependence. It is clear from these results that the introduction of CO_3^{2-} groups into HA results in an increase in the cross-polarization efficiency for some of the PO_4^{3-} groups in the crystal lattice. Concurrently, there are some changes in the PO_4^{3-} shift anisotropies. Note, for example, that the sideband intensities are larger than those in pure HA and that a second set of sidebands is clearly present in both cross-polarization spectra. Moreover, in the sample containing 14.5% CO_3^{2-}, the first set of sidebands shows a slight asymmetry. The fact that the Bloch decay and cross-polarization spectra differ indicates that there are at least two types of phosphate groups in the lattice. Finally, in the proton-suppression spectra only ~20% of the intensity remains after 1 ms of dipolar coupling.

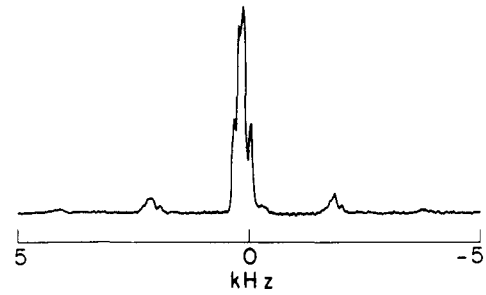

FIGURE 3: Bloch decay ^{31}P MASS NMR spectrum of type A carbonatohydroxyapatite. Replacement of the OH^- groups by CO_3^{2-} results in four resolved splittings of the centerband. Since the OH^- groups are no longer present, no cross-polarization or dipolar-suppression spectra are presented.

Complete substitution of CO_3^{2-} for the OH^- groups yields a type A CA. A type A CA Bloch decay spectrum is presented in Figure 3. Although this sustitution does not greatly affect the ^{31}P MASS NMR shift anisotropies, it does lead to nonequivalence between the PO_4^{3-} groups. The centerband in this spectrum is split into four lines, and the general features of the centerband appear in the sidebands. Since there are no protons in this sample, we do not present cross-polarization or dipolar-suppression spectra.

Amorphous Calcium Phosphate (ACP). ^{31}P MASS NMR spectra obtained from the ACP prepared as described by Heughebaert & Montel (1982) are shown in Figure 4. These spectra show several interesting features. First, since ACP contains water, one might expect that the line shapes observed in the Bloch decay and in the cross-polarization spectra to be different. However, as can be seen from Figure 4a, b, they are virtually identical within experimental error. At the moment, we have no satisfactory explanation for this observation. We have observed similar spectra from a variety of ACP samples prepared under different conditions (i.e., various Ca/PO_4^{3-} ratios and/or different pHs). Secondly, in contrast to the behavior observed in the hydroxyapatites, proton coupling completely suppresses the ^{31}P signal after 1 ms of dipolar coupling. This observation arises from the overall proton density; chemical analysis of ACP suggests that it contains water.

Octacalcium Phosphate (OCP). Part 1 of Figure 5 shows the spectra obtained from a sample of OCP. The spectra are

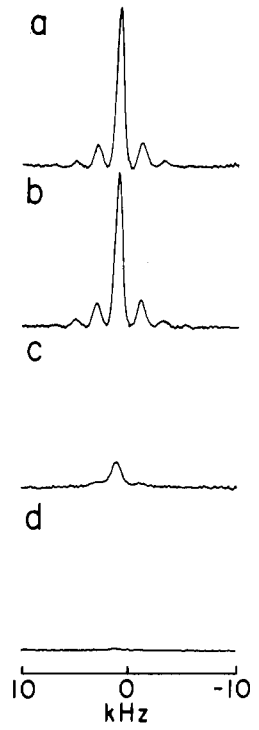

FIGURE 4: Proton-decoupled ^{31}P MASS NMR spectra of amorphous calcium phosphate (ACP). Experimental conditions for (a)–(d) are given in Figure 2. Note that in contrast to the hydroxyapatite samples (Figure 2), the signal is completely suppressed after 1 ms of dipolar coupling.

in agreement with the features found in the crystal structure. Specifically, the Bloch decay spectrum shows a strong centerband with relatively weak sidebands at approximately the same position observed in the spectra obtained from apatitic PO_4^{3-} groups. In addition, a shoulder with about half the intensity is apparent on the right side of the centerband, and the sidebands are skewed in this direction as well. In the cross-polarization spectrum, the intensities of the two lines are reversed, and the rotational sidebands are clearly much stronger. Finally, in the suppression experiments, the high-field line decays more rapidly than the low-field line. All of these observations are consistent with the presence of protonated and unprotonated phosphate groups and with the chemical composition of OCP. Specifically, the Bloch decay and suppression experiments yield the spectrum of unprotonated PO_4^{3-} groups, while the cross-polarization spectrum is typical of the protonated species (HPO_4^{2-}). The isotropic shifts for the two species are given in Table I and are in agreement with those reported previously (Rothwell et al., 1980). Rothwell et al. (1980) also described a similar, differential, cross-polarization behavior from OCP.

Monetite (*$CaHPO_4$*). The ^{31}P MASS NMR spectra of monetite are shown in part 2 of Figure 5. They reveal the presence of two chemically nonequivalent phosphate groups. However, a comparison of these spectra with those obtained from OCP reveals a number of easily discernible differences. First, although in both the Bloch decay and cross-polarization spectra the centerband and sideband intensities are approximately the same for both species, the shape of the sidebands is asymmetric. This indicates a reasonably large shift anisotropy for both HPO_4^{2-} groups. However, even though all of the phosphate groups are protonated in monetite, it is apparent that the protons are not attached in the same manner as they are in OCP. This fact can be derived from the dipolar suppression spectra since the low-field line decays more rapidly than the high-field line. Finally, another important difference

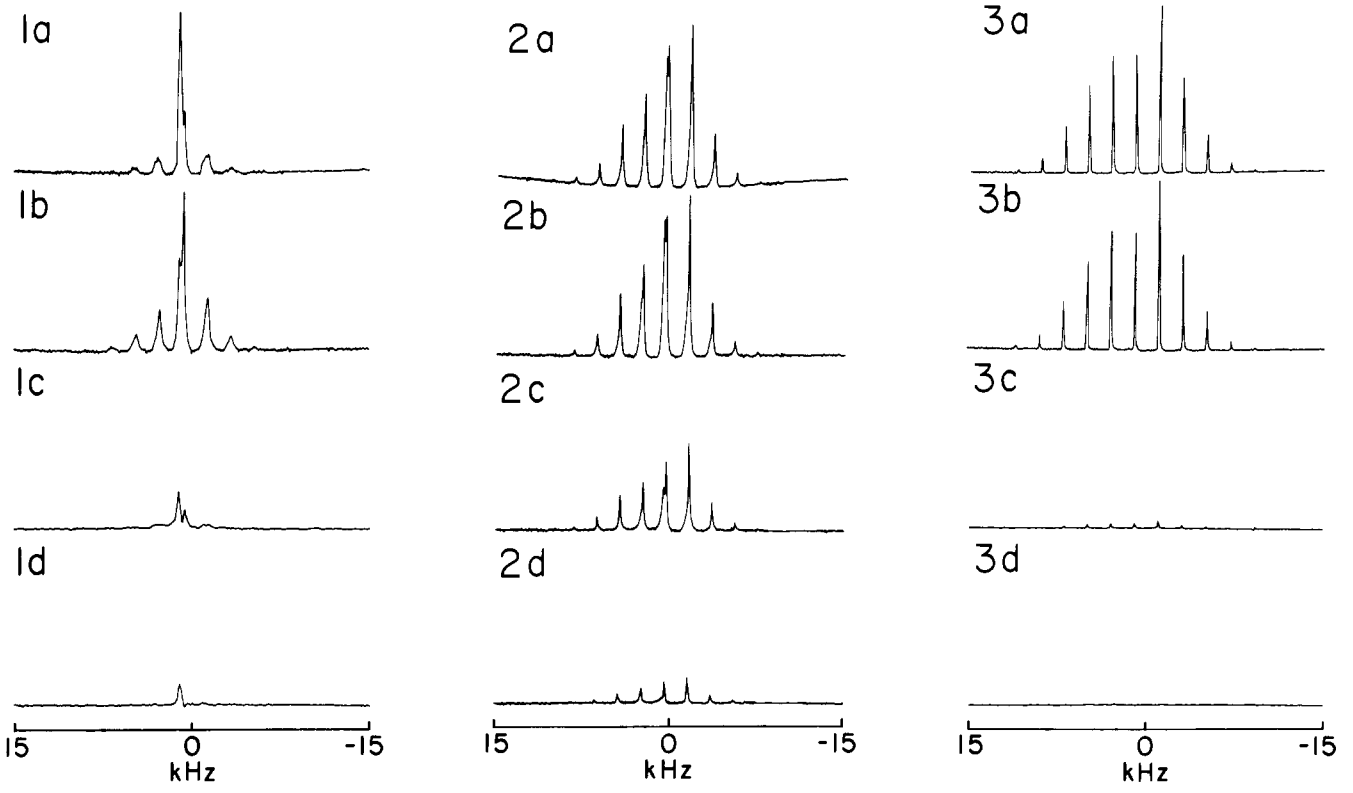

FIGURE 5: Proton-decoupled ^{31}P MASS NMR spectra of calcium phosphate minerals containing protonated phosphate moieties. Experimental conditions for (a)–(d) are given in Figure 2. (1) Octacalcium phosphate. Note the presence of two lines corresponding to the PO_4^{3-} and HPO_4^{2-} groups in the sample and the change of their relative intensities from (a) to (b). (2) Monetite. Two signals are observed, and considerable intensity persists after 1 ms of dipolar coupling. (3) Brushite. A single HPO_4^{2-} species is present, and the spectrum is strongly suppressed by dipolar coupling due to the relatively high proton density.

between the OCP and MON spectra is the fact that considerable spectral intensity remains after 1 ms of dipolar coupling. There are two possible explanations for this observation. Although an unusually long ^1H–^{31}P distance in MON could account for this behavior, its crystal structure essentially eliminates this possibility. The more probable explanation is that the overall proton density of MON is lower than that of OCP, since unlike OCP, MON does not contain water of hydration.

Brushite ($CaHPO_4 \cdot 2H_2O$). The ^{31}P MASS NMR spectra of BRU are shown in part 3 of Figure 5. Since there is only one species of phosphate present in this compound, the line shapes in the Bloch decay and cross-polarization spectra are identical. However, when they are compared on an absolute intensity scale, there is a 6-fold difference in the intensities. This is much more than the theoretical cross-polarization enhancement factor and is due to the fact that the ^{31}P T_1 is much longer than the 60-s recycle delay employed in the Bloch decay experiment. In addition, the dipolar suppression pulse sequence results in a complete disappearance of the ^{31}P signal at 1 ms. This is similar to the behavior observed for OCP but is different from the results obtained from monetite. The isotropic shift for BRU is given in Table I.

Discussion

We have examined a number of synthetic crystalline and noncrystalline $CaPO_4$ solids by ^{31}P MASS NMR in order to have a set of standards with which to compare ^{31}P MASS NMR data obtained from bone and other mineralized tissues. The synthetic samples were selected to include essentially all of the model compounds that have been postulated to exist in biological tissues impregnated with a solid phase of $CaPO_4$ [for reviews, see Posner (1969), Brown & Chow (1976), and Glimcher et al. (1981)]. In general, the data demonstrate that isotropic and anisotropic chemical shifts together with proton-suppression techniques can be used to differentiate the synthetic $CaPO_4$ compounds from one another. For example, hydroxyapatites display isotropic shifts that are 1.5 ppm downfield from $CaPO_4$ solid phases containing HPO_4^{2-} groups. Furthermore, the ^{31}P MASS NMR spectra of the hydroxyapatites have no sidebands, whereas BRU and MON exhibit strong sideband patterns. In addition, these latter compounds cross-polarize much more efficiently than apatites and exhibit a substantially different spectral behavior in the proton-suppression experiments. Moreover, when a $CaPO_4$ phase contains both an apatite phase and a phase that contains HPO_4^{2-} groups, the sideband pattern characteristic of the HPO_4^{2-} groups appears. Even more striking examples of this behavior are seen in the spectra of OCP and MON in which cases the line intensities and sideband patterns are strongly dependent on whether or not cross-polarization is used.

As is described in the accompanying paper (Roufosse et al., 1984), the use of the ^{31}P MASS NMR techniques reported in this paper has not only permitted us to exclude certain $CaPO_4$ solid phases as major or minor phases in bone, but they have also made it possible to define much more clearly than heretofore the nature of the mineral phases in bone.

Acknowledgments

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Registry No. MON, 21063-37-6; BRU, 14567-92-1; OCP, 14096-86-7; CA, 66524-19-4; HA, 1306-06-5; calcium phosphate, 10103-46-5.

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