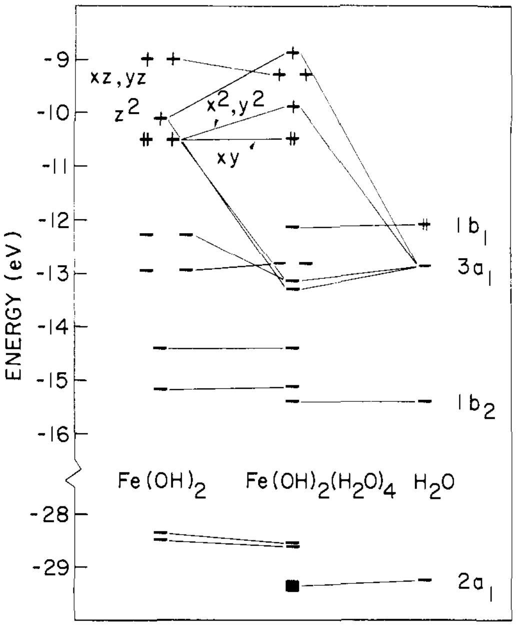

Figure 11. Bonding of water to $Fe(OH)_2$ to form $Fe(OH)_2(H_2O)_4$.

Our calculated destabilization energy is 4.7 eV. The proton solvation energy has been calculated to be ~5 eV,[3b] which will counter this energy increase. For the stripping of the four water molecules in $Fe(OH)_2(H_2O)_4$ we calculate an energy input of 1.4 eV, but cohesion of two $Fe(OH)_2$ molecules from above is 0.8 eV. Thus the net calculated energy for the reaction 2 is +0.3 eV. The cohesive energy of bulk $Fe(OH)_2$ will overcome this small barrier, and though the entropy decrease will favor maintaining the reactants in (4), at high pH or sufficiently anodic potentials $Fe(OH)_2$ forms.

Our total calculated ligand binding energies in $FeOH(H_2O)_5^+$ and $Fe(OH)_2(H_2O)_4$ are 6.1 and 6.3 eV, respectively. The $Fe(OH)_2$ ligand bonds are accounting for 78% of the stability in $Fe(OH)_2(H_2O)_4$, with the four water molecules weakly coordinated, with an average energy of 0.4 eV. In $Fe(OH)_2(H_2O)_n$, where $n = 3$, 2, and 1, the average H_2O coordination energies are 0.18, 0.22, and 0.56 eV, respectively. Consequently, $Fe(OH)_2(H_2O)_4$, once it loses the first H_2O ligand, will lose the others readily and precipitate $Fe(OH)_2$. The weakness of the coordination of 4 H_2O ligands to $Fe(OH)_2$ has associated with it the absence of orbital energy level stabilizations indicated in Figure 11. The two axial hydroxyl ligands have tied up and "saturated" the Fe $d_{x^2-y^2}$ and d_{z^2} orbitals so that there is a weaker interaction with equatorial H_2O ligands: Compare Figures 9 and 11.

Concluding Comments

We have determined a clear-cut mechanism for anodic iron dissolution to form $(FeOH)_{aq}^+$: surface OH weaken Fe-surface bonds and $FeOH^+$ forms and leaves the surface, becoming rapidly solvated, forming $FeOH(H_2O)_5^+$.

$(FeOH)_{aq}^+$ will hydrolyze, forming $Fe(OH)_2(H_2O)_4$ which is weakly stable and will precipitate as solid $Fe(OH)_2$. This is probably a step in anodic passive film formation at low pH. At high pH the dissolution step does not seem to occur,[4b] and the mechanism may involve surface reactions.

Thermodynamics must ultimately be taken into account in any complete description of iron dissolution and passivation in an aqueous environment. Entropy changes can dominate reactions. Detailed considerations of thermodynamics are beyond the scope of our quantum chemical study. However, the structural, energetic, and mechanistic hypotheses in our work corroborate a number of hypotheses in the literature.

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Phosphorus NMR Study of Solid Amorphous Calcium Phosphate

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Abstract: We have characterized solid synthetic amorphous calcium phosphate (ACP) by ^{31}P NMR, using magic angle spinning, proton enhancement via cross polarization, and variable temperature. With no cross polarization, the spinning sidebands of ACP are stronger than those of unprotonated phosphate in hydroxylapatite, but weaker than those of dibasic calcium phosphates. Cross polarization over a wide range of mixing times causes no change in the appearance of ACP spectra from 25 to -120 °C. In contrast, mixtures of hydroxylapatite with dibasic calcium phosphates, which, without cross polarization mimic the appearance of ACP spectra, show large differential enhancements of sideband intensities when cross polarized. Octacalcium phosphate behaves similarly to the mixtures. Vacuum drying of ACP at 450 °C removes tightly bound water. The sidebands of dried ACP are undiminished, but no cross polarization signal can be obtained. Similar drying of hydroxylapatite affects neither the sideband intensities nor the cross polarization. We conclude that the strength of ACP sidebands is due to a characteristic structural distortion of unprotonated phosphate and *not* to a mixture of protonated and unprotonated phosphates. Structural models of ACP and the implications for ^{31}P NMR studies of bone mineral are briefly discussed.

Introduction

It has long been known that formation of hydroxylapatite (HA) in vitro can occur via an amorphous precursor substance, commonly referred to as amorphous calcium phosphate (ACP).[1] When calcium phosphate is precipitated from sufficiently concentrated aqueous solution at or above neutral pH, the initial product is amorphous, as judged by X-ray diffraction, electron microscopy, and infrared spectroscopy.[2] This amorphous pre-

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cipitate transforms spontaneously to HA by a process of solution and renucleation, in aqueous slurries at physiological pH and temperature.[3] On this basis it has been proposed that formation of biological apatites, e.g., bone and tooth mineral, may also occur through an amorphous precursor, similar to or identical with synthetic ACP.[2,4,5]

Attempts to refine this hypothesis of biological mineralization have been hampered by uncertainties concerning the nature of synthetic ACP. On one hand, it is widely believed that ACP constitutes a well-defined compound of unique molecular structure, and a detailed model, involving $Ca_9(PO_4)_6$ clusters, has been proposed.[2] On the other hand, it has been argued that no ACP compound exists, and that the amorphous preparations are in fact poorly crystalline mixtures of apatite and protonated (dibasic) calcium phosphates,[6] e.g., monetite ($CaHPO_4$) and brushite ($CaHPO_4 \cdot 2H_2O$). This disagreement reflects the inherent difficulty of characterizing *any* amorphous substance as well as contributing a source of ambiguity to the interpretation of experiments on biological mineral.

For instance, recent work by electron diffraction indicates the presence of amorphous material in early mineral deposits from embryonic chick bone,[7] while an NMR study suggests that the earliest mineral deposit is brushite, which gradually transforms into apatite.[8] The apparent contradiction between the diffraction and NMR results might be resolved if synthetic ACP were shown to be, in fact, a mixture of apatite and brushite. In any case, further characterization of ACP is clearly desirable.

Phosphorus NMR, utilizing magic angle spinning (MAS) and cross polarization (CP) techniques, provides a useful method for analyzing calcium phosphate solids. The common calcium phosphate compounds (in polycrystalline form) are readily distinguished from each other by their characteristic spinning sideband patterns.[9] Mixtures of these compounds are readily detected provided that the isotropic chemical shifts of the different components are sufficiently well resolved; in such cases the spectra consist of interleaved patterns of sidebands from each component.[9,10] However, if the isotropic shifts are not resolved, the sidebands of the components superimpose and their intensities add. It is not then obvious whether one has a mixture or an unknown compound whose sideband intensities fortuitously match those of a given mixture. ACP is a case in point (vide infra).

One then has recourse to cross polarization,[11-13] (CP) i.e., signal enhancement via double resonance transfer of NMR polarization to phosphorus nuclei from surrounding protons. Since the signal enhancement experienced by a given phosphorus depends upon the number and spatial disposition of nearby protons, one can produce differential enhancement of sideband intensities in mixtures or in pure compounds with chemically inequivalent phosphate groups by varying the cross polarization conditions. If all phosphates in the sample are chemically and structurally equivalent no differential enhancement is produced, and the spectra with and without cross polarization are essentially indistinguishable. Such effects were dramatically demonstrated in octacalcium phosphate (OCP), which contains both protonated and unprotonated phosphate groups in the crystallographic unit cell, and were also used to show the presence of protonated phosphates in calcium deficient apatites.[9]

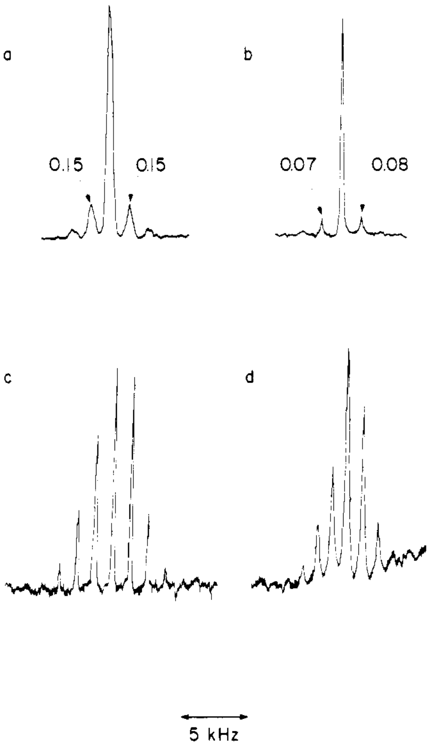

Figure 1. Proton-decoupled ^{31}P MAS spectra at 20 °C. The numbers above the first-order sidebands give the intensities relative to the center peak: (a) amorphous calcium phosphate, spinning rate = 1.35 kHz, pulse interval 60 s; (b) hydroxylapatite (Ca/P = 1.33), spinning rate = 1.40 kHz, pulse interval 60 s; (c) brushite, spinning rate = 1.33 kHz, pulse interval 10 s; (d) Monetite, spinning rate = 1.25 kHz, pulse interval = 10 s.

We have studied the ^{31}P NMR of synthetic ACP, of apatite–brushite and apatite–monetite mixtures, and of octacalcium phosphate, under a variety of conditions of cross polarization and at several temperatures. Our results show that ACP is almost certainly *not* a mixture of apatitic and protonated calcium phosphate phases, and therefore strongly suggest the existence of a unique ACP compound. Furthermore, our work shows that cross polarization is a desirable adjunct to any ^{31}P NMR study of biological mineral if ambiguities are to be avoided.

Experimental Section

Calcium Phosphate Compounds. ACP was prepared by the method of Blumenthal *et al.*,[14] sieved to 325 mesh, and stored in a desiccator over $CaSO_4$. ACP so prepared has a Ca/P ratio of 1.50 ± 0.02 and contains about 20% by weight tightly bound water which is not removed by desiccation at room temperature. A portion of the ACP was dried at 450 °C under vacuum for 24 h to remove the tightly bound water. X-ray powder diffraction patterns of dried and undried ACP showed no discernible differences.

Octacalcium phosphate was a gift of Dr. W. E. Brown of the National Bureau of Standards. Commercial samples of brushite ($CaHPO_4 \cdot 2H_2O$) and monetite ($CaHPO_4$) (Alfa Inorganics) were checked by X-ray powder diffraction. Commercial hydroxylapatite (Baker "Tricalcium Phosphate") was not characterized. All commercial samples were used as received. A nonstochiometric apatite (Ca/P = 1.33) and a chemically analyzed brushite sample were kindly provided by Dr. James Yesinowski of Procter and Gamble Co. Regardless of origins, all brushite and apatite samples gave identical NMR spectra.

NMR Spectroscopy. All spectra were obtained on a home-built double resonance spectrometer operating at 68.4 MHz for ^{31}P and 170 MHz for ^{1}H. The magic angle spinner was of the Beams-Andrew type. Samples for spectroscopy consisted of 0.2 to 0.3 g of polycrystalline or amorphous powder, packed firmly in Delrin rotors with press-fit Teflon caps.

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Spinning speeds were determined from the sideband spacing in spinning spectra. For low-temperature experiments, the spinner gas (N_2) was cooled by passage through a copper coil immersed in liquid N_2 and reheated downstream with a Nichrome wire heater as required. The spinner gas was vented through the long exhaust part of a glass Dewar which encased the entire probe head and provided thermal insulation. Temperature of the sample was measured with a copper–constantan thermocouple in the gas stream.

All ^{31}P spectra were taken with proton decoupling during data acquisition. Alternation of spin temperature was used in some of the cross polarization work to eliminate a glitch at the ^{31}P carrier frequency. The cross polarization contact time, i.e., the time during which the Hartmann–Hahn matching condition[11,13] is fulfilled, was varied from 0.1 to 20 ms, depending upon the aims and requirements of individual experiments.

Results

Figure 1a shows the room temperature MAS spectrum of ACP. Figures 1b, 1c and 1d show the spectra of HA (Ca/P ratio 1.33), brushite ($CaHPO_4{\cdot}2H_2O$), and monetite ($CaHPO_4$) for reference. The numbers above the first-order sidebands give their intensities relative to the central peak. The isotropic chemical shift of ACP is 1.7 ppm downfield of 85% H_3PO_4, which is the same as that observed for HA, but the ACP sidebands are roughly twice as intense as those of HA. The ACP linewidth is 7 ppm, compared to 2 ppm for HA. Although the HA shown here is calcium deficient, its spectrum under these conditions is virtually identical with that of stoichiometric HA.[9]

The strong sidebands of the dibasic calcium phosphates, monetite and brushite, reflect the large chemical shift anisotropy due primarily to protonation of the phosphate; in HA the phosphates are unprotonated but presumably distorted from tetrahedral symmetry, which leads to small but easily observable sidebands.

The sideband intensities of ACP are intermediate between those of HA and the protonated phosphates. Two explanations of the sidebands intensities of ACP may be given. (1) ACP contains only unprotonated phosphates, but with perhaps greater distortion from tetrahedral symmetry than found in HA. (2) ACP is a mixture of apatite and dibasic calcium phosphates, and the increased sideband intensities are due to the superposition of spectra from protonated as well as unprotonated phosphates. Those two possibilities are examined in experiments described below.

Figures 2a and 2c give the spectra without cross polarization of 2:1 mixtures (by mass) of HA and brushite, and HA and monetite. Figure 2e gives the spectrum of ACP without cross polarization, for comparison. The mole ratios of protonated to unprotonated phosphate are roughly 1:2 in the mixture spectra (Figures 2a and 2c). While it is clear by inspection that the sideband intensities of the mixtures do not match perfectly those of ACP, one could probably produce a ternary mixture whose sideband intensities without cross polarization were very close to those of ACP. The broader lines of ACP might then be ascribed to local structural inhomogeneities, which would obscure the partial resolution of the HA and monetite sidebands in Figure 2c.

Figures 2b, 2d, and 2f show the cross polarized spectra of the 2:1 HA–brushite mixture (CP contact time 1 ms), the 2:1 HA–monetite mixture (CP contact time 0.5 ms) mixture, and ACP (CP contact time 3 ms). Comparing Figures 2a and 2b, one sees that the appearance of the HA/brushite mixture changes drastically due to the large signal enhancement experienced by brushite relative to HA. Similarly dramatic changes in the HA–brushite are obtained over a range of CP contact times from 0.5 to 5 ms. The HA monetite spectrum also shows an unmistakable increase in sideband intensities (cf. Figures 2c and 2d), although not so large as in HA–brushite. The monetite mixture was also studied at CP contact times in the range 1 to 7 ms; the differential enhancements are smaller but easily detectable. *In sharp contrast to the mixtures, the ACP sideband intensities are virtually unchanged in the cross-polarized spectra.* (Compare Figures 2e and 2f.)

A comparison of sideband intensities with and without cross polarization was also performed for octacalcium phosphate, which contains both protonated and unprotonated phosphate groups in the crystallographic unit cell. Unmistakable differential enhancements of sideband intensity were obtained with CP contact times 0.5 and 1 ms. The visual appearance of the spectra (not shown herein), is similar to that of the monetite apatite mixture (Figures 2c and 2d), showing that differential sideband enhancement can occur in an intimate mixture of protonated and unprotonated phosphates as well as in mechanical admixtures of phases.

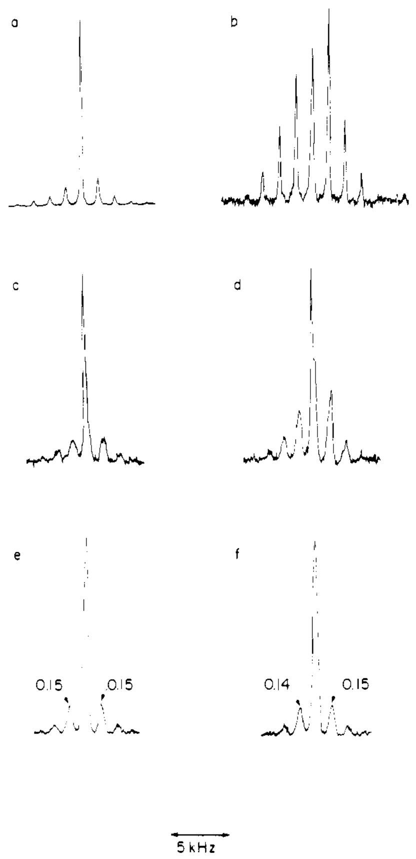

Figure 2. Proton-decoupled ^{31}P MAS spectra with and without cross polarization, at 20 °C: (a) 2:1 mixture by mass hydroxylapatite (Ca/P = 1.33) and brushite; spinning rate = 1.32 kHz, pulse interval 20 s, not cross polarized; (b) same as (a), but cross polarized with cross polarization contact time 1 ms; (c) 2:1 mixture by mass of commercial hydroxylapatite and monetite; spinning rate = 1.36 kHz, pulse interval 20 s, not cross polarized; (d) same as (c) but cross polarized with cross polarization contact time 0.5 ms; (e) amorphous calcium phosphate, spinning rate = 1.35 kHz, pulse interval 60 s, not cross polarized; (f) same as (e) but cross polarized with cross polarization contact time 3 ms and pulse interval 20 s.

The cross polarization of ACP at very short contact times was studied systematically. Figure 3 shows the ACP spectra at contact times of 0.1, 0.3, 0.7, and 1.0 ms. A modest differential enhancement is seen at 0.1 ms, but at 0.3 ms and above, the spectra are identical with those obtained at much longer CP contact times, e.g., 3 and 5 ms, and they are not readily distinguishable from spectra without cross polarization.

The data presented thus far show that any simple model of ACP as mixture of apatitic and dibasic calcium phosphate phases cannot be correct. The small enhancement of ACP sidebands at very short contact times may indicate the presence of small amounts of protonated phosphate, although not in sufficient quantity to explain the sideband intensities without cross polarization.

Spectra of ACP with and without CP were recorded at −70, −100, and −120 °C. The sideband intensities are equal with and without CP in all cases, within experimental error. Figure 4 shows the spectra at −120 °C. The overall increase in sideband intensity at lower temperatures is due to a slower spinning speed, not an increase in chemical shift anisotropy. These experiments were done to explore the possibility that ACP contains protonated phosphate groups which reorient rapidly at room temperature. The failure to observe differential sideband intensity enhancements

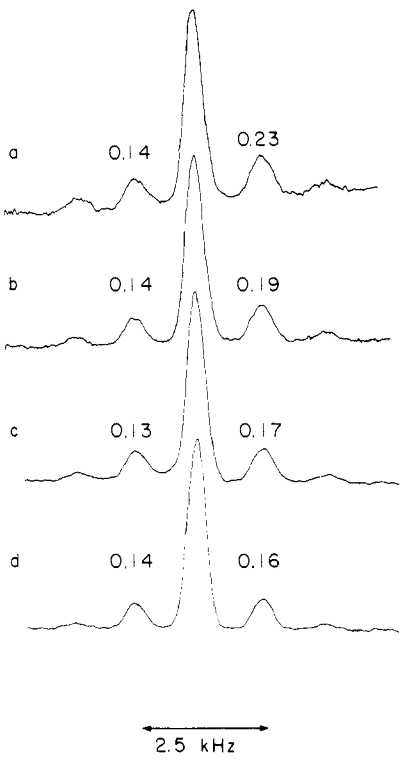

Figure 3. Proton-decoupled ^{31}P MAS spectra of amorphous calcium phosphate with different cross polarization contact times, at 20 °C, spinning rate = 1.28 kHz, pulse interval 4 s. The numbers above the first-order sidebands give the intensities relative to the center peak: (a) contact time 0.1 ms; (b) contact time 0.3 ms; (c) contact time 0.7 ms; (d) contact time 1.0 ms.

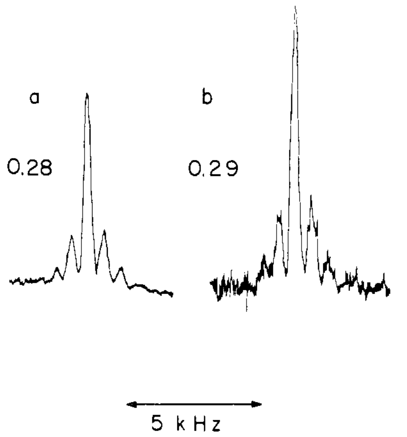

Figure 4. Proton-decoupled ^{31}P MAS spectra of amorphous calcium phosphate at −120 °C. The numbers give the averaged intensity of the first-order sidebands relative to the center peak: (a) cross polarized with 1 ms contact time, pulse interval 10 s, spinning rate = 800 Hz; (b) not cross polarized, pulse interval 60 s, spinning rate = 800 Hz.

at low temperature suggests but does not prove the absence of such protonated phosphates.

Figure 5a gives the spectrum without cross polarization of ACP which was vacuum dried at 450 °C to remove tightly bound water. The spectrum is quite similar to that of normal ACP except for a small decrease in the intensity of the outermost (second-order) sidebands. The absolute signal amplitude is roughly equal to that of normal ACP with the same spectrometer settings. However, the cross polarization behavior of dried ACP is greatly altered. Figures 5b and 5c show the free induction decays (single transient of ACP and dried ACP, both recorded with identical spectrometer settings for cross polarization. No CP signal is observed for the dried ACP. The comparison of ACP and dried ACP was repeated several times to ensure that the process of changing sample and restarting the spinner did not alter the probe tuning and thereby the CP conditions. Several variations of proton and phosphorus channel radiofrequency fields were also tried, including mismatches of the Hartmann–Hahn condition. In all cases, normal ACP gave good CP signals with a single shot, while dried ACP gave no discernible signal. We conclude that the failure to obtain a CP signal from dried ACP reflects the properties of the sample itself and is no way related to the spectrometer settings.

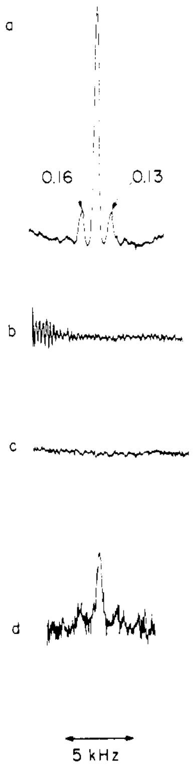

Figure 5. Comparison of dried and undried amorphous calcium phosphate at 20 °C: (a) proton-decoupled MAS spectrum of dried amorphous calcium phosphate, spinning rate = 1.36 kHz, pulse interval 60 s, not cross polarized; (b) single transient free induction decay of normal (undried) amorphous calcium phosphate with 3-ms cross polarization contact time and proton decoupling. Spinning rate = 1.33 kHz; (c) single transient-free induction decay of dried amorphous calcium phosphate, with cross polarization contact time and RF power as in (b); spinning rate = 1.36 kHz, proton decoupled; (d) cross-polarized spectrum of dried amorphous calcium phosphate, from average of 100 transients with 40-s pulse interval, 1-ms cross polarization contact time, and proton decoupling. See text for further results.

A sample of commercial hydroxylapatite was subjected to the same drying procedure employed for ACP. Comparison of dried and undried HA showed, as expected, no difference in cross polarization.

The entire series of experiments just described was completed within a week of the actual drying of the ACP sample. At that time no attempt was made to search for small CP signals by signal averaging. A series of experiments with signal averaging was tried several months later on the same sample which had been stored in a desiccator over $CaSO_4$ without vacuum. It was then found that a small signal could be observed, after averaging about 100 transients. Delay times of 10, 20, and 40 s were tried to determine whether the CP signal strength was dependent on the proton T_1. In all cases the signal obtained was reduced about 10-fold in intensity from the CP signal to normal ACP. Such a small signal would not appear above noise level in a single transient, so the results with averaging are consistent with those without. On the other hand, it is possible that signal was due to a small amount of rehydration during storage. The spectrum with 40 s delay, given in Figure 5d, shows suggestions of sidebands equal in intensity to those of the spectrum without CP, but the poor signal to noise precludes a definitive comparison.

Discussion

A number of conclusions concerning ACP may be drawn from the ^{31}P NMR results. In some instances the evidence of NMR is definitive; in others, the accumulated evidence from other techniques is more compelling. In what follows, we list the conclusions and review the supporting evidence.

ACP Is Not a Poorly Crystalline Hydroxylapatite. The spinning sidebands of ACP are much larger than those of any hydroxylapatite studied to date;[8,9] furthermore, ACP loses its cross polarization signal when vacuum dried at 450 °C, whereas HA does not. In any case, many other experiments have already established that ACP and HA are chemically distinct. Kinetic studies of HA formation from ACP show that the conversion requires uptake of hydroxide and involves dissolution of ACP with subsequent reprecipitation of HA.[3,15] In a thermochemical study, ignition of ACP at 600 °C gave tricalcium phosphate (TCP) and some pyrophosphate, whereas apatite did not give TCP at 600 °C.[16] Computer simulation of X-ray diffraction patterns of microcrystalline HA[17] and X-ray radial distribution function analysis of HA and ACP[2] also show that ACP is distinct from HA.

ACP Is Not a Mixture of Apatitic and Dibasic Calcium Phosphate Phases. This follows directly from the comparison of the cross-polarized spectra of ACP with those of the apatite–dibasic phosphate mixtures. While it is possible to match the sidebands of ACP and mixture spectra without cross polarization, the cross-polarized spectra distinguish clearly between ACP and the mixtures.

Further evidence for the above stated conclusion comes from the study of dried ACP. The spectra show large sidebands (relative to HA), but a much reduced cross polarization effect. If the large sidebands were due to the presence of protonated phosphate, then strong CP should result, in consequence of the proximity of protons and phosphorus. The observation of a spectrum which does not cross polarize yet still displays large sidebands argues strongly that these sidebands cannot be due to protonated calcium phosphate phases.

In the above discussion, we have used the term "calcium phosphate phases" advisedly, since it implies the existence of homogeneous domains whose structure and molecular dynamics are identical with those of the corresponding pure compounds. Conceivably, ACP could contain substantial amounts of protonated phosphate not in the form of any known phase. To argue for this possibility, one would have to postulate that the cross polarization due to protonated phosphate is largely obliterated by rapid molecular reorientation. If such were the case, one would expect to observe differential enhancement of sidebands at low temperature, whereas in fact one does not, at least to −120 °C. Furthermore, the comparison of octacalcium phosphate spectra with and without cross polarization shows that preferential enhancement of sidebands from protonated phosphate can occur in an intimate mixture of protonated and apatitic phosphate.

The observation of differential enhancements in OCP shows that cross polarization is fast compared to phosphorus–phosphorus spin diffusion between neighboring protonated and unprotonated phosphates. This should be true for *any* calcium phosphate, since spin diffusion rates depend on ^{31}P–^{31}P distances which are constrained in all cases by the size and packing geometry of the phosphate tetrahedra. Thus the failure to observe differential enhancements in ACP is not a consequence of spin diffusion and constitutes further evidence against the proposition that ACP contains protonated and unprotonated phosphate.

In sum, most of our observations are consistent with the view that the phosphate in ACP is chemically and structurally homogeneous and that ACP is therefore a unique, and well-defined, compound. The small sideband enhancement observed at very short CP contact times may reflect some minor heterogeneity.

Tightly Bound Water Molecules Are Probably Not of Intrinsic Importance in the Structure of ACP. It is well known that ACP contains 10–20% by weight of tightly bound water, which is removed by vacuum drying at elevated temperature.[2] X-ray radial distribution functions of dried and undried material suggest that drying does not alter the atomic arrangement of calcium and phosphorus. The sideband intensities of dried ACP suggest that its chemical shift anisotropy is similar to or identical with that of normal ACP. Inasmuch as the anisotropy is a sensitive indicator of local structure, the NMR and X-ray evidence are in accord. The loss of cross polarization upon drying indicates that the cross polarization arises from water protons.

A detailed model structure has been proposed for ACP,[2] and it is of interest to examine that model in light of our NMR results. The basic structural unit of the model is a $Ca_9(PO_4)_6$ cluster of diameter 9.5 Å. The clusters are presumed to pack randomly with respect to each other, forming the large 300 to 800 Å spheres which are observed by electron microscopy of ACP.[2,18] The tightly held water of ACP is believed to reside in the interstices between clusters. Thus, the only protons associated with ACP would be those of the interstitial water molecules. The complete loss of cross polarization which is found experimentally in thoroughly dried ACP is a predictable consequence of the structural model.

Finally, it is worth emphasizing that synthetic ACP is of interest primarily as a model compound for early bone mineral. A recent ^{31}P study has shown that the sideband intensities of early bone mineral from chick embryo may be closely matched by the sidebands of apatite–brushite mixtures, but no mention was made of cross polarization.[8] Our results with ACP suggest that comparisons of spectra with and without cross polarization studies are desirable in any future investigations of bone mineral by ^{31}P NMR.

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Registry No. ACP, 7758-87-4.

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