NONINVASIVE ANALYSIS OF BONE MINERAL CONTENT

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INTRODUCTION

Several pathological conditions in which bone mineralization is deficient have been observed. These can arise from a number of conditions including abnormalities in hormonal regulation, renal cortical damage and lack of adequate calcium and vitamin D in the diet. Osteoporosis, the most common condition, is associated with aging in both sexes and with alterations in hormonal regulation during menopause. In general terms, onset of the condition may involve a series of events that begin at the age when bone mass ceases to increase [i.e., about 30-40 years]. A number of factors may be involved, including regulation of calcitonin, parathormone, and 1,25-dihydroxyvitamin D levels. The 1α-hydroxylase activity of the kidney that is involved in conversion of vitamin D to its active form may decrease as part of the aging process and/or in response to lower secretion of estrogen. The resulting decrease in intestinal absorption of calcium places an increased demand on the skeletal system. The mineral content of skeletal tissue decreases, especially in trabecular bone (i.e., bone of the type found in the spine, hip, wrist and heel), and there is a subsequent increase in the incidence of fractures. The skeletal complications of osteoporosis become apparent in women as early as 40-50 years of age, but somewhat later in men. Fractures of the hip can be life-threatening in the aged, and the annual cost associated with them is more than 3 billion dollars in the United States alone.

There is at the present time a clinical need for a rapid assay of the mineral content of bone. Procedures that require the taking of a bone biopsy sample are very painful and distressing to the patient, and the risk of infection is high. A more desirable approach would be to perform a noninvasive assay of the bone while it is still in the patient. The best procedures available at the present time are dual-photon absorptiometry and quantitative computed tomography (1). There is some question, however, of whether routine screening of healthy women of child-bearing age should be done with ionizing radiation.

In this work, we demonstrate the feasibility of quantifying the mineral content of a stationary sample of bone with ^{31}P NMR spectroscopy. These experiments differ from previous reports (2-7) in that cross polarization (CP), magic angle sample spinning (MAS) and proton decoupling, which cannot be applied readily to a clinical assay, are not used. Instrumentation such as that in Figure 1 is considered.

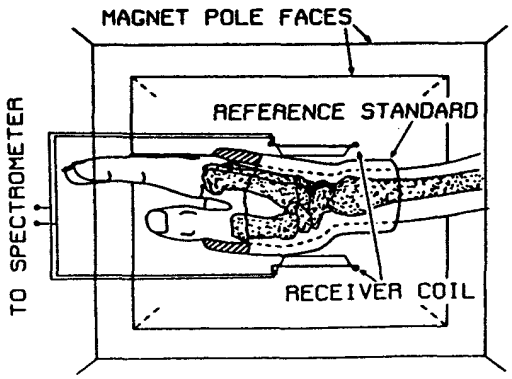

Figure 1. Schematic view of the wrist of a patient and a reference standard in the magnet of an NMR spectrometer.

EXPERIMENTAL

Hydroxyapatite was obtained from Bio-Rad. Brushite and potassium hexafluorophosphate [KPF_6] were from Aldrich. Bone (i.e., femur) and skeletal muscle were excised from a member of the bovine species prior to analysis. All materials were used without further purification or drying. ^{31}P NMR spectra were recorded with a Nicolet NT-150 spectrometer equipped with a 20 mm high-resolution (i.e., liquids) probe.

RESULTS AND DISCUSSION

The predominant mineral forms in adult bone are hydroxyapatite [$Ca_{10}(OH)_2(PO_4)_6$] and apatite in which hydroxide is substituted by carbonate (i.e., carbonatoapatite). The early steps of apatite deposition are not fully understood, but it appears that brushite [$CaHPO_4 \cdot 2H_2O$] is deposited first. This is believed to redissolve and be converted to amorphous calcium phosphate (a noncrystalline association of ions), which in turn yields octacalcium phosphate [$Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$]. The final step is considered to be conversion of octacalcium phosphate to apatite, the least soluble mineral of this group (3,8-10).

The MAS ^{31}P NMR spectrum of apatites consists of a single peak with small first-order spinning side bands at moderate sample spinning rates (2,3) and is unaffected by proton decoupling (7). In sharp contrast, the protonated mineral forms and the phospholipids of membranes exhibit multiple spinning sidebands (2,3), and dipolar broadening decreases resolution and signal-to-noise of their spectra in the absence of high-power resonant decoupling (7). Thus, one can readily distinguish PO_4^{3-} of apatites in fresh, intact bone from HPO_4^{2-} of the more soluble calcium phosphate minerals and the phosphate headgroups of the phospholipids in cellular membranes on the basis of the intensities of spinning sidebands and the effects of proton decoupling in the MAS ^{31}P NMR spectra (7).

For ^{31}P NMR to be used clinically, however, the measurement must be made within a stationary patient. The above results suggest that, with only the minor motional averaging expected in intact biological tissues, apatites should yield a resonance that is broader than those from metabolites dissolved in cytosol but narrower than those from either phosphate head groups of membranes or the more soluble, protonated mineral forms. The phosphate groups of apatites exhibit less apparent anisotropy of shielding tensors and negligible dipolar broadening compared to the protonated phosphates of phospholipids and the other mineral forms. Experimental results bear out these theoretical expectations (Fig. 2).

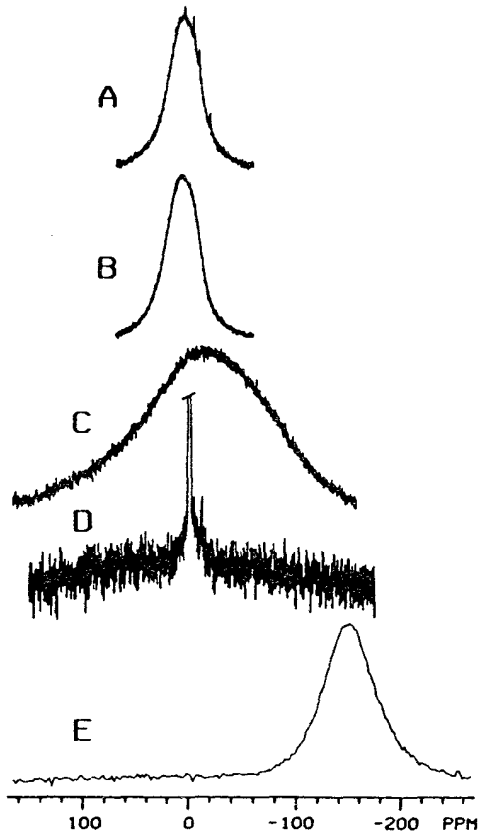

Figure 2. Undecoupled ^{31}P NMR spectra of stationary samples of (A) solid bone immersed in a solution of 4mM ATP, 50mM Tris and 150mM KCl, (B) powdered hydroxyapatite, (C) powdered brushite, (D) excised skeletal muscle, and (E) powdered KPF_6. Hydrolysis of ATP and other cytosolic phosphoesters is apparent from the narrow inorganic phosphate peak at +3.2 ppm in A and D.

These observations indicate that the apatite content of bone can be measured in a stationary patient without interference from the head-groups of membrane phospholipids in skeletal muscle. The protonated mineral forms, which are believed to be involved in the early stages of mineral deposition, will yield broad, unresolved lines that contribute to the overall spectral intensity. However, these are only a minor part of the mineral in adult bone. They have been observed in the MAS ^{31}P NMR spectra of lyophilized, powdered chicken bone (6) but are not readily detected in intact mouse bone (7).

The relaxation properties of bone make quantitative measurements difficult. The spin-lattice relaxation time, T_1, of apatite is measured in seconds, but the spin-spin relaxation time, T_2, is measured in milliseconds. Thus the total polarization of the sample cannot be measured directly from the area of the recorded free induction decay [FID]. This T_2-dominated decay (heavy line in Fig. 3) provides only an indication of the initial height, rather than the area, of the time-domain signal from spin-lattice relaxation (narrow line in Fig. 3).

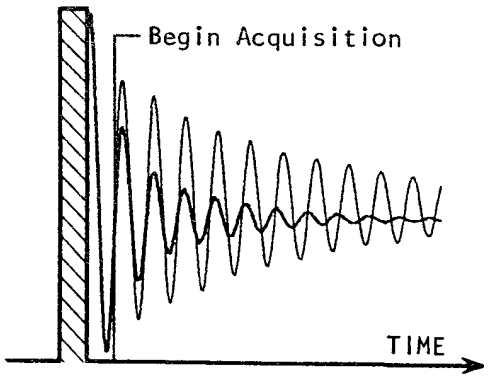

Figure 3. Spectra are obtained with a simple observe pulse. A short delay occurs between the pulse and signal acquisition. The FID that is obtained (indicated by the heavy line) is dominated by a very short T_2. The narrow line is a schematic indication of the decay of polarization according to the much longer T_1 and cannot be detected by this simple pulse sequence. Times are not drawn to scale.

We have taken the tack of using a quantitative reference standard for these measurements. The reference standard must fulfill several criteria. First, the material must not be toxic or highly reactive if it is to be used in the clinical setting. Second, the signal from the reference must not overlap that of the bone mineral. Third, the line width and intensity of the reference signal should be similar to that from bone. A corollary is that the T_1 and T_2 values of the reference material should be the same as that of apatite. This limits the constraints on the analog-to-digital converter. Furthermore, this decreases the strong dependence of the observed intensity of the T_2-dominated FID on minor variations in the length of the delay between the end of the observe pulse and the beginning of data acquisition (Fig. 3). These requirements indicate the use of a solid reference material. Thus, a forth requirement is that a solid reference material yield a spectrum with the above characteristics in the absence of magic angle sample spinning and/or proton decoupling. It also would be advantageous if the signal intensity of the reference material were insensitive to high-power proton decoupling. We have found solid KPF_6 to fulfill these requirements relatively well.

Insight into how precisely bone mineral content might be measured with this reference material is provided in Figs. 4 and 5. A layer of KPF_6 in the bottom of a stationary 20 mm NMR tube was positioned such that it resided in the bottom portion of the receiver coil, and a ^{31}P NMR spectrum was recorded (Fig. 4F). Then apatite was poured on top of the KPF_6 in five separate portions. After each addition another spectrum was recorded (Fig. 4E-A). The final addition (Fig. 4A) was made such that all of the last aliquot was outside the receiver coil. The relative peak areas in each spectrum were determined by integration, and the ratios of peak areas thus obtained were plotted versus the weight of apatite in the NMR tube at the time each spectrum was recorded (Fig. 5). Peak-area ratios are found to be linearly related

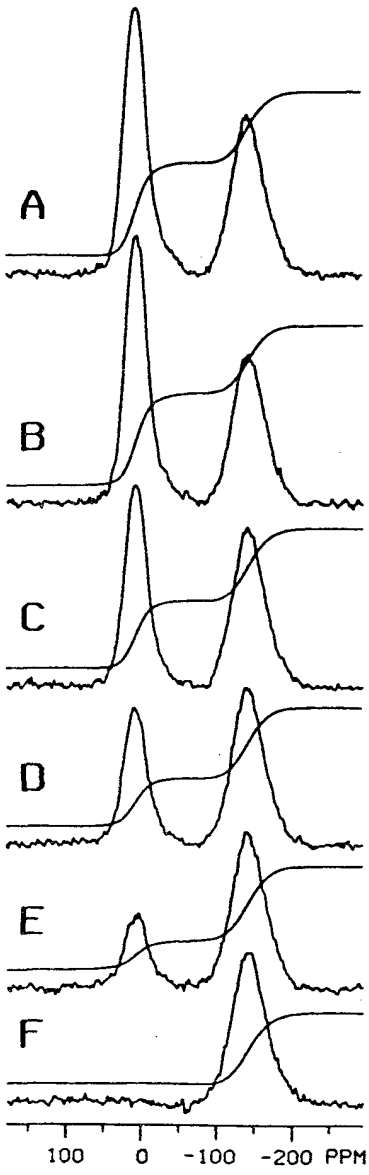

Figure 4. ^{31}P NMR spectra of varying amounts of powdered hydroxyapatite [(A) 1.9g - (F) 0g] layered on top of powdered KPF_6 [2g] in a stationary NMR tube. Each spectrum is the result of only a single acquisition. Proton decoupling was not used. Spectral integrations used to plot the peak-area ratios in Fig. 5 are represented by the step curves.

to the weight of hydroxyapatite within the receiver coil of the NMR spectrometer. The error level for a single measurement appears to be in the range of 3%.

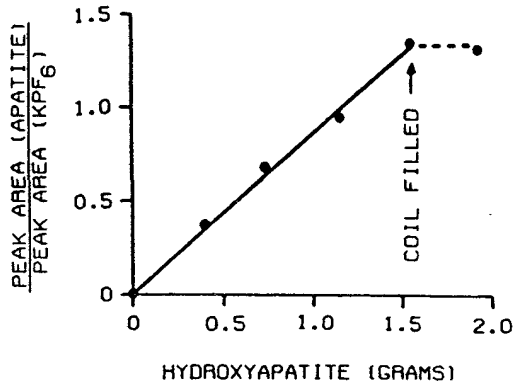

Figure 5. Plot of the ratios of peak areas in the ^{31}P NMR spectra of Fig. 4 vs. the weight of powdered hydroxyapatite in the NMR tube during each acquisition. Each point is the result of a single measurement.

Preliminary experiments suggest that this assay can be performed with receiver coils of larger radius which accomodate, for example, the wrist as suggested schematically in Fig. 1. ^{31}P NMR spectra of solid bone in juxtaposition with a flexible reference standard of powdered KPF_6 in polyethylene were recorded with a home-built 55 mm receiver coil (11). This coil was the largest our magnet could reasonably accomodate. A sample of bone equivalent in volume to an average wrist yielded a spectrum with reasonable signal-to-noise from a single acquisition (11).

For routine screening of osteoporosis, bone mineral content needs to be measured with a precision of about 1%. The above results suggest that this probably can be achieved by further optimizing the instrumental conditions and/or averaging the results of more than one measurement. Although the noise level of the spectra can be improved greatly by averaging multiple acquisitions, a delay between pulses of several minutes is required for the peak areas to be quantitative (Fig. 6). Such a time constraint is not very practical in the clinical setting. Another alternative is to make single-acquisition measurements of trabecular bones in different parts of the body. This would provide statistical reliability to the measurement, while

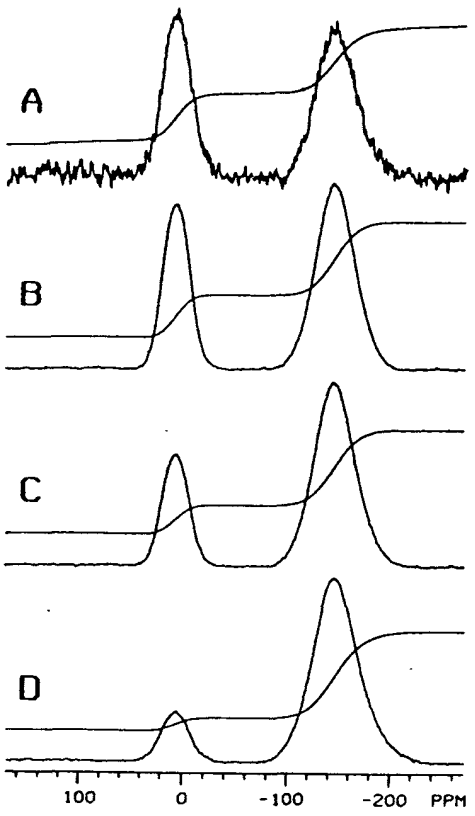

Figure 6. ^{31}P NMR spectra of stationary bone surrounded by a solid matrix of KPF_6 in polyethylene. The number of pulses used to record each spectrum were (A) 1, and (B-D) 100. The delays between pulses were (B) 60 sec, (C) 26 sec, and (D) 5 sec. The step curves are digital integrations of relative peak areas.

avoiding the time constraints caused by the slow spin-lattice relaxation of ^{31}P in the solid state. The difficulty that mineral is lost from different bones at different rates also would be overcome to some extent.

Consensus has not yet been reached as to which trabecular bone (i.e., wrist, heel, spine or hip) should be analyzed. It seems likely that diagnosis will include analysis of the spine and hip, whereas routine screening will be performed with the extremities, as was recently suggested (12). This has obvious ramifications for the use of ^{31}P NMR in the diagnosis and routine screening of osteoporosis. Analysis of the spine and hip would need to be performed in a whole-body magnetic resonance imaging system with the ability to record NMR spectra. Measurement of bone mineral content in the extremities could be performed in a magnet with a much smaller gap, but a permanent magnet would need to be used to make the equipment economically acceptable for the average physician's office. Thus, the clinical assay may need to be performed in a magnet of only one-tenth the field used for this study. This should not be a serious constraint, however. Since the frequency separations between the shielding tensors and between the dipolar coupling tensors are larger at higher magnetic field strengths, the linewidths of ^{31}P peaks from minerals in stationary bone will cover a narrower frequency range in lower-field magnets. The apparent widths of the peaks will be narrowed, and the requirements for frequency response of the rf transmitter and receiver can be eased. Since the decreased sensitivity of NMR at lower magnetic field strength should be offset by the apparent narrowing of these broad resonances, the signal-to-noise should remain similar to that reported above.

Two major stumbling blocks have impeded efforts toward prevention of osteoporosis (12). First, the cause of osteoporosis must be understood for treatment or preventive measures to be most effective. Second, a method to measure the presence and extent of osteoporosis is needed not only for early diagnosis, but also to assess treatment. NMR spectrometry could be used repeatedly to assess the patient's response to treatment since the technique is noninvasive and does not use ionizing radiation. The ability to make repetitive measurements could prove important for determining the cause of mineral loss in individual patients.

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