Concentration of hydroxyl groups in dental apatites: a solid-state ^{1}H MAS NMR study using inverse $^{31}P \rightarrow {}^{1}H$ cross-polarization†

Joanna Kolmas and Waclaw Kolodziejski*

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The concentration of structural hydroxyl groups in the apatite mineral of enamel, dentin and cementum of healthy human teeth was estimated by reference to stoichiometric hydroxyapatite to be 73 ± 3, 18 ± 2 and 18 ± 1%, respectively.

Mineral of bone and hard dental tissues is composed of apatites, compounds with a crystal structure similar to that of calcium hydroxyapatite (HA):[1] $Ca_{10}(OH)_2(PO_4)_6$. In enamel, dentin and cementum, the apatite mineral is accompanied by an organic matrix and water (Table 1). The differences in the chemical composition of the hard dental tissues reflect their functions and tailor their biological and mechanical properties.

Apart from the inorganic/organic ratio, calcified tissues have other, probably inter-related, chemical features which are crucial for their performance in a living organism.[1g,4] Features involving the mineral part are: nanocrystallinity,[4a,b] ionic substitution into the apatite crystal lattice resulting in its non-stoichiometry,[1b,4c] deficiency of the crystal lattice in the OH groups,[4d–f] crystal surface structure[4e,g–j] and hydration,[4j,k] possible presence of interstitial water[4j,k] and various phenomena at the apatite mineral/organic matrix interface.[4j,l,m]

We have measured the relative concentrations of the structural OH groups in the mineral of hard dental tissues‡ using an unusual solid-state NMR technique: $^{31}P \rightarrow {}^{1}H$ cross-polarization.§

Typical synthetic and biological apatites crystallize in the hexagonal space group $P6_3/m$.[5] The intracrystalline OH groups, hereafter called structural hydroxyl groups, are located at the edges of unit cells in the — O–H O–H O–H O–H — columns, parallel to the *c*-axis. The oxygen atoms are too distant (0.344 nm) from each other to form O–H···O hydrogen bonds, so that the ^{1}H NMR peak of the structural OH groups from apatites, under magic-angle spinning (MAS), appears at 0 ppm (Fig. 1).[1g,4e]

Biological apatites are in fact carbonatoapatites, with two kinds of substitutions occurring in the crystal lattice:[1b] CO_3^{2-} for OH^- (type A) and CO_3^{2-} for PO_4^{3-} (type B). In dental apatites, OH^- ions are partially replaced by the F^- ions.[1b] Because of those substitutions, and possibly the nanocrystallinity, the material is deficient in structural OH groups.[4d–f] One may conjecture that the OH groups can be counted by integrating the peak at 0 ppm in a conventional ^{1}H MAS NMR spectrum. In practice, for biological apatites there is usually a massive resonance of water at *ca.* 5.4 ppm and organic peaks are spread all over the proton spectrum,[1g,4e] obscuring the relatively small peak from the structural OH groups in dentin (Fig. 1c) and cementum (not shown, spectrum fairly similar). For bone, this peak has been better visualized using 2D HETCOR NMR.[4e,f] Using this technique, the content of the structural OH groups was estimated at 21% of the stoichiometric value for HA.[4f] The concentration of the structural OH groups in apatites of the hard dental tissues was previously completely unknown.

Cross-polarization (CP) is routinely used to enhance low-intensity solid-state NMR peaks from dilute spins S by the polarization transfer from abundant spins I, usually protons.[6] Since the CP process is mediated by the I–S dipolar couplings, it proceeds faster and gives higher signal intensity if the spins involved are closer in space. In view of this and for relaxation reasons, peak intensities in the CP spectra are different from the intensities in the Bloch-decay (BD) spectrum (a conventional pulse-acquisition experiment) and vary with the contact time over which the polarization transfer is performed. In the case of similar populations of spins I and S, CP is possible in both directions, that is in the I → S and S → I modes. The inverse S → I CP experiments are rare and rather difficult so that only a few examples of $^{31}P \rightarrow {}^{1}H$ CP have been published.[7] However, the technique is worth trying, because unwanted peaks from protons distant from ^{31}P become effectively eliminated and the OH peak exposed (Fig. 1).

Performance of our inverse CP experiments, considering number of transients§ and the signal-to-noise ratio (Fig. 1), decreased in the following series, consistent with the apatite content: HA > enamel > dentin > cementum (the latter not shown). It has been subsequently found that this series also reflects the structural OH concentration. We admit that the inverse-CP experiments are time-consuming, because they require long repetition times (see ESI material) and suffer from loss of sensitivity compared to the BD experiments. The inverse CP peaks for HA and enamel are broader than their BD counterparts (Fig. 1), because the former were measured under much slower

Table 1 Chemical composition (wt%) and the hydroxyl content (% in reference to the stoichiometric HA) of the studied tissues

	Enamel	Dentin	Cementum
Apatite mineral[a]	98.1	74.1	71.3
Organic matrix[a]	1.2	21.2	25.4
Water[a]	0.7	4.7	3.3
Hydroxyl content[b]	73 ± 3	18 ± 2	18 ± 1

[a] Thermogravimetric analysis in air at a heating rate of 2 K min^{-1} (DuPont 910 Thermogravimetric Analyzer). Water released up to 473 K was determined. [b] Solid-state NMR (see text).

Medical University of Warsaw, Faculty of Pharmacy, Department of Inorganic and Analytical Chemistry, ul. Banacha 1, 02-097 Warszawa, Poland. E-mail: waclaw@farm.amwaw.edu.pl; joanna.kolmas@wp.pl; Fax: +48 (0)22 5720 784; Tel: +48 (0)22 5720 784

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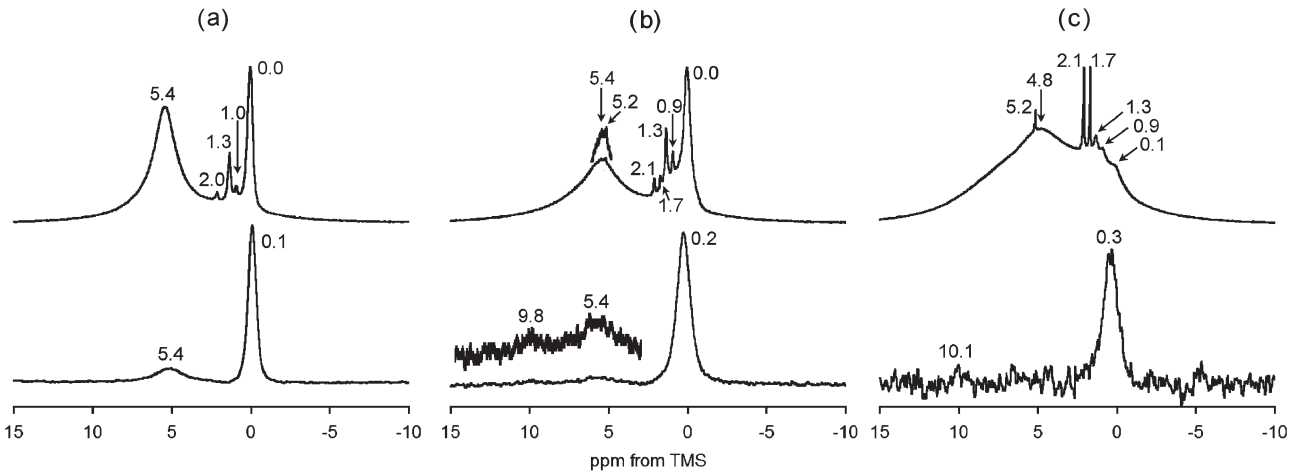

Fig. 1 Proton MAS NMR spectra of (a) HA100, (b) enamel and (c) dentin. Upper and lower spectra were acquired with the Bloch-decay and $^{31}P \rightarrow {}^{1}H$ CP techniques, respectively. For the inverse CP, the contact time of 2 ms was used. The spectra are scaled to the same maximum intensity. Magnified insets for enamel reveal spectral details. Spinning sidebands are outside the presented spectral region.

MAS. Another reason may be that CP accesses sites differently from BD. For HA, the $^{31}P \rightarrow {}^{1}H$ CP signal at 0 ppm was sufficiently strong to study its CP kinetics (Fig. 2). The CP intensity varied with the contact time according to the non-classical kinetic model[6c] with the CP time constant of 0.25 ms and the relaxation time $T_{1\rho} = \infty$ for ^{31}P. This dependence assured us that the $^{31}P \rightarrow {}^{1}H$ CP experiment had been adjusted properly and assisted us in selecting the contact time of 2 ms for subsequent quantitative analysis.

The OH counting for enamel and synthetic apatite was possible using ^{1}H BD NMR under ultra-high MAS. Thus, in the initial analytical step, we acquired the BD spectra of enamel and HA800 together with the spectra of adamantane and NH_4Cl, which we used as two independent standards of the absolute ^{1}H signal intensity. For all the measurements, we applied the same acquisition and processing parameters. The recycle delay was selected to be sufficiently long to comply with the relaxation requirements of the four materials. We used peak areas from computer fittings/deconvolutions (program NUTS, Acorn NMR). For NH_4Cl, it was necessary to take into account first spinning sidebands. For other samples, they were negligible. In the calculations, we took into account a mass of the materials in rotors as well as the mineral content in enamel (Table 1). The analytical procedure, including the NMR measurements, was repeated three times. The materials were measured in an alternating sequence imposing probe retuning before each spectrum acquisition. In this way, the OH content in enamel was estimated at 73 ± 3% of the stoichiometric HA value. For HA800, we got 94 ± 1%, which result looks correct considering the 90% value obtained by Arends *et al.*[8] for well-defined pure HA, carefully prepared and characterized as a standard for comparative studies.

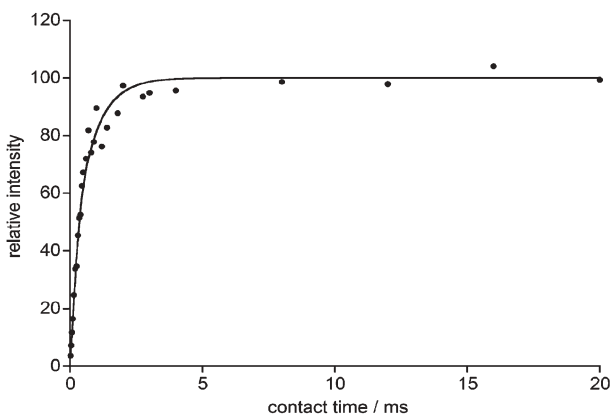

Fig. 2 Kinetics of the inverse CP for the 0.1 ppm peak of HA100.

The principal analytical procedure involved inverse CP experiments on enamel, dentin and cementum. Unfortunately, a simple approach using an internal standard added to a sample has not been possible. Note that there is no salt of phosphorous acid which fulfils the following three conditions: (1) contains protons, (2) easily undergoes inverse CP and (3) its ^{1}H MAS NMR spectrum does not overlap with that of apatite. In such circumstances, enamel with the previously determined OH content served as the absolute intensity standard for dentin and cementum. The reliability of this procedure has been confirmed by the study of the CP kinetics in the dental tissues (see ESI material). Again, peak areas were computed. For enamel, it was necessary to include the first spinning sidebands. For other samples, they were negligible. As before, the calculation involved a load of the substances in rotors and their mineral content, which is different for the dental tissues (Table 1). In order to improve accuracy, the measurements were repeated 6, 3 and 3 times for enamel, dentin and cementum, respectively, with the sample switching. Because of similar magnetic susceptibility of the materials, only slight adjustments of probe tuning were required. We found that dentin and cementum both contain 18% of the apatite structural OH groups compared to the stoichiometric value for HA (Table 1). This number is very close to the bone result of 21%.[4f] It follows that the structure of apatites of dentin and cementum is probably similar to that of bone.

The inverse-CP technique also provides information indispensable for the assignment of the ^{1}H apatite peaks. Consider that a reasonable water CP peak appears only for HA100 (Fig. 1). Then, note that ^{1}H BD lines of water for hard dental tissues are evidently

broader than that for HA100. Furthermore, in the dentin BD spectrum the water peak is moved to 4.8 ppm, possibly due to weaker or less extensive hydrogen bonding. This may suggest the presence of two different pools of water. Our interpretation is that in HA100, and to a certain extent in enamel, water is adsorbed on the crystal surface of apatite. In contrast, in the hard dental tissues water must be generally remote from ^{31}P, thus the $^{31}P \rightarrow {}^{1}H$ CP to water is inefficient. We infer that, at least in dentin and cementum, water is predominantly located in the organic matrix. Otherwise, it would be necessary to assume for dentin and cementum that water on the apatite crystal surface and/or in the apatite/organic matrix interfacial region is very mobile. However, such water would produce sharp proton BD peaks, contrary to the presented experimental results. Our interpretation and the increase of the water BD peak from enamel to dentin are in accordance with the water and organic matrix contents reported in Table 1. Another interesting observation is that the inverse-CP peaks at 0 ppm for enamel and dentin are broader than that from HA100 (Fig. 1). We believe that this is caused by a greater disorder of the structural OH groups in crystals of the dental apatites than of the synthetic HA. Finally, we note that inverse CP reveals small peaks at *ca.* 10 ppm in the spectra of enamel and dentin (Fig. 1). They probably come from HPO_4^{2-} ions participating in strong hydrogen bonds. A similar peak was found at 10.4 ppm in the proton BD spectrum of brushite ($CaHPO_4{\cdot}2H_2O$), acquired with MAS at 40 kHz.[1g,4e]

The ^{1}H BD spectra (Fig. 1) contain minor, sharp extra peaks in the 0–5 ppm region. The peaks at 5.2, 2.1 and 1.7 ppm, considering their increased intensity from enamel to dentin, are from the organic matrix. Consequently, the corresponding protons do not cross-polarize from ^{31}P of the apatite mineral. The peaks at 2.0, 1.3 and 1.0 (0.9) ppm, best seen for HA100, are from proton sites in apatites. Their assignment is a matter of a dispute in the literature.[4k,9,10] We want only to stress that the corresponding ^{1}H sites do not cross-polarize from ^{31}P, so the proper explanation should be consistent with this fact. We feel that the experimental evidence is at present insufficiently conclusive to solve this problem.

In summary: (1) We have applied an unusual solid-state NMR technique, that is inverse $^{31}P \rightarrow {}^{1}H$ CP, to the study of synthetic and dental apatites. Whole hard dental tissues were studied. (2) We have counted the structural OH groups in those materials. (3) The dental apatites are deficient in the structural OH groups, compared to the stoichiometric content in $Ca_{10}(OH)_2(PO_4)_6$. (4) The concentration of the structural OH groups in the apatites of dentin and cementum is very close to that of bone. (5) Pure synthetic hydroxyapatite is also slightly dehydroxylated. (6) The inverse-CP technique provides new structural information and can aid spectral assignment.

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Notes and references

‡ Human hard dental tissues (enamel, dentin and cementum) were isolated from *ca.* 120 healthy molars, extracted for orthodontic reasons. Separation of cementum from dentin was verified using optical and IR microscopes. The samples were then dried in air and powdered. Hydroxyapatite (HA) was synthesized by the wet chemical method.[2] Sample HA100 was dried at 373 K and sample HA800 was calcined at 1073 K. Those materials had specific surface areas of 85.3 and 15.5 $m^2\ g^{-1}$, respectively. Purity of HA was checked by powder XRD and IR. Adamantane and NH_4Cl, used as proton NMR intensity standards, were of the analytical grade. Before use, NH_4Cl was exhaustively dried in a vacuum oven.

§ All the measurements were done at 298 K using a Bruker Avance 400 WB spectrometer with the resonance frequencies of 400 and 162 MHz for ^{1}H and ^{31}P, respectively. The $^{31}P \rightarrow {}^{1}H$ CP experiment was set on HA100. We used a 4 mm double-bearing Bruker CP/MAS probe with a MAS rate of 7 kHz and a $\pi/2$ ^{31}P pulse of 3.15 μs. For HA, 32 transients with a recycle delay of 30 s were used. For the hard dental tissues, 104 transients with a recycle delay of 600 s were used. The inverse CP spectra for the quantitative analysis were recorded with a contact time of 2 ms. The ^{1}H BD NMR spectra were acquired using a 2.5 mm high-speed double-bearing Bruker MAS probe. A probe background was carefully subtracted.[3] We used a MAS rate of 30 kHz, a $\pi/2$ ^{1}H pulse of 1.65 μs, 16 transients with a recycle delay of 50 s. Exponential apodization with LB = 5 Hz was applied for all the BD FIDs, while for the inverse CP we used LB of 5 and 50 Hz for HA and the hard dental tissues, respectively.

1 (*a*) S. Man, *Biomineralization*, Oxford University Press, Oxford, 2002; (*b*) R. Z. LeGeros, in *Hydroxyapatite and related materials*, ed. P. W. Brown and B. Constanz, CRC Press, Boca Raton, 1994, pp. 3–28; (*c*) H. Aoki, *Science and medical applications of hydroxyapatite*, Takayama Press, Tokyo, 1991; (*d*) R. Z. LeGeros, *Monographs in oral science 15. Calcium phosphates in oral biology and medicine*, S. Karger, Basel, 1991; (*e*) R. A. D. Williams and J. C. Elliott, *Basic and Applied Dental Biochemistry (Churchill Livingstone Dental Series)*, Longman, Edinburgh, 1989; (*f*) E. P. Lazari, *Dental Biochemistry*, Lea & Febiger, Philadelphia, 1976; (*g*) W. Kolodziejski, *Top. Curr. Chem.*, 2005, **246**, 235.

2 A. Slosarczyk, E. Stobierska, Z. Paszkiewicz and M. Gawlicki, *J. Am. Ceram. Soc.*, 1996, **79**, 2539.

3 Q. Chen, S. S. Hou and K. Schmidt-Rohr, *Solid State Nucl. Magn. Reson.*, 2004, **26**, 11.

4 (*a*) J. D. Pasteris, B. Wopenka, J. J. Freeman, K. Rogers, E. Valsami-Jones, J. A. M. van der Houven and M. J. Silva, *Biomaterials*, 2004, **25**, 229; (*b*) B. Wopenka and J. D. Pasteris, *Mater. Sci. Eng., C*, 2005, **25**, 131; (*c*) Y. Wu, M. J. Glimcher, C. Rey and J. L. Ackerman, *J. Mol. Biol.*, 1994, **244**, 423; (*d*) C. Rey, J. L. Miquel, L. Facchini, A. P. Legrand and M. J. Glimcher, *Bone*, 1995, **16**, 583; (*e*) A. Kaflak-Hachulska, A. Samoson and W. Kolodziejski, *Calcif. Tissue Int.*, 2003, **73**, 476; (*f*) G. Cho, Y. Wu and J. L. Ackerman, *Science*, 2003, **300**(5622), 1123; (*g*) Y. Wu, J. L. Ackerman, H.-M. Kim, C. Rey, A. Barroug and M. J. Glimcher, *J. Bone Miner. Res.*, 2002, **17**, 472; (*h*) M. Jarlbring, D. E. Sandström, O. N. Antzutkin and W. Forsling, *Langmuir*, 2006, **22**, 4787; (*i*) C. Jäeger, T. Welzel, W. Meyer-Zaika and M. Epple, *Magn. Reson. Chem.*, 2006, **44**, 573; (*j*) E. E. Wilson, A. Awonusi, M. D. Morris, D. H. Kohn, M. M. J. Tacklenburg and L. W. Beck, *J. Bone Miner. Res.*, 2005, **20**, 625; (*k*) E. E. Wilson, A. Awonusi, M. D. Morris, D. H. Kohn, M. M. J. Tacklenburg and L. W. Beck, *Biophys. J.*, 2006, **90**, 3722; (*l*) C. Jäeger, N. S. Groom, E. A. Bowe, A. Horner, M. E. Davies, R. C. Murray and M. J. Duer, *Chem. Mater.*, 2005, **17**, 3059; (*m*) G. Goobes, P. S. Stayton and G. P. Drobny, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2007, **50**, 71.

5 K. Sudarsanan and R. A. Young, *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.*, 1969, **25**, 1534.

6 (*a*) A. Pines, M. G. Gibby and J. S. Waugh, *J. Chem. Phys.*, 1973, **59**, 569; (*b*) E. O. Stejskal and J. D. Memory, *High resolution NMR in the solid state. Fundamentals of CP/MAS*, Oxford University Press, Oxford, 1994; (*c*) W. Kolodziejski and J. Klinowski, *Chem. Rev.*, 2002, **102**, 613.

7 (*a*) R. C. Crosby, R. L. Reese and J. F. Haw, *J. Am. Chem. Soc.*, 1988, **110**, 8550; (*b*) W. Kolodziejski and J. Klinowski, *Chem. Phys. Lett.*, 1997, **206**, 597; (*c*) W. Kolodziejski and J. Klinowski, *J. Phys. Chem.*, 1997, **101**, 3937; (*d*) J. Kolmas, Ph. D. Thesis: *Solid-state NMR and IR studies of synthetic and dental apatites*, Medical University of Warsaw, 2006.

8 J. Arends, J. Christoffersen, M. R. Christoffersen, H. Eckert, B. O. Fowler, J. C. Heughebaert, G. H. Nancollas, J. P. Yesinowski and S. J. Zawacki, *J. Cryst. Growth*, 1987, **84**, 515.

9 J. P. Yesinowski and H. Eckert, *J. Am. Chem. Soc.*, 1987, **109**, 6274.

10 D. E. Sandström, M. Jarbling, O. N. Antzutkin and W. Forsling, *Langmuir*, 2006, **22**, 11060.

