Article

Homogeneous Nucleation of Hydroxyapatite, $\text{Ca}_5\text{OH}(\text{PO}_4)_3$, at 37 °C

Hans Erik Lundager Madsen

Chemistry Department, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen Ø, Denmark; helm@chem.ku.dk

Received: 20 May 2020; Accepted: 6 July 2020; Published: 11 August 2020

Abstract: Precipitation of the calcium phosphate hydroxyapatite, $\text{Ca}_5\text{OH}(\text{PO}_4)_3$, is studied by simple mixing of reagent solutions and measurement of light scattering (turbidimetry) at six different wavelengths from 300 to 800 nm. Measured turbidities are analyzed using Mie's theory of light scattering from small particles. Results are interpreted in terms of classical theory of homogeneous nucleation, and from this the surface free energy of crystals is determined. The low value thus found is explained as the effect of protonation of the strongly basic anions hydroxide and phosphate at the crystal surface. Relatively large particles registered by turbidimetry are shown to be not monocrystals, but aggregates of nanocrystals.

Keywords: calcium phosphate; hydroxyapatite; light scattering; homogeneous nucleation; surface free energy; surface protonation

1. Introduction

Apatite, $\text{Ca}_5(\text{OH}, \text{F})(\text{PO}_4)_3$, is by far the most abundant phosphate mineral in Nature. It constitutes the mineral phase of bone and teeth in vertebrates. Numerous publications have appeared over the last 150 years dealing with the physical and chemical properties of this substance, both of natural and synthetic origin, including its crystallization and dissolution [1–5]. Nevertheless there are still unsolved issues in relation to surface properties of the crystals, in particular as these influence the crystallization process. An example is that the initial product, persistent for some time, is characterized as amorphous hydrous tricalcium phosphate, $\text{Ca}_3(\text{PO}_4)_2 \cdot x\text{H}_2\text{O}$ [6]. This assumption is, naturally, based on the absence of X-ray reflections. Other investigations using high-resolution transmission electron microscopy and electron microdiffraction have, however, shown that such a precipitate consists of nanocrystals with apatite structure, even when the sample was taken as short as 15 s after mixing of reagents [7]. This means that certain previous findings should be reinterpreted [8]. A relatively recent communication invoking nonclassical theory of calcium phosphate crystallization points in this direction as well [9].

In the present paper we present a method of determination of kinetics of homogeneous nucleation of hydroxyapatite (HAp) crystals at 37 °C using measurement of light scattering (turbidimetry) on suspensions of crystals during the process of precipitation. The results are used for determination of surface free energy of crystals and interpretation of earlier findings from precipitation experiments.

2. Materials and Methods

2.1. Apparatus

pH was measured with a Radiometer PHM 240 pH-meter (Radiometer, Copenhagen, Denmark), using a Metrohm Solitrode combination electrode (Metrohm Nordic ApS, Glostrup, Denmark). For calibration ISO standard buffers were used: potassium tetraoxalate, 0.05 m, pH = 1.679, potassium hydrogen phthalate, 0.05 m, pH = 4.005, and potassium dihydrogen phosphate + disodium hydrogen

phosphate, both 0.025 M, pH = 6.865, all values applying to 25 °C. Calibration was, however, carried out at the experimental temperature of 37 °C, using tabulated values for this temperature.

Turbidity spectra for determination of nucleation rates were recorded with a World Precision Instruments/Ocean Optics model SD2000 fiber optic spectrometer (World Precision Instruments Ltd., 1 Hunting Gate, Hitchin, Hertfordshire SG4 0TJ, UK) with a spectral range of 250–850 nm and a resolution of 1.9 nm. The light source was a World Precision Instruments D₂-Lite combined deuterium and tungsten/halogen lamp. Samples were placed in standard 1 cm quartz cuvettes inserted in an Ocean Optics CUV-UV cuvette holder connected to a circulation thermostat. Both instruments, the pH meter and spectrometer, were connected to a serial port of a PC for recording of measurements.

A few precipitates were characterized by high-resolution transmission electron microscopy and selected-area electron microdiffraction using a Philips EM 430ST transmission electron microscope (Philips, Eindhoven, Netherlands) operating at 300 kV.

2.2. Reagents

All solutions were prepared from Merck analytical reagent chemicals except potassium hydrogen phthalate (Fluka analytical reagent) and potassium tetraoxalate (BDH AnalaR reagent). As solvent we used demineralized water further purified by passing through a filter with activated carbon and a Silhorko Silex 1 mixed-bed ion exchange column.

Solutions for the precipitation experiments were the same as those used in the Solution Growth Facility of the EURECA space flight [10]. They were as follows:

A: 0.05356 M CaCl₂ + 0.05840 M KCl

B: 0.00671 M KH₂PO₄ + 0.04685 M K₂HPO₄

C: 0.16 M KCl.

2.3. Procedures

2.3.1. pH Recording

A mixture of A and C was pipetted into a test tube and a mixture of B and C was pipetted into another test tube. The total volume in either test tube was 3 mL. The two test tubes were placed in the thermostat, and after a few minutes the contents of one were poured into the other, the pH electrode was inserted and the recording was started. The following day the final pH was noted. Experimental conditions for turbidimetry were selected on the basis of these results: precipitation should be clearly delayed, but at least observable on the pH-meter.

2.3.2. Turbidimetry

The spectrometry program on the PC to which the spectrometer was connected, was set to record simultaneously absorbances (i.e., turbidities) at 300, 400, 500, 600, 700 and 800 nm. The experiments were similar to those above, except that the total volume was only 3 mL. Recording was stopped when the maximal absorbance had reached a value of about 2, causing the signal-to-noise ratio to be too low for reliable measurement.

2.3.3. Calculations

Supersaturations and amounts precipitated as functions of time and at the end of an experiment were calculated from initial concentrations and recorded pH, using literature values of equilibrium constants [4,11–14]. A previously described computer program, based on the Newton–Raphson iteration and recently revised such as to run under Microsoft Windows 10, was used for these calculations [15,16]. Ion activity coefficients are obtained from the Debye–Hückel equation using Kielland's tabulated ion radius parameters [17].

Turbidity data from spectroscopy were evaluated using the Mie theory for light scattering from small particles [18,19]. We have no explicit expression for calculating particle size or number density from turbidity data. Instead, the method is based on the extinction efficiency factor:

$$Q_{\text{ext}} = \frac{\tau}{\pi N a^2} \quad (1)$$

where τ is the turbidity, corresponding to absorbance in a cuvette of unit length, N is the number of particles per unit volume and a is the radius of a particle. Q_{ext} , in turn, is a function of a , the wavelength λ and the refractive indices of particles and the surrounding medium, taken as 1.65 for crystals and 1.33 for solution. In the present study, Q_{ext} was calculated from these latter parameters using the method of phase angles [19]. For each set of six turbidity values at the selected wavelengths recorded at a given time, a is adjusted until the dependence of calculated values of Q_{ext} on λ agrees with the variation of τ with λ . The calculation involves the summation of more than 20 spherical Bessel functions, but with a suitable recursion formula readily implemented in the computer program this is a relatively simple task [20]. Knowing a we obtain N from (1), and the increase of N with time gives the rate of nucleation J .

3. Results

Out of the precipitation experiments with different mixtures of solutions A, B and C listed above in the subsection Reagents, six showed behavior that is useful for turbidimetry. In the more concentrated systems with higher supersaturations instantaneous precipitation was observed, and so the rate of nucleation could not be measured, and at the other extreme precipitation was too faint to be measurable with adequate precision. The compositions are listed in Table 1 together with initial values of pH and supersaturations $\log \beta$ as well as the calculated pH of solutions saturated with respect to HAp, pH(eq).

Table 1. Compositions of mixtures for turbidimetry. Solution C is added up to a total volume of 3 mL.

Exp. No.	A/mL	B/mL	pH	$\log \beta$	pH(eq)
1	0.25	0.25	7.556	14.32	5.462
2	0.20	0.20	7.566	13.66	5.570
3	0.30	0.10	7.538	13.55	5.364
4	0.15	0.15	7.576	12.79	5.709
5	0.10	0.30	7.592	12.76	6.808
6	0.25	0.05	7.545	12.36	5.521

β is the *saturation ratio*, here defined as

$$\beta = \frac{a(\text{Ca}^{2+})^5 a(\text{HPO}_4^{2-})^3}{a(\text{H}^+)^4 K_{\text{sp}}} \quad (2)$$

where the solubility product constant K_{sp} , following Bjerrum [21,22], refers to the process $\text{Ca}_5\text{OH}(\text{PO}_4)_3 + 4\text{H}^+ \rightleftharpoons 5\text{Ca}^{2+} + 3\text{HPO}_4^{2-} + \text{H}_2\text{O}$. The value is thus

$$pK_{\text{sp}} = 7.50.$$

Graphs of results of pH recording as described in Section 2.3.1 are shown in Figure 1. Storage of values was discontinued when more than 12 h had elapsed, or pH had decreased by less than 0.01 in 1 h.

Figure 1. Graphs of pH vs. time for the six experiments listed in Table 1.

Figure 2 shows at left a graph of a few measured turbidities in the experiment with 0.30 mL A and 0.10 mL B, resulting in initial pH = 7.538 and $\log \beta = 13.55$, cf. Table 1. The graph at right shows calculated particle number density N as a function of time. Particle radius a shows only small variations around $\approx 0.4 \mu\text{m}$.

Figure 2. Measured turbidities and particle number variation for 0.30 mL A and 0.10 mL B.

The rate of nucleation is now determined from the slope of the first part of the graph up to the steep rise, which is most probably caused by secondary nucleation; the final decrease is likely due to agglomeration and sedimentation. The classical expression for homogeneous nucleation rate J per unit volume of solution as worked out by Becker, Döring, Volmer, Zel'dovich and Frenkel is:

$$J = k_2 N_1^2 Z \exp \left[-\frac{16\pi\gamma^3\Omega^2}{3(kT)^3 \ln^2 \beta} \right] \quad (3)$$

where k_2 is a second-order rate constant for assembling of growth units, N_1 is the number density of single growth units in solution, and Z is the Zel'dovich factor accounting for the fact that the distribution of crystal embryos is not the equilibrium distribution. Different, but equivalent expressions for Z are found in the literature; one of them is [23]:

$$Z = \frac{1}{8\pi\Omega} \left(\frac{kT}{\gamma} \right)^{3/2} \ln^2 \beta \quad (4)$$

Further, γ is the surface free energy, and Ω is the volume of a formula unit in the crystal. As Z is proportional to $\ln^2 \beta$, a plot of $\ln(J / \ln^2 \beta)$ vs. $1 / \ln^2 \beta$ should have the same slope as that of $\ln(J / Z)$, and with this we find γ using (3). The plot is shown in Figure 3.

Figure 3. Plot of nucleation rate according to Equation (3).

From the lattice parameters [24] we have $\Omega = 2.64 \times 10^{-28} \text{m}^3$ and thus

$$\gamma = 68.1 \pm 2.9 \text{ mJ/m}^2$$

The standard deviation indicated is the result of the regression analysis.

Final pH values in the turbidity experiments fell in the range $\text{pH} = 5.81 \pm 0.06$ with resulting supersaturations $\beta_{\text{final}} = 180 \pm 50$.

4. Discussion

We note that the measured values from one experiment, No. 5, deviated significantly from the rest. After the initial decrease pH was approximately constant and not much higher than the value calculated for the saturated solution of HAp, given in the last column of Table 1. The simple explanation is that in this case the initial total phosphate concentration was three times as high as that of calcium, so that only a minor fraction of phosphate went into the precipitate. As a consequence the solution was still buffered at a relatively high pH. Experiment No. 4 showed similar behavior, but less pronounced (Figure 1). In the remaining cases final pH was 0.4–0.5 higher than that calculated for each individual saturated solution, but close inspection of Figure 1 reveals that precipitation was still taking place at a low rate when recording had ended.

The above findings should now settle the question of composition and structure of the precipitate. No other assumption than that of HAp would lead to the observed agreement.

The value found for γ is significantly lower than those found in other investigations. In particular, it is unusually low for an anhydrous salt of di- and trivalent ions, being even lower than for monetite, CaHPO_4 [25]. Another discrepancy results if we insert values into the Gibbs–Kelvin equation:

$$kT \ln \beta = \frac{2\gamma\Omega}{r} \quad (5)$$

where r is the radius of the nucleus. Inserting the final saturation ratio $\beta_{\text{final}} = 180$ and $r = a = 0.4 \text{ }\mu\text{m}$, we find $\gamma = 17 \text{ J/m}^2$. Such a high value is, of course, impossible. The problem arises from the assumption that the particles registered by turbidimetry are monocrystals, whereas they are, in fact, aggregates or agglomerates of nanocrystals as shown in Figure 4.

Figure 4. Transmission electron microscope image (left) and electron microdiffraction pattern (right) of aggrete from precipitated hydroxyapatite.

If, instead, we insert our experimental value from turbidimetry into (5) together with a value of β_{initial} from Table 1 and calculate r from the equation, we end up with a value yielding a volume of the nucleus $< \Omega$, i.e., equally unacceptable. The problem is, no doubt, that we do not know with adequate precision the actual size and shape of individual crystal nuclei. Another explanation could be that of a nonclassical mechanism [9].

Further, we have the question of the low value of γ . Estimates by Boistelle and Lopez-Valero [26] led to an order of magnitude of $\approx 150 \text{ mJ/m}^2$. An explanation could be that the two anionic components, PO_4^{3-} and OH^- , are both strong bases with pK_a of the corresponding acids equal to 12.30 and 13.62, respectively, at 37°C . In neutral solution these anions at the crystal surfaces will certainly take up protons, and this reduction of surface charge density will undoubtedly reduce surface free energy. The process could be written as:

This corresponds (apart from crystal water) to formation of octacalcium phosphate (OCP) with similar surface free energy to that found in the present study for HAp [26]. The case of “amorphous tricalcium phosphate” may be given a similar interpretation with the difference that two formula units are involved: both hydroxides and no phosphate are protonated, and a calcium ion is liberated:

In the previous investigation based on determination of critical supersaturation for nucleation [8] it was assumed that the nucleus could be regarded as whitlockite, $\text{Ca}_3(\text{PO}_4)_2$. The value found for β_{crit} was 3900, and using the classical expression

$$J = A \exp \left[-\frac{16\pi\gamma^3\Omega^2}{3(kT)^3 \ln^2 \beta} \right] \quad (6)$$

with $J = 1$ and $A = 10^{23}$, both in $\text{dm}^{-3}\text{s}^{-1}$, we found $\gamma = 81 \text{ mJ/m}^2$. This is considerably closer to the expected value. It could be interesting to check the values on the assumption that the precipitate is HAp. This yields $\gamma = 62.3 \text{ mJ/m}^2$. However, the actual value of the critical supersaturation is rather uncertain in this case.

As a final aspect, important in relation to the biological role of calcium phosphate, we may consider substitution of hydroxide by fluoride in apatite crystals. As the fluoride ion is a very weak base, it will not take up protons from a neutral solution, and so the two processes described above

will not take place with fluorapatite (FAP). Hence we can expect a higher surface free energy of FAP relative to HAP and consequently a lower nucleation rate, resulting in larger crystals. This is likely part of the explanation of the stabilizing effect of fluoride on tooth enamel.

5. Conclusions

Based on previous findings that nanoparticles of calcium phosphate precipitated in neutral solution consist, in fact, of nanocrystals of hydroxyapatite, it was shown that such crystals follow classical theory of homogeneous nucleation. An unusually low value of surface free energy was found, but this may be interpreted as resulting from surface protonation of the strongly basic anions phosphate and hydroxide.

Funding: This research was funded by the Danish Space Board.

Acknowledgments: The author is highly thankful to Elena I. Sovorova for investigations by electron microscopy and to Tanja Madsen for skillful technical assistance.

Conflicts of Interest: The author declares no conflict of interest.

References

1. Warington, R. On the decomposition of tricalcic phosphate by water. *J. Chem. Soc.* **1873**, *26*, 983–989 [[CrossRef](#)]
2. Bassett, H. The phosphates of calcium. Part IV. The basic phosphates. *J. Chem. Soc.* **1917**, *111*, 620–642. [[CrossRef](#)]
3. Bjerrum, N. Calciumorthophosphate. *Mat. Fys. Medd. Dan. Vid. Selsk.* **1958**, *31*, 1–70.
4. Moreno, E.C.; Gregory, T.M.; Brown, W.E. Preparation and solubility of hydroxyapatite. *J. Res. Natl. Bur. Stand.* **1968**, *72A*, 773–782. [[CrossRef](#)] [[PubMed](#)]
5. Christoffersen, M.R.; Dohrup, J.; Christoffersen, J. Kinetics of growth and dissolution of calcium hydroxyapatite with variable calcium to phosphate ratio. *J. Cryst. Growth* **1998**, *186*, 283–90. [[CrossRef](#)]
6. Čadež, V.; Erceg, I.; Selmani, A.; Jurašin, D.D.; Šegota, S.; Lyons, D.M.; Kralj, D.; Sikirić, M.D. Amorphous calcium phosphate formation and aggregation process revealed by light scattering techniques. *Crystals* **2018**, *8*, 254. [[CrossRef](#)]
7. Suvorova, E.I.; Klechkovskaya, V.V.; Komarov, V.F.; Severin, A.V.; Melikhov, I.V.; Buffat, P.A. Electron microscopy of biomaterials based on hydroxyapatite. *Crystallogr. Rep.* **2006**, *51*, 881–887. [[CrossRef](#)]
8. Madsen, H.E.L.; Lopez-Valero, I.; Lopez-Acevedo, V.; Boistelle, R. The formation product of amorphous tricalcium phosphate at 37 °C. *J. Cryst. Growth* **1986**, *75*, 429–434. [[CrossRef](#)]
9. Habraken, W.J.E.M.; Tao, J.; Brylka, L.J.; Friedrich, H.; Bertinetti, L.; Schenk, A.S.; Verch, A.; Dmitrovic, V.; Bonnans, P.H.H.; Frederik, P.M.; et al. Ion-association complexes unite classical and non-classical theories for the biomimetic nucleation of calcium phosphate. *Nat. Commun.* **2013**, *4*, 1507. [[CrossRef](#)] [[PubMed](#)]
10. Madsen, H.E.L.; Christensson, F.; Polyak, L.E.; Suvorova, E.I.; Kliya, M.O.; Chernov, A.A. Calcium phosphate crystallization under terrestrial and microgravity conditions. *J. Cryst. Growth* **1995**, *152*, 191–202. [[CrossRef](#)]
11. Smith, R.M.; Martell, A.E. *Critical Stability Constants, Vol. 4: Inorganic Complexes*; Plenum Press: New York, NY, USA, 1976.
12. Martell, A.E.; Smith, R.M.; Motekaitis, R.J. *NIST Critically Selected Stability Constants of Metal Complexes Database*; version 5.0, 8.0; NIST Standard Reference Database; Standard Reference Data Program, National Institute of Standards and Technology, US Department of Commerce: Gaithersburg, MD, USA, 1998, 2004; Volume 46.
13. Grzybowski, A.K. The standard potential of the calomel electrode and its application in accurate physicochemical measurements. II. Thermodynamics of the second ionization of orthophosphoric acid. *J. Phys. Chem.* **1958**, *62*, 555–559. [[CrossRef](#)]
14. Bjerrum, N.; Unmack, A. Elektrometrische Messungen mit Wasserstoffelektroden in Mischungen von Säuren und Basen mit Salzen. Die Dissoziationskonstanten von Wasser, Phosphorsäure, Citronensäure und Glycin. *Mat. Fys. Medd. Dan. Vid. Selsk.* **1929**, *9*, 1–208.

15. Lundager Madsen, H.E. Aspects physicochimiques de la lithiase urinaire. *Néphrologie* **1984**, *5*, 151–154. [[PubMed](#)]
16. Berland, Y.; Olmer, M.; Grandvuillemin, M.; Lundager Madsen, H.E.; Boistelle, R. In vitro and clinical study of oxalate influence on calcium oxalate crystal formation. *J. Cryst. Growth* **1988**, *87*, 494–506. [[CrossRef](#)]
17. Kielland, J. Individual activity coefficients of ions in aqueous solutions. *J. Am. Chem. Soc.* **1937**, *59*, 1675–1678. [[CrossRef](#)]
18. Mie, G. Beiträge zur Optik trüber Medien, speziell kolloidaler Metallösungen. *Ann. Phys.* **1908**, *25*, 377–445. [[CrossRef](#)]
19. van de Hulst, H.C. *Light Scattering by Small Particles*; Wiley: New York, NY, USA, 1957; Dover: New York, NY, USA, 1981; Chapters 9–10.
20. Lundager Madsen, H.E. Turbidimetric study of fluorite nucleation in solution. *J. Colloid Interface Sci.* **2007**, *307*, 469–476. [[CrossRef](#)] [[PubMed](#)]
21. Bjerrum, N. Undersøgelser over kalciumfosfaters opløselighed. In *Nordiska (19. Skandinaviska) Naturforskarmötet i Helsingfors*. 1936. pp. 344–348.
22. Bjerrum, N. Investigations on the solubility of calcium phosphates. In *Selected Papers*; Munksgaard: Copenhagen, Denmark, 1949; pp. 245–248.
23. Lundager Madsen, H.E. Crystallization of heavy-metal phosphates in solution—III. nucleation and growth of $\text{Cd}_5\text{H}_2(\text{PO}_4)_4 \cdot 4\text{H}_2\text{O}$ at 37 °C. *J. Cryst. Growth* **2002**, *244*, 349–358. [[CrossRef](#)]
24. Nriagu, J.O. Phosphate minerals: Their properties and general modes of occurrence. In *Phosphate Minerals*; Nriagu, J.O., Moore, P.B., Eds.; Springer: Berlin, Germany, 1984; pp. 54–55.
25. Lundager Madsen, H.E.; Thorvardarson, G. Precipitation of calcium phosphate from moderately acid solution. *J. Cryst. Growth* **1984**, *66*, 369–376. [[CrossRef](#)]
26. Boistelle, R.; Lopez-Valero, I. Growth units and nucleation: The case of calcium phosphates. *J. Cryst. Growth* **1990**, *102*, 609–617. [[CrossRef](#)]

© 2020 by the author. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).