Biochimica et Biophysica Acta, 628 (1980) 244—248
© Elsevier/North-Holland Biomedical Press

BBA Report

BBA 21525

AN OCTACALCIUM PHOSPHATE INTERMEDIATE IN THE SYNTHESIS OF CHROMATOGRAPHIC HYDROXYAPATITE

MICHAEL SPENCER
King's College Department of Biophysics, 26—29 Drury Lane, London WC2B 5RL (U.K.)
(Received November 13th, 1979)

Key words: Octacalcium phosphate; Hydroxyapatite

Summary

In the laboratory synthesis of hydroxyapatite for liquid column chromatography, different procedures give varying amounts of a transient intermediate, identified by X-ray diffraction as octacalcium phosphate. The intermediate is most abundant in a recently-developed method involving conversion from brushite at neutral pH; well-crystallized hydroxyapatite is then produced without the boiling previously found necessary. The results are relevant to hydroxyapatite biosynthesis, where a similar intermediate has been postulated. Methods are described for the laboratory synthesis of octacalcium phosphate.

There is increasing acceptance of the idea that the biosynthetic pathway of hydroxyapatite often involves octacalcium phosphate as an intermediate [1—4], though much of the evidence is indirect. The two compounds crystallize in lattices having strong similarities [5, 6] and although hydroxyapatite is the more stable compound under physiological conditions, the growth of octacalcium phosphate in vitro is sometimes initially more rapid [3, 5]. This paper reports the identification of an octacalcium phosphate intermediate in the laboratory synthesis of hydroxyapatite for liquid column chromatography and discusses the relevance of the results to physiological problems. All the methods to be discussed use, as starting material, a suspension of calcium monohydrogen phosphate dihydrate (brushite), which has also been found in association with octacalcium phosphate and hydroxyapatite in dental calculus [7].

The oldest and most widely-used method of making chromatographic hydroxyapatite is that of Tiselius et al. [8—10], who first crystallized brushite from solution and then converted it to hydroxyapatite by boiling with an excess of alkali. The method gives material comprising sintered masses of submicroscopic, calcium-deficient hydroxyapatite crystals, retaining the gross morphology and higher flow-rate of the brushite. The specific surface area and binding capacity for macromolecules are greatly increased by the conversion. However, the boiling stages are difficult to control and there is a tendency for the aggregates to break up and give an unacceptably low flow-rate. There have been various attempts to devise alternative procedures.

In the course of work reported in detail elsewhere [11—13], we have monitored intermediate stages of a number of procedures by powder X-ray diffraction and other methods [12]. In the Tiselius method, where brushite conversion is performed at high pH, there is a rapid initial conversion to hydroxyapatite with no detectable intermediate, but the crystallite size is very small and the binding of macromolecules relatively weak. The function of the boiling, which is repeated during later washes, appears to be to ripen the hydroxyapatite crystallites to a larger size (Table I) so that the binding strength is enhanced; it is found that binding strength levels off when the smallest dimension of a crystal face approximates to the size of the bound molecule [11]. Conversion at room temperature gives only small crystallites and weak binding.

A later procedure due to Main et al. [14, 15] employs conversion by boiling at pH 8, alkali being added to maintain constant pH, followed by washing in phosphate buffer. In this case, X-ray diffraction shows the transient appearance of octacalcium phosphate, though the proportion of octacalcium phosphate to hydroxyapatite in an intermediate sample is small. Table I shows that the product differs from that of the Tiselius procedure in that its crystallite size is greater without the need for extra boiling.

An improved method developed in this laboratory [12, 16] utilizes conversion at neutral pH, and here there is a clearly-defined intermediate phase of well-crystallized octacalcium phosphate, present at times in a proportion comparable to that of hydroxyapatite; indeed, lowering the pH below 6.5 inhibits the final conversion to hydroxyapatite, and a stable preparation of octacalcium phosphate is obtained (Table I). Conversion to hydroxyapatite at pH 7 can be carried out entirely below 100° C, thus avoiding the mechanical stresses of boiling, and Table I shows that the crystallites are as large as those obtained after extensive boiling at higher pH.

It is clear, therefore, that as one approaches physiological pH values there is an increasing tendency (at least at temperatures approaching 100° C) to favour the production of octacalcium phosphate as an intermediate between brushite and hydroxyapatite. The crystallite size of hydroxyapatite depends on the degree of crystallization of the octacalcium phosphate, and a slight fall in pH can favour octacalcium phosphate so strongly that it forms larger crystals resistant to conversion. This may well be relevant in the more acidic of oral environments [7].

Even in the absence of added alkali, which is needed for quantitative conversion, there is a strong tendency for octacalcium phosphate to form when brushite is heated [5]. If a suspension of washed brushite at a calcium concentration of 0.2 M is heated to approx. 80° C, there is a rapid reaction in which the pH value falls to between 4 and 5; X-ray diffraction indicates that a small amount of octacalcium phosphate has been formed, and light micro-

TABLE I

PROPERTIES OF HYDROXYAPATITE AND INTERMEDIATE COMPOUNDS

Intermediate compounds were identified by powder X-ray diffraction, using JCPDS file data.
For details of analytical techniques, see Ref. 11. All preparations were composed initially of brushite, obtained by mixing solutions of calcium chloride and sodium phosphate [12]. HA, hydroxyapatite; OCP, octacalcium phosphate; brushite, calcium monohydrogen phosphate dihydrate.

Approx. pH of conversion	Alkali	Stage in preparation	Components	HA crystallite size L_{002} (nm)	Molar ratio CA/P (± 0.03)
>13 (Refs. 8—10)	NaOH	Heated to 50°C	brushite, HA	—	—
		Heated to 100°C	HA	45	1.47
		Boiled 60 min and washed X 5 in boiling phosphate buffer	HA	53	1.56
8 (Refs. 14,15)	NH_4OH	Heated to 50°C	brushite, weak OCP	—	—
		Heated to 100°C	HA	49	1.54
		Boiled 30 min and washed X 7 in cold phosphate buffer	HA	62	1.55
7 (Ref. 11)	NH_4OH or NaOH	At start of alkali addition, 90°C	brushite, OCP	—	—
		At end of alkali addition, 80°C	OCP + HA	—	—
		Washed in hot water and cooled overnight to 20°C	HA	63	1.52
6 (Ref. 11)	NH_4OH	Alkali added at 65—75°C, washed and cooled as above	OCP	—	1.37

scopy shows the growth of very thin crystals that greatly increase the settled volume of the preparation. Octacalcium phosphate must form from dissolving brushite by the following reaction (neglecting the equilibrium between ionic species):

$$8CaHPO_4 \rightarrow Ca_8H_2(PO_4)_6 + 2H_3PO_4$$

The final pH arises from a new balance between the orthophosphate species derived from liberated phosphoric acid and excess brushite. The solubility of brushite increases with falling pH, so the reaction is in a sense autocatalytic; it stops only when the pH falls below the value at which octacalcium phosphate can form. Initiation of crystallization does, however, require a pH value above 6. Fig. 1 shows that a suspension of brushite can remain stable until a small injection of alkali initiates octacalcium phosphate crystallization, after which the pH falls well below the initial value. An unexpected process of this kind may perhaps be relevant in vivo where local pH values are subject to variation.

The synthesis of microcrystalline octacalcium phosphate, which would merit further study in this connection, has been reported by some earlier workers [5, 17, 18]. In the present work, octacalcium phosphate was produced by two alternative methods. Slow addition at room temperature of 0.5 M $CaCl_2$ to an excess of 0.5 M sodium phosphate buffer, with addition of NaOH to maintain constant pH, gave a product with the powder diffraction pattern of octacalcium phosphate when the pH was between 7 and 8; strong central scatter indicated a low degree of crystallinity. At pH 9.5 a mixture of octacalcium phosphate and hydroxyapatite was obtained, while below pH 6 the product was brushite. A better-crystallized octacalcium phosphate

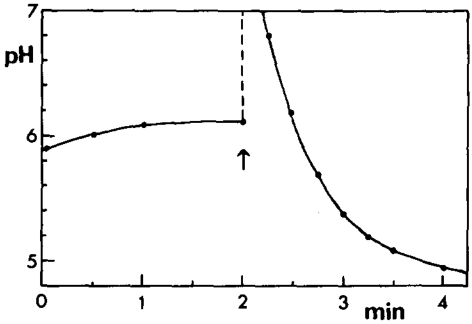

Fig. 1. Partial hydrolysis of brushite to octacalcium phosphate. Brushite free of octacalcium phosphate was crystallized by pumping 2 l 0.5 M $CaCl_2$ at 55 ml • min^{-1} into 2.4 l 0.5 M sodium phosphate buffer, the pH being kept at 4.5 ± 0.4 by controlled addition of 20% (w/v) NaOH. Mixing was carried out at room temperature with vigorous agitation. A 500-ml aliquot of the suspension was allowed to settle and the supernatant decanted. The slurry of crystals (containing approx. 1 • 10^{-3} mol calcium) was added to 500 ml water pre-heated on a hotplate magnetic stirrer, giving the mixture a temperature of 70—80°C. At the time indicated by the arrow, 0.5 ml 20% NaOH (approx. 2.5 • 10^{-3} mol) was added. In other experiments (not illustrated), the brushite was pre-washed by resuspending twice in 500 ml cold water, and the hot mixture was stabilized to approx. pH 6 by adding 0.5 ml 1M HCl. Addition of alkali then caused a larger initial rise of pH due to the absence of excess soluble phosphate, but the pH fell subsequently to the same value after 4 min. The same phenomenon was observed when washed brushite was mixed with hot water without acid stabilization, but in this case the initial pH was approx. 7 and it began to fall immediately, reaching pH 5 after 1.5 min.

was obtained by alkali treatment of a heated brushite suspension at pH 6.5 or below, as described above. Experience so far suggests that, by varying the temperature and pH of reaction, it should be possible to produce octacalcium phosphate of various degrees of crystallinity.

The results described here neither prove nor disprove the hypothesis [5, 17, 19] that calcium-deficient hydroxyapatite is really a mixture of lamellae comprised of octacalcium phosphate and stoichiometric hydroxyapatite. The chromatographic behaviour of hydroxyapatite sheds no light on the matter either, since the Ca/P ratio appears unrelated to the binding properties for macromolecules [11]. Such properties are thought to depend either on charge interactions with surface calcium or phosphate ions [20] or, for polyphosphates, on binding to exposed hydroxyl sites [21, 22]. The work does, however, emphasize the complexity of the equilibria affecting hydroxyapatite formation, and suggests that octacalcium phosphate should receive more attention. There are also indications from DNA-binding studies [11] that it might be of interest as a chromatographic medium.

I am grateful to Drs. J.C. Elliott, W.B. Gratzer, M. Grynpas and S.L. Rowles for useful discussions. This work was supported by the Medical Research Council.

References

1 Elliott, J.C. (1973) Clin. Orthopaed. 93, 313—345
2 Tomažič, B. and Nancollas, G.H. (1975) J. Colloid Interface Sci. 50, 451—461
3 Eanes, E.D. and Meyer, J.L. (1977) Calcif. Tiss. Res. 23, 259—269
4 Tomson, M.B. and Nancollas, G.H. (1978) Science 200, 1059—1060
5 Brown, W.E., Smith, J.P., Lehr, J.R. and Frazier, A.W. (1962) Nature 196, 1050—1055
6 Taves, D.R. (1963) Nature 200, 1312—1313
7 Barone, J.P. and Nancollas, G.H. (1977) J. Colloid Interface Sci. 62, 421—431
8 Tiselius, A., Hjertén, S. and Levin, Ö. (1956) Arch. Biochem. Biophys. 65, 132—155
9 Levin, Ö. (1962) Methods Enzymol.5, 27—31
10 Jenkins, W.T. (1962) Biochem. Preps. 9, 83—85
11 Spencer, M. and Grynpas, M. (1978) J. Chromatogr. 166, 423—434
12 Spencer, M. (1978) J. Chromatogr. 166, 435—446
13 Spencer, M., Neave, E.J. and Webb, N.L. (1978) J. Chromatogr. 166, 447—454
14 Main, R.K., Wilkins, M.J. and Cole, L.J. (1959) J. Am. Chem. Soc. 81, 6490—6495
15 Atkinson, A., Bradford, P.A. and Selmes, I.P. (1973) J. Appl. Chem. Biotechnol. 23, 517—529
16 Spencer, M. (1978) British Patent application No. 30646/78
17 Glimcher, M.J. and Krane, S.M. (1968) in Treatise on Collagen (Gould B.S., ed.) 2B, p. 103, Academic Press, New York, NY
18 Füredi-Milhofer, H., Bilinski, H., Brečević, L., Despotović, R., Filipović-Vinceković, N., Oljica, E. and Purgarić, B. (1975) Colloques Internationaux C.N.R.S. No. 230, 303—310
19 Joris, S.J. and Amberg, C.H. (1971) J. Phys. Chem. 75, 3172—3178
20 Bernardi, G., Giro, M.-G. and Gaillard, C. (1972) Biochim. Biophys. Acta 278, 409—420
21 Taves, D.R. and Reedy, R.C. (1969) Calcif. Tissue Res. 3, 284—292
22 Kawasaki, T. (1978) J. Chromatogr. 151, 95—112