DOI: 10.1002/ejic.200700654

In Vitro Formation of Nanocrystalline Carbonate Apatite – A Structural and Morphological Analogue of Atherosclerotic Plaques

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Keywords: Carbonate apatite / Crystallization / Calcification / Nanocrystalline / Calcium

The in vitro formation of carbonate apatite in solutions with ion concentrations comparable to those in human serum was studied. The composition and morphology of the resulting apatite precipitate displayed a hierarchical assembly of elongated plate-shaped nanocrystals of carbonate apatite analogous to previously characterized bioapatites formed in vivo. The main conclusion is that so-called bioapatites may form in vitro and that precipitation inhibitors most likely are essential for the prevention of spontaneous calcification at normal human serum ion concentrations.
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Introduction

Biological mineralization of solid calcium phosphates occurs in humans both under normal conditions, such as in bone and teeth formation, and under pathological conditions, such as vascular calcification related to advanced atherosclerosis,[1] diabetes[2] and chronic renal failure,[3] where the latter also includes end-stage renal disease pertinent to patients on hemodialysis.[4] Giachelli[5] recently published a comprehensive review on possible mechanisms behind vascular calcification, in which several mechanisms were discussed.

From a purely solution chemistry point of view, vascular calcification is expected: normal human serum calcium and inorganic phosphate concentrations are such that supersaturation exists with respect to hydroxyapatite, $Ca_5(PO_4)_3OH$, (OHAp),[6] octacalcium phosphate (OCP), and carbonate apatite (dahllite);[7] the latter is often denoted bioapatite, a name suggesting that it can be formed only in vivo. Investigations of atherosclerotic plaques show that bioapatite is an apatite with a significant amount of carbonate included[7,8] (cf. Table 2). Also, comparisons between synthetically made and biological apatites showed that phosphate, to a varying degree, was exchanged for carbonate in the latter type.[9]

Biomineralization of apatites has been host to renewed interest. The main reason is the hierarchical crystallization and observation of nano- and mesocrystalline features, especially in combination with composite formation. Spherical precipitates were identified and shown to consist of bundles of small crystallites.[11–13]

Despite supersaturation, spontaneous calcification under normal human serum conditions does not occur. This indicates that inhibitors must be present to maintain metastability. With regard to pathological vascular calcification, a pathway involving a lack of inhibition seems to be the most important of the mechanisms listed by Giachelli.[5] Both in vitro and mice in vivo experiments support this mechanism.[14] In vivo mice experiments with a reduced concentration of calcification inhibitors in the serum were shown to give calcification;[15,16] the "knock-out mice" that lacked inhibitors were severely calcified despite having normal mice serum ionized calcium and inorganic phosphate concentrations. However, applied to human vascular calcification, the mice results are inconclusive. The reason is that even though mice serum contains the same total calcium concentration as human serum, the inorganic phosphate concentrations are two to three times higher.[16,17] From a solution chemistry point of view, this mice and human serum phosphate concentration difference is essential, as it is related to the details of calcium phosphate precipitation; more precisely, this value is related to supersaturation with respect to brushite, $CaHPO_4 \cdot 2H_2O$. A number of in vitro studies have shown that once this species is formed,[18] it is transformed to the less-soluble OCP and in turn transformed into OHAp at physiological pH. Even formation of bioapatite as the end product is possible; according to LeGeros, OCP is transformed into carbonated apatite in the presence of hydrogen carbonate.[19] However, in normal human serum the calcium ion and inorganic phosphate concentrations are so low that the saturation limit for brushite is never exceeded. This was verified experimentally by Eidelman et al.,[20] who showed that brushite dissolves in ultrafiltered human serum, and by model equilibrium calculations.[21]

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Supporting information for this article is available on the WWW under http://www.eurjic.org or from the author.

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Table 1. Composition of solutions, concentrations in mmol/L; calcium–phosphate product, $Ca \times P_i$; calcium–phosphate molar ratio in precipitate (Ca/P, ppt), number of vessels and vessel material [glass (G) or polypropylene (PPye)].[a]

	2×44, G (low hydrogen carbonate)		4×25 PPye, 4×5 G				25 PPye	Normal human serum[27]
P_i	1.30	1.84	0.99	1.29	1.59	1.90	1.90	0.84–1.45
Ca, total	1.30	1.30	1.56	1.56	1.56	1.56	1.64	2.1–2.6
$(Ca \times P_i) \times 10^6$ [mol/L]^2	1.7	2.4	1.5	2.0	2.5	3.0	3.1	(1.8–3.8)
$NaHCO_3$	0.18	0.11	24.7	24.7	24.9	24.6	24.9	22–26
Na^+, total	145	146	146	148	148	146	146	135–145
K^+								3.5–5.0
Mg^{2+}								0.7–1.0
Cl^-	145	145	124	125	125	123	122	100–108
iCa = $[Ca^{2+}]$, calcd.	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.14–1.30
$CaHPO_4$(aq.), calcd.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	–
$CaHCO_3^+$, calcd.	–	–	0.3	0.3	0.3	0.3	0.3	–
pH, at preparation	7.67	7.41	7.38	7.36	7.16	7.21	7.43	7.35–7.45
Sign of 1:st ppt (weeks)	no precipitate		6	3	3	3	1–2	–
pH, after ppt			7.43	7.43	7.43	7.42	7.37	
Ca/P, ppt	–	–	1.85	1.79	1.76	1.73	1.73	–

[a] For calculated values, see Supporting Information.

To test the mechanism based on the lack of inhibition in the case of human serum, we here report a study of salt solutions supersaturated with respect to OHAp, OCP, and carbonate apatite (cf. bioapatite), but not brushite, and with temperature, buffer (sodium hydrogen carbonate), and thus pH, ionic medium, and strength as close as possible to human serum conditions (cf. Table 1). These conditions ensure that the chemical potentials of all individual ionic species involved in the hypothetical precipitation process are close to those in human serum.

Our study is an extension of an earlier investigation, in which we characterized a carbonated apatite formed in solution with inorganic phosphate corresponding to hyperphosphatemic concentrations and determined the stoichiometry to be $Ca_5(PO_4)_3HCO_3 \cdot 4H_2O$.[22]

In the experiments reported here, phosphate was varied from human normal to hyperphosphatemic concentrations. To the best of our knowledge there is no experimental study published about in vitro formation of carbonate apatite (analogous to bioapatite) in solutions with calcium, phosphate, and hydrogen carbonate concentrations comparable to those in human serum. Even though the solutions in the experiments reported by Boskey et al. contained equally low concentrations of calcium and phosphate, hydrogen carbonate was not present thus excluding carbonate apatite as a potential mineral deposit.[23]

For the sake of completeness we have included experiments in a physiological sodium chloride medium containing very low hydrogen carbonate concentrations aimed at giving precipitation of a carbonate-free apatite, i.e. OHAp.

Results

The time from preparation of the solution until a precipitate was visually observed in the polypropylene tubes amounted to 2–3 weeks for all phosphate concentrations, except P_i = 0.99 mmol/L, for which the delay time was 5–6 weeks. After seven weeks all tubes contained precipitate. The precipitate observed in the glass flasks occur as relatively large half-spheres, few in number, and with a diameter of about 1 mm, similar to those found in glass flasks with an initial phosphate concentration of 1.8 mmol/L reported earlier and displayed in Figure 1.[21] Those in the polypropylene tubes occur as considerably smaller and more numerous half-spheres (Figure 2). However, for P_i = 0.99 mmol/L, in a few polypropylene tubes we observed precipitates consisting solely of a few half-spheres with a diameter of about 0.3 mm.

In an earlier publication we showed that the precipitates formed in glass flasks with an initial phosphate concentration of 1.8 mmol/L consist of a hydrated carbonate apatite. A plausible formula was suggested, $Ca_5(PO_4)_3(HCO_3) \cdot 4H_2O$.[22] Our results reported here (cf. Figures 1, 2, and 3), show that the precipitates in the polypropylene tube are also calcium phosphates that contain carbon.

The EDS spectrum of a half-sphere removed from a glass flask with an initial phosphate concentration of 1.8 mmol/L is displayed in Figure 1. A comparison between that and the EDS spectra in Figures 2 and 3 shows that the atomic composition of the precipitates in the polypropylene tubes is approximately the same as that in the glass flask with an initial phosphate concentration of 1.8 mmol/L. Signals from calcium, phosphorus, oxygen carbon, and sodium are present, but those of chlorine are not. The IR spectrum in Figure 4 was not assigned in detail but only compared to the published and assigned IR spectra of OCP by Fowler et al.,[24] and to those of OHAp and high-temperature carbonate apatite by Nelson et al.;[25] our IR spectrum was also compared to that of calcified vascular deposits by Tomazic.[7] The spectrum by Nelson et al. with 6.7 wt.-% carbonate and that by Tomazic compare favorably with regard to both the positions and shapes of the bands and to the relative magnitude of the strong carbonate and phosphate bands. It should be noted that the strong band observed in the OHAp spectrum at 631 cm^{-1} and assigned to hydroxy

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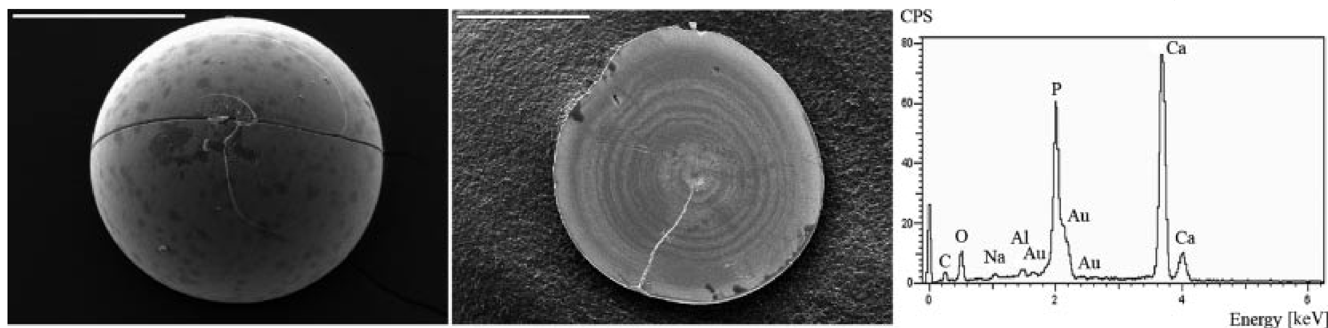

Figure 1. Scanning electron micrographs of two gold-coated samples grown in a glass bottle. The diameter is approximately 1 mm. The scale bar corresponds to 500 µm. The EDS spectrum is from the central to the right part of the flat surface of the sample. Original solution phosphate concentration: 1.8 mmol/L.

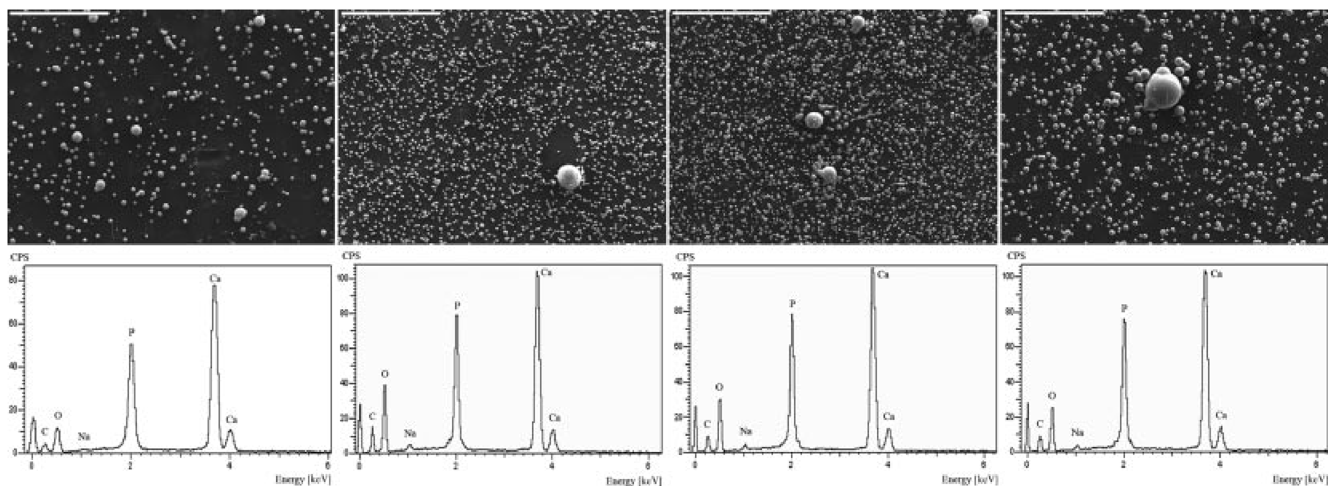

Figure 2. Scanning electron micrographs (top) and accompanying EDS spectra (bottom) of carbonated apatite samples grown in polypropylene tubes. The scale bars correspond to 500 µm. The EDS spectra are from the central to the right part of the flat surface of the samples. Original solution phosphate concentrations from left to right: 0.99, 1.29, 1.59, and 1.90 mmol/L.

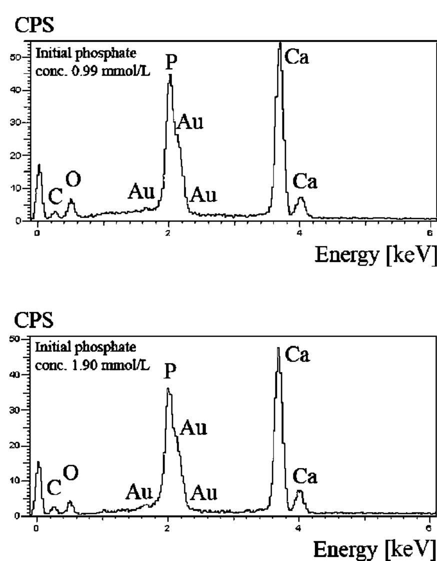

Figure 3. EDS spectra from gold-coated samples grown in polypropylene tubes. Original solution phosphate concentrations: 0.99 (top) and 1.90 (bottom) mmol/L. Note the presence of the carbon peak.

(OH) liberation is hardly discernible in our spectrum,[25] which indicates a low amount of OH. Tomazic made a similar observation concerning his reported IR spectrum of cardiovascular deposits.[7] We can safely conclude that the precipitate formed in vitro and reported here has approxi-

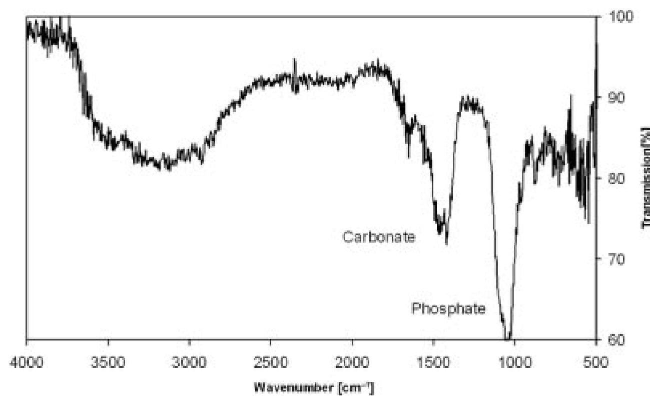

Figure 4. FTIR spectrum of precipitate grown in a polypropylene tube. Original solution phosphate concentration: 1.90 mmol/L.

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Table 2. Composition (wt.-%) and Ca/P molar ratio of apatites and human vascular calcium phosphate deposits.

Calcium phosphate stoichiometry	Ref.	Ca	PO_4	CO_3	Ca/P
OHAp, $Ca_5(PO_4)_3OH$		39.9	56.7	–	1.67
In vitro precipitated carbonate apatite, $Ca_5(PO_4)_3HCO_3{\cdot}4H_2O$	[22]	32.4	46.0	9.7	1.67
Human atherosclerotic thoracic and abdominal aortas 4 samples. Recalculated assuming 15% protein.	[8]	33.5–36.6	47.3–50.5	7.2–14.2	1.61–1.77
Human atherosclerotic cardiovascular deposits (average) $Ca_{8.66}Mg_{0.22}Na_{0.32}H_{0.14}(PO_4)_5(CO_3)_{1.22}(OH,F,Cl)_{0.80}$	[7]	37.4	51.2	7.9	1.73

mately the same relative amounts of carbonate and phosphate as the cardiovascular deposits investigated by Tomazic.

The Ca/P molar ratios based on chemical analysis of the precipitates in the polypropylene tubes are consistently higher than those of OHAp and $Ca_5(PO_4)_3(HCO_3){\cdot}4H_2O$ (Ca/P = 1.67; cf. Table 1). The Ca/P molar ratio varies with the initial phosphate concentrations in the solution: the lower the phosphate concentration, the higher the ratio. This indicates that the composition of the precipitates to some extent varies as far as the relative amount of calcium and phosphate is concerned. Thus, from the electroneutrality requirement we can conclude that even the carbonate content may vary. An upper limit for the carbonate content can be calculated. For example, if Ca/P = 1.75 = 7/4, the electroneutrality condition requires one carbonate, stoichiometry $Ca_7(PO_4)_4CO_3$ (Ca/C = 7) or two hydrogen carbonates $Ca_7(PO_4)_4(HCO_3)_2$ (Ca/C = 3.5), and with Ca/P = 1.80 = 9/5, either two carbonates, $Ca_9(PO_4)_5(CO_3)(HCO_3)$ (Ca/C = 4.5) or, if also hydroxide is included, one carbonate $Ca_9(PO_4)_5(CO_3)OH$ (Ca/C = 9). The wt.-% calcium, phosphate, and carbonate of the in vivo calcified vascular deposits from atherosclerotic plaque $Ca_5(PO_4)_3(HCO_3){\cdot}4H_2O$ and OHAp are summarized in Table 2.

With respect to OHAp, which is assumed to form in the solutions with a very low amount of hydrogen carbonate, it does not show any tendency to precipitate in vitro after more than 16 months, neither under the prevailing conditions occurring in a so-called physiological salt solution (150 mmol/L sodium chloride and pH 7.4), nor in the presence of physiological amounts of hydrogen carbonate, where solely a carbonate apatite is formed. This is consistent with the finding that the composition of mineral deposits reported by Tomazic[7] and Schmid et al.[8] cannot be represented by the OHAp stoichiometry.

Discussion

In a water solution with inorganic phosphate concentrations corresponding to normal human serum values, spontaneous precipitation of calcium phosphate occurs. Higher phosphate concentrations, corresponding to human hyperphosphatemic conditions, are not necessary to induce precipitation, even though they may accelerate it. The composition of the in vitro formed carbonate apatite precipitate agrees strikingly well with the findings of Tomazic[7] and Schmid et al.[8] with regard to calcified atherosclerotic plaque in a number of human blood vessels (cf. Table 2). The morphological characteristics of the precipitated carbonate apatite in vitro and in vivo are also comparable: small – a few µm – spherically shaped deposits depicted in Figures 2 and 5 are very similar to those found in human vascular deposits, see Schmid et al.,[8] Tomazic,[7] and Mohr.[26] The spherical shape thus exists both in calcification in vivo and in vitro. From the high magnification image displayed in Figure 5 it can be concluded that the spherical particles are built from units consisting of elongated plate-shaped "crystals", with a size of about a few hundred nm. Figure 5 gives the impression that there is successive assemblage of these small units first into rather irregular shaped aggregates and then, with an increasing number of units, a spherical shape. These results are in good agreement with old electron microscopy investigations and those observed for fluorapatites grown in gelatin media.[10–12]

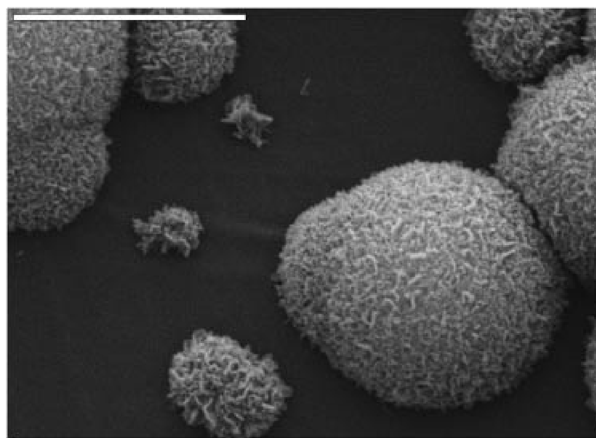

Figure 5. Assemblage of spherically shaped particles from small plate-shaped particles. The scale bar corresponds to 5 µm. Sample grown in a polypropylene tube. Original solution phosphate concentration: 1.90 mmol/L.

Our study shows that from a thermodynamic point of view we can assume that the in vitro and in vivo conditions required to induce precipitation are similar: the chemical potentials of the relevant individual ionic species are highly related and the precipitate formed in vitro is strikingly similar to that formed in vivo vascular deposits. We cannot, however, say anything about the details of the chemical reactions leading to in vitro precipitation, although we can exclude an "inorganic" mechanism assuming brushite as precursor. We can also exclude biological mechanisms that actively promote calcification, even though we obviously cannot exclude such mechanisms in vivo. From our study,

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we can also conclude that the absence of calcification in human serum with normal clinical levels of calcium and phosphate ions unconditionally requires at least one species that prevents the precipitation process to take place. From our work and the above-mentioned in vitro[14] and mice in vivo[14,15] experiments it follows that calcification is solely due to the presence of calcium, phosphate, and hydrogen carbonate ions and decreased levels of those specific proteins that act as calcification inhibitors. Other serum components may influence the rate of the precipitation process, but not the final outcome.

Our study shows that spontaneous calcification occurs at normal human serum ion concentrations of calcium, inorganic phosphate, and hydrogen carbonate and a lack of calcification inhibitors is the simplest explanation to pathological calcification. Biological processes regulating the serum concentration of these inhibitors are of vital importance for the prevention of vascular calcification.

Experimental Section

All concentrations were chosen to give pH and ionic medium and strength similar to those in human serum, cf. Table 1. The expected solid deposit components, calcium, phosphate, and carbonate (from the buffer) were included, and carbon dioxide was present in the atmosphere surrounding the solutions with a partial pressure either corresponding to serum conditions or equal to that of air. Two hydrogen carbonate concentrations, one high physiological and one low were used, cf. Table 1.

False indications of precipitation due to bacterial growth were suppressed and bacterial growth was never observed. Solution preparation was designed to prevent precipitation of calcium phosphate or calcium carbonate at the time of mixing. The tubes and flasks were either stored in a climate chamber at 37.0 ± 0.2 °C, 5% CO_2(g), and 99% relative humidity (high hydrogen carbonate concentration) or in a heat chamber at 37 ± 1 °C with 65% relative humidity (low hydrogen carbonate concentration). The calcium and phosphate ion concentrations were determined by reflectance spectrophotometry with a KODAK Ektachem DTII (Eastman Kodak Company, Rochester, New York, US) instrument.

All tubes were periodically visually checked for precipitation and a few removed for pH, calcium, and phosphate ion analysis. Precipitates were dissolved and the calcium content was determined by atomic absorption spectrometry with a Varian SpectrAA 220FS with a hollow cathode lamp at 422.7 nm, and phosphate spectrophotometrically in the form of its molybdenum complex at 882 nm with a Perkin–Elmer Lambda 20 spectrophotometer. Precipitates were also characterized by energy-dispersive spectrometry (EDS) in a scanning electron microscope (SEM) and with Fourier transformation infrared (FTIR) spectroscopy. A Philips SEM 515, operated at 20 kV was used for imaging, and calcium, phosphorus, oxygen, and carbon contents were detected with a Link ISIS EDS. An IR transmission spectrum for the qualitative determination of carbonate and phosphate was recorded with a Perkin–Elmer Spectrum BX FTIR instrument with a spectral range from 450 to 4400 cm^{-1}.

Supporting Information (see footnote on the first page of this article): Full description of the experimental procedures.

Acknowledgments

This work was supported by the Swedish Research Council.

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Received: June 25, 2007
Published Online: August 3, 2007

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