

Synthesis of triclinic calcium pyrophosphate crystals

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Abstract This paper presents a method for preparing crystals of triclinic calcium pyrophosphate (*t*-CPPD). A calcium pyrophosphate intermediate is first prepared by reaction of potassium pyrophosphate and calcium chloride. Samples of the intermediate are dissolved in hydrochloric acid and urea added. Upon heating to 95–100 °C, hydrolysis of the urea causes the pH to rise and *t*-CPPD crystallises out. Purity of the product was ascertained by chemical and physical analysis. Where large crystals are required an unstirred system is used, while smaller crystals are produced by stirring the reaction mixture.

Introduction

A feature of several arthropathies is the formation within the articular tissues of crystalline material, which may be poorly crystallised apatite, possibly associated with other calcium orthophosphates such as octacalcium phosphate and whitlockite [1], or

calcium pyrophosphate dihydrate (CPPD, $\text{Ca}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$). The latter is associated with the acute inflammatory condition of pseudogout and also with chronic osteoarthritis. CPPD occurs in monoclinic and triclinic forms (*m*-CPPD and *t*-CPPD), usually as a mixture. There is evidence [2] that in pseudogout the proportions of the two forms varied between acute and interval periods.

Since the amount of crystalline material formed in vivo is small, work on synthetic CPPD is an essential aspect of understanding crystal deposition arthropathies, allowing investigation of such properties as solubility, crystal growth and dissolution and mineral/cell interactions. *t*-CPPD can be reliably prepared by the method of Brown et al. [3], as modified by Mandel et al. [4]. However, this method has certain disadvantages, which we discuss later, and in the present paper we describe a simpler alternative method which overcomes these disadvantages.

Materials and methods

General principle

There are two stages in the process. In the first, a gel-like calcium pyrophosphate intermediate is prepared by reacting equimolar quantities of soluble calcium and pyrophosphate salts. In the second, the intermediate is redissolved in acid, urea is added and the mixture heated. The heat-induced hydrolysis of urea causes a rise in pH, until the solution has a high enough supersaturation for *t*-CPPD to precipitate. This technique is well established in analytical chemistry as a method for preparing crystalline precipitates of

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hydroxides and basic salts which otherwise tend to be amorphous [5].

Materials

Analytical grade reagents were purchased from Aldrich or Sigma (Poole, Dorset, U.K.). Pure water, conductivity $<0.05 \mu\text{S}/\text{cm}$, was prepared by reverse osmosis and ion-exchange (Purite Ltd., Thame, Oxfordshire, UK).

Preparation of calcium pyrophosphate intermediate

Fifty mmol (16.5 g) anhydrous potassium pyrophosphate was dissolved in about 800 mL water and the solution stirred using a magnetic stirrer. Fifty mmol calcium chloride, as 50 mL of a 1 mol/L solution, was added dropwise and stirring continued for 1 h after addition was complete. The resulting gelatinous precipitate was collected on a sintered glass filter using a Buchner flask evacuated by a water pump. It was washed on the filter, by twice resuspending in about 300 mL water and refiltering, and then washed once with acetone. Finally, it was transferred to an evaporating dish and dried overnight at 37°C . The dried precipitate was coarsely crushed with a mortar and pestle and stored in an airtight container.

Synthesis of *t*-CPPD

We describe two methods. In the first, the reaction mixture is not stirred and large crystals are formed. The second method was designed to produce small crystals and uses a stirred reaction mixture.

Method 1

A total of 8.16 g calcium pyrophosphate intermediate was added to about 850 mL water, 75 mL of 1.0 mol/L HCl added and the suspension stirred until the solid had dissolved (about 30 min). The solution was made up to 1 L with water. About 100 mL of this solution was placed in a 250 mL Erlenmeyer flask, 1.5 mL of 1.0 mol/L urea solution added and the flask placed on a steam bath for 3 h. After filtering the mixture through a No. 1 filter paper, the crystals were washed with acetone and dried on the paper at 37°C .

Method 2

A total of 3.26 g calcium pyrophosphate intermediate was added to about 850 mL water, 30 mL 1.0 mol/L HCl added and the suspension stirred until the solid had

dissolved. The solution was then made up to 1 L with water. 250 mL of this solution was placed in a 500 mL Erlenmeyer flask, together with 10.8 mL of 1 mol/L urea solution and a magnetic stirrer bar. The mixture was heated to 95°C and stirred at this temperature for 3 h, on a stirrer/hotplate. The crystals were finally separated, washed and dried as in Method 1.

Chemical analysis

Samples of the calcium pyrophosphate intermediate and of the final products were dissolved in HCl and the solutions analysed for calcium, potassium and inorganic phosphorus as orthophosphate and pyrophosphate. Calcium and potassium analyses were performed by atomic absorption spectroscopy. For calcium, 0.2% w/v $\text{LaCl}_3 \cdot 4\text{H}_2\text{O}$ was used to suppress interference by phosphates and, for potassium, 0.1% w/v NaCl was used to suppress ionisation. For analysis of pyrophosphate, 60% w/v perchloric acid was added to each solution, to a concentration of 1.0 mol/L. The solutions were heated for 3 h at 100°C in a steam bath to hydrolyse the pyrophosphate, cooled and analysed for orthophosphate by a phosphomolybdate method [6], using standards of sodium pyrophosphate heated with perchloric acid at the same time as the samples. Samples of the solutions which had not been perchlorate-treated were analysed for orthophosphate as the phosphomolybdate complex [6] and also by ion chromatography (BioLC system: Dionex UK Ltd., Crawley, Surrey, UK), using an AS5 column eluted with 1.7 mmol/L NaHCO_3 /1.8 mmol/L Na_2CO_3 .

For infra-red spectroscopy, samples were ground with dried KBr, formed into pellets and spectra obtained in a Nicolet 205 Fourier-transform infra-red (FTIR) spectrometer.

Crystal structure

X-ray diffraction

Patterns for one Method 1 preparation and three Method 2 preparations were obtained using a powder diffractometer consisting of an Enraf-Nonius FR590 X-ray generator, Ge 111 cut monochromator ($\text{CuK}\alpha_1$ radiation, wavelength 1.5406 \AA) and curved position-sensitive detector (INEL CPS-120), calibrated with $\text{Pb}(\text{NO}_3)_2$.

Rietveld refinement

The program GSAS (General Structure Analysis System) [7] was used to refine the structures with form

factors for neutral atoms. The background was modelled using Chebyshev polynomials of the first kind. The March–Dollase model for preferred orientation was used in all refinements, but differed little from unity, with the exception of one specimen prepared by Method 1, which demonstrated some extreme preferred orientation. The peakshape used was type 3 varying GU, GW, LX and trns. GU and GW are coefficients which model the Gaussian contribution to the peakshape, LX a coefficient which models the Lorentzian contribution and is also related to the size of the diffraction domains within the crystal, and trns a variable which models and corrects for apparent sample shift due to specimen transparency to X-rays. Initial atomic parameters came from the single crystal X-ray work of Mandel [8]. The space group was P-1. The occupancies, atom positions, unit cell, peak profile, and background parameters were varied. The occupancies of the Ca atom sites had a fixed value of 1.0 throughout the refinements. Likewise the anisotropic atomic displacement parameters given by Mandel [8] were used but not varied. Neither the atom occupancy nor their positional parameters of the H atoms were varied.

Microscopy

Suspensions of crystals in DPX resin were examined by ordinary and polarised-light microscopy. Measurements of crystal lengths and widths were made using a calibrated eyepiece scale. For scanning electron microscopy, crystals were suspended in ethanol and a drop of each suspension placed on a stub covered with double-sided adhesive tape. After draining off the ethanol and air-drying, the specimens were sputter-coated with gold and examined in a Cambridge 90B Stereoscan in the secondary emission mode at an accelerating voltage of 25 kV.

Results

The mean composition of four samples of intermediate prepared in the first step in this procedure (% w/w \pm 1 SD) was: Ca 20.8 \pm 2.3; P 22.2 \pm 1.5; K 4.0 \pm 1.3. On

examination by polarizing microscopy, the intermediate was found to contain fine needle-shaped crystals embedded in an isotropic, apparently amorphous material. The intermediate yielded an X-ray diffraction pattern but this did not correspond to any of the salts investigated by Brown et al. [3].

Once prepared, the intermediate could be used to prepare *t*-CPPD for at least several months. If, during the second, heated step, the final pH was higher than about 5.0, the product usually contained other types of crystal besides the characteristic parallelepipedal crystals of *t*-CPPD, indicating a mixed product. Some preparations consisted entirely of a single type of crystals which were not *t*-CPPD. This limited the starting concentration of urea which could be used if a pure preparation of *t*-CPPD were to be obtained, and hence controlled the possible yield. With the procedures given here, the final pH of the mother liquor (after cooling) was 3.3–3.5 in Method 1 and 4.8–5.0 in Method 2.

The mean composition of 17 samples of *t*-CPPD (% w/w \pm 1 SD) was: Ca 26.3 \pm 1.8, P 21.3 \pm 1.1. Stoichiometric *t*-CPPD contains 27.6% w/w Ca and 21.4% w/w P. Potassium was detected (at <1% w/w) only in two samples. In acid solutions of the crystals which had not been subjected to perchlorate hydrolysis, orthophosphate was detected in small, variable quantities by the phosphomolybdate method [6], which includes a 2 h incubation at 37 °C under strongly acidic conditions (1 mol/L sulphuric acid). However, none was detected by ion chromatography, in which the eluent has a pH of about 10. We therefore concluded that the phosphomolybdate procedure caused partial acid hydrolysis of pyrophosphate.

Typically, Method 1 yielded up to 0.6 g *t*-CPPD per g calcium pyrophosphate intermediate, while Method 2 yielded about 0.3 g *t*-CPPD per g intermediate. The reason for the difference in yields is not known.

All *t*-CPPD preparations consisted of colourless, transparent, positively birefringent crystals. The crystals were parallelepipedal in form, with sharp edges and corners. Information on size (length and width) and shape (length/width ratio) is given in Table 1. Method 1 produced large crystals, 45–190 μ m long, with considerable variation in length/width ratio. Scanning

Table 1 Dimensions of *t*-CPPD crystals in one sample prepared by Method 1 and two samples prepared by Method 2. Light microscopy of 40 crystals of each preparation: Means \pm 1 SD

	Method 1 (not stirred)	Method 2 (moderate heating and stirring)	Method 2 (rapid heating and stirring)
Length (μ m)	100.8 \pm 35.8	12.3 \pm 3.9	17.6 \pm 9.4
Width (μ m)	13.9 \pm 6.0	3.4 \pm 1.2	1.03 \pm 0.6
Length/width ratio	9.6 \pm 8.1	3.9 \pm 1.4	19.4 \pm 7.4

electron microscopy showed that these crystals formed clusters in which there was extensive intergrowth (Fig. 1). Crystals produced by Method 2 were much smaller than those produced by Method 1 (Fig. 2, Table 1). When rapid heating and high stirring rates were used, the crystals were more elongated and more heterogeneous in size, including some that were too small to measure reliably by light microscopy (Fig. 2).

All preparations of crystals gave FTIR spectra identical with that of *t*-CPPD prepared by the method of Mandel et al. [4] (Fig. 3). The crystals yielded sharp X-ray diffraction patterns characteristic of well-crystallised *t*-CPPD, but with additional weak *t*-CPPD reflections to those reported by Mandel et al. [4]. No other phases were detected. Rietveld refinements [9] were performed on the X-ray diffraction patterns of one Method 1 preparation (H45) and three Method 2

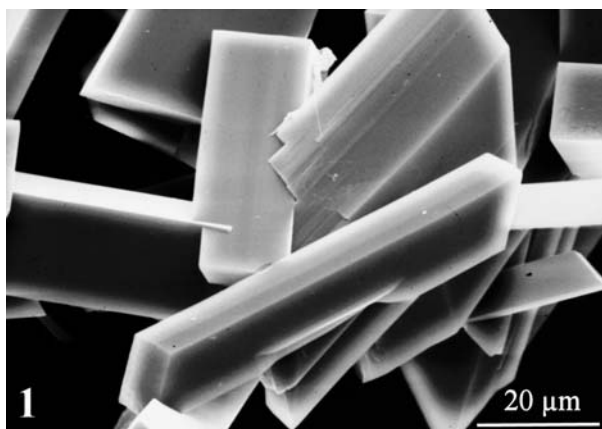


Fig. 1 Scanning electron micrograph of large, intergrown *t*-CPPD crystals prepared by Method 1

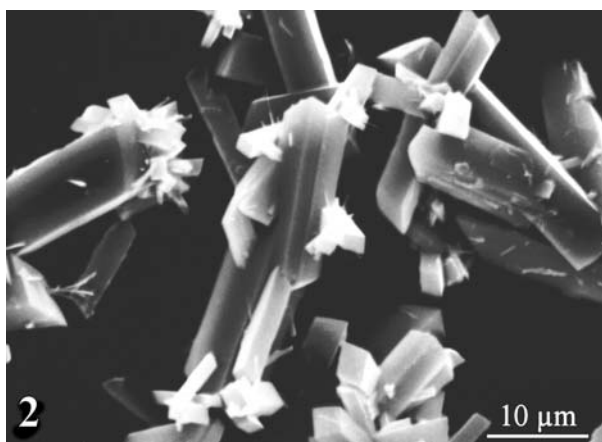


Fig. 2 Scanning electron micrograph of small *t*-CPPD crystals with a heterogeneous size distribution prepared by Method 2, with rapid heating and stirring

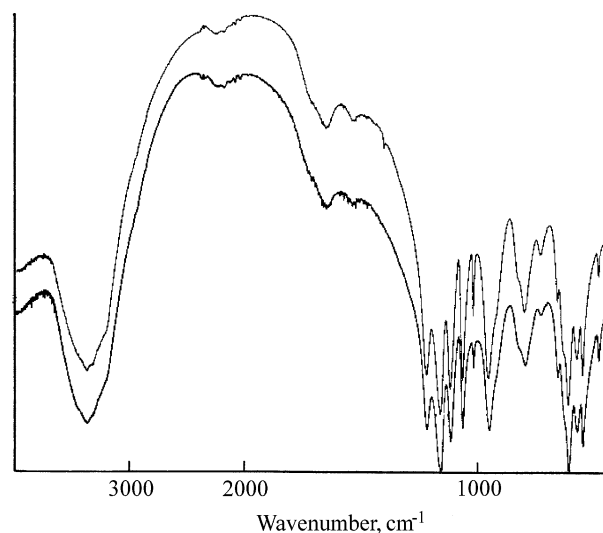


Fig. 3 FTIR spectra of (upper) *t*-CPPD prepared by method of Mandel et al. (1988) [4]; (lower) *t*-CPPD prepared by Method 1

preparations (H61, H63, H65). The refinement of H45 was very problematic as the sample exhibited severe preferred orientation. Consequently the unit cell parameters were obtained, but the peak intensities were never successfully modelled. No extra peaks from other phases were detected in the background, but it was impossible to identify any contaminant peaks overlapping with the *t*-CPPD peaks because of the fitting problems. In contrast good refinements of H61, H63 and H65 were obtained. Figure 4 shows an example of the H61 data and the Rietveld fit to the data. Atom occupancies varied within a few percent of 1.0 and generally the atom positions were within one or two estimated standard deviations (esd's) of the values given by Mandel [8]. The unit cell parameters obtained are compared to those of Mandel in Table 2. The differences from Mandel's results may be real, or may just be due in part to systematic errors, which are not accounted for in the model and certainly are not accounted for in our quoted esd's.

Discussion

In the method of Brown et al. [3] and its modification by Mandel et al. [4], an acidic intermediate, calcium dihydrogen pyrophosphate (CDPP), is first synthesised by adding $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$ to hot pyrophosphoric acid and *t*-CPPD is produced by crystallisation from a saturated solution of this material. While we have found the modified method [4] to be reliable, it has disadvantages. (1) Production of CDPP involves heating concentrated phosphoric acid to 215 °C for more

Fig. 4 X-ray powder diffraction pattern of H61 for 10 to 40° 2 θ . (+ + + +) experimental and (---) fitted. The tick marks are the expected peak positions, beneath each of which is the difference between the experimental and fitted patterns

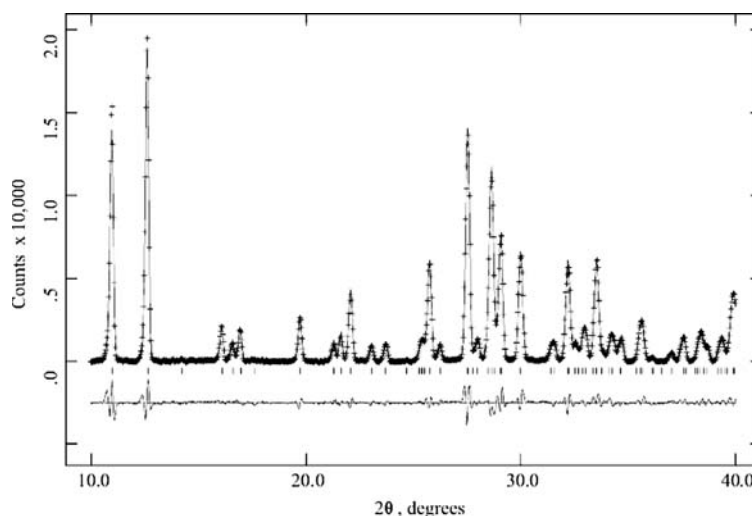


Table 2 Rwp values and unit cell parameters (in Ångstroms and degrees), obtained by Rietveld refinement, for the *t*-CPPD samples compared to the single crystal data from Mandel [7]. Estimated standard deviations are given in parenthesis

	Rwp	<i>a</i> (Å)	<i>b</i> (Å)	<i>c</i> (Å)	α (degrees)	β (degrees)	γ (degrees)
Mandel	–	7.365(4)	8.287(4)	6.691(4)	102.96(1)	72.73(1)	95.01(1)
<i>Method 1 (unstirred)</i>							
H45	–	7.360(4)	8.286(4)	6.688(4)	102.87(2)	72.76(2)	94.95(1)
<i>Method 2 (stirred)</i>							
H61	0.0617	7.3569(2)	8.2738(3)	6.6860(2)	102.819(2)	72.708(2)	94.950(2)
H63	0.0640	7.3653(5)	8.2732(6)	6.6856(5)	102.781(2)	72.667(2)	94.942(2)
H65	0.0530	7.3570(4)	8.2746(4)	6.6857(4)	102.836(1)	72.708(1)	94.950(1)

than 1 h, and this presents a hazard. (2) The intermediate is unstable, and we have found it best to use it within a day of preparation. (3) The yield of CPPD is small, typically about 0.03 g per g $\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot 2\text{H}_2\text{O}$ in our experience. The method described here overcomes these drawbacks

Our observations on the calcium pyrophosphate intermediate produced in the first part of our procedure suggest that it is composed of a phase(s) different from *t*-CPPD. The low potassium content of the intermediate indicates that in the second step the crystals form in an environment with few impurities, so that the final product shows little or no contamination with potassium.

The yield of *t*-CPPD crystals is limited mainly by the amount of urea which can be used, since this controls the final pH and this in turn must be limited, if formation of other phases is to be avoided.

In attempts to produce the monoclinic form, we have conducted numerous experiments in which we have systematically modified the solution in the second step, e.g. by including magnesium ions, which favour

m-CPPD formation in low-temperature systems [10, 11]. None of these experiments were successful but it remains possible that the method could be adapted for preparation of *m*-CPPD.

The refinements showed that the Method 2 preparations examined were pure *t*-CPPD as no impurities could be seen in the diffractograms. The Method 1 preparation was also probably pure *t*-CPPD but this could not be definitely confirmed by Rietveld refinement.

We have found, like Mandel et al. [4], that smaller crystals can be produced by stirring the reaction mixture [12]. The mean lengths of *t*-CPPD crystals isolated from synovial fluid are typically 1–5 μm in length [2], so the small crystals prepared by our Method 2, although still larger than the size in vivo, would be more suitable than those prepared by Method 1 for many studies related to arthritis research. It seems clear that stirring rate and heating rate have a marked influence on the size and morphology of the resulting crystals and standardisation of these factors would be essential, to ensure consistency of crystal size

and shape between batches. In view of the fact that microscopical studies have assigned needle-like crystals to *m*-CPPD and rhomboidal crystals to *t*-CPPD [2], it is of interest that some of the crystals in our stirred preparations were more elongated than the crystals produced in our unstirred preparations or by previously reported methods.

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