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Calcium Phosphate Phase Transformations in Serum

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Summary. A better knowledge of the pathological calcification mechanisms should provide a rational basis for their control. In the present study, dicalcium phosphate dihydrate (DCPD, $CaHPO_4 \cdot 2H_2O$) was used as a source of calcium and phosphate ions to investigate the mechanism of formation of more basic and more insoluble calcium phosphates in ultrafiltered serum (u.f.s.). DCPD crystals were suspended in u.f.s. at 37°C by constant stirring; samples were removed periodically for calcium and phosphate analysis and pH measurement. Occasionally, samples of solids were removed for X-ray diffraction. The experiments were carried out both with and without a 5.5% CO_2 atmosphere. After initially becoming saturated with DCPD, the u.f.s. composition changed and became saturated with respect to octacalcium phosphate (OCP, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$). At this point OCP crystals were detected in the solid phase by X-ray diffraction. Further stirring changed the composition so that it became undersaturated with both DCPD and OCP and shifted toward, but did not reach, a value so low as to be saturated with hydroxyapatite (OHAp, $(Ca_5(PO_4)_3OH)$. The presence of CO_2 in the atmosphere slowed down, but did not prevent, the above sequence of events. The above results strongly suggest that calcifications, beneficial and pathological, that take place in serum may involve OCP as a precursor, which hydrolyzes *in situ* to a more basic apatitic product. The results also indicate that direct formation of OHAp in u.f.s. is a very slow process and may occur only rarely. The process appears to be similar in whole serum.

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Key words: Dicalcium phosphate dihydrate — Ultrafiltered serum — Octacalcium phosphate — Hydroxyapatite — Ion activity products.

The exact nature of the inorganic constituent in calcified cardiovascular deposits is not known. There is agreement that it consists mostly of calcium phosphates, but the reported Ca/P ratios, structural nature of the products, and names used to identify them vary widely from one investigator to the next: Harasaki et al. [1], Ca/P = 2.3, "calcium phosphate" by X-ray diffraction analysis; Coleman et al. [2], Ca/P = 1.88, "hydroxyapatite"; Bigi et al. [3], Ca/P = 1.5, "β-tricalcium phosphate; Yu [4], Ca/P = 0.3–1.5, "carbonate apatite"; Etz et al. [5], Ca/P = 1.75, "OCP-hydrolyzate."

The calcified deposits on heart valves and implants are in contact with blood and those in the walls of aortas are in close interaction with blood. In contrast to bone, which may involve active pumping of calcium and phosphate ions, cardiovascular deposits probably have a more passive relationship with blood. Thus, the level of saturation of blood is more likely to reflect to some degree the solubility of such deposits. Therefore, it is important to study the stability and phase transformations of different calcium phosphates in blood in order to understand the mechanisms of formation of calcified deposits.

There is increasing evidence that octacalcium phosphate (OCP, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$) occurs as a precursor in the formation of many biominerals [6–8]. We present here evidence, both structural and thermodynamic, that OCP is involved in the formation of an apatitic product in ultrafiltered human serum (u.f.s.) and in whole serum. Since most pathological biomineralizations take place in direct contact with serum or at least have passive

relationships with serum, these observations may have implications regarding a broad range of calcifications.

Materials and Methods

Materials

DCPD was prepared by ammoniation of an aqueous solution equilibrated with monocalcium phosphate monohydrate ($Ca(H_2PO_4)_2 \cdot H_2O$) and commercial DCPD according to the procedure described by Moreno et al. [9]. OCP was prepared by hydrolysis of DCPD in distilled water added dropwise over a period of about 3 months at room temperature [10]. Both the DCPD and OCP yielded their characteristic X-ray diffraction patterns (Philips X-ray diffractometer). Carbon dioxide was mixed with nitrogen in a 5.5:94.5 ratio by volume in order to get a mixture of 5.5% CO_2.

Ultrafiltration

Human serum was obtained from freshly collected blood which was allowed to clot and then centrifuged at room temperature. Then it was filtered through a high-performance thin-channel ultrafiltration Amicon system equipped with an XM-50 Amicon Diaflow filter with nominal exclusion of 50,000 molecular weight. This process removes very nearly all the proteins from the serum [11, 12]. (There are essentially no low molecular weight proteins in the range of 0–50 K molecular weight in serum.) The ultrafiltrate contains the inorganic constituents (ionized and complexed calcium, inorganic phosphate, magnesium, bicarbonate, pyrophosphate, etc.) and most of the inhibitors present in serum [12–14]. After the ultrafiltration the pH of the u.f.s. was in the range of 8–8.5 because of partial CO_2 loss during the processing and delivery.

Analytical Methods

Calcium was determined spectrophotometrically as the Ca-Arsenazo III complex, and phosphate was determined as the phospho-molybdate malachite green complex according to the procedure described by Vogel et al. [15]. The validity of the calcium and phosphate analysis procedures was confirmed in a previous study with ultrafiltered plasma by the method of known additions and by comparing the results from different methods (i.e., atomic absorption [16] for calcium and that of Murphy and Riley [17] for phosphate).

Hydrolysis Procedure

The experiments were carried out in polypropylene test tubes which were mounted either in jacketed Pyrex cells through which thermostated water was circulated or in a constant temperature bath. The test tubes were equipped with a special cap with close-fitting holes for a combination pH electrode and with three tubes for introducing the solids, taking samples, and supplying the CO_2 mixture in some experiments. Three to 5 ml of u.f.s. were stirred in the reaction test tube until a uniform temperature was obtained. The initial pH was measured, and then DCPD crystals were added to the stirred, thermostated u.f.s. or serum through the feeding funnel. The hydrolysis was followed by continuously or periodically recording the pH of the solution and by periodically removing samples (during continuous stirring) of the u.f.s. for analysis of calcium and phosphate and the solid for X-ray diffraction. When the pH was recorded continuously, the electrode was recalibrated periodically.

The apparent ion activity products (IAPs) for the various phases of calcium phosphate in the u.f.s. were calculated from the calcium and phosphate concentrations and pH using an iterative procedure. The ion activity coefficients were calculated through the use of the expanded Debye-Huckel equation [18]. The formation of ion pairs, $CaPO_4^-$, $CaHPO_4^0$, $CaH_2PO_4^+$, and $CaOH^+$ are taken into account as well as the dissociation of H_3PO_4 and H_2O [18] and the presence of 0.13 M/L NaCl in serum. The program takes into account the formation of $CaCO_3^0$ and $CaHCO_3^+$ complexes [19] in experiments that were done under 5.5% CO_2 atmosphere. The extent of ion pair formation between Na and PO_4 is not well known; therefore it was not taken into account [20].

The ion activity products are defined as follows:

$$IAP(DCPD) = (Ca^{2+})(HPO_4^{2-})$$

$$IAP(OCP) = (Ca^{2+})^4(H^+)(PO_4^{3-})^3$$

$$IAP(OHAp) = (Ca^{2+})^5(PO_4^{3-})^3(OH^-)$$

The negative logarithms of the IAP and K_{sp} values (pIAP and pK_{sp}, respectively) were calculated, and the differences between them ($\Delta pK = pK_{sp} - pIAP$) were used to define the degrees of saturation of each of the above calcium phosphate phases. Negative values for ΔpK indicate undersaturation, positive values supersaturation, and zero values saturation. X-ray patterns of the solid phases and chemical potential plots, $-\log[(Ca^{2+})(OH^-)^2]$ vs. $-\log[(H^+)^3(PO_4^{3-})]$, were used to help identify the equilibrating solid phase [21].

Results

The pH, the phosphate and calcium concentrations of the solutions, and the ΔpK values for relevant calcium phosphate phases as functions of time for a typical DCPD hydrolysis experiment in u.f.s. (30 mg DCPD/ml u.f.s.) are shown in Figure 1. Addition of DCPD crystals to u.f.s. in a closed system (i.e., without addition of CO_2) caused an immediate decrease in pH (from around 8.7 to 8.0) and an increase in calcium and phosphate concentrations (Fig. 1). These three quantities remained relatively constant during the first 20 minutes. In the next 3 hours, the calcium concentration decreased, and the phosphate concentration increased slowly. During the same period the pH was constant (7.97 $\pm$ 0.06, n = 18 from five experiments) for approximately 1.5 hours, and then a slight increase was noticed (pH = 8.15 at most). During the time interval from 6 minutes to 3.5 hours, the u.f.s. was very close to saturation of DCPD, as indicated by a mean ion activity product (IAP) for DCPD of $(2.55 \pm 0.09) \times 10^{-7}$ (n = 17 from five experiments) vs. the K_{sp} of DCPD of $(2.3 \pm 0.06) \times 10^{-7}$ [22].

After about 3.5 hours of near constancy, the pH

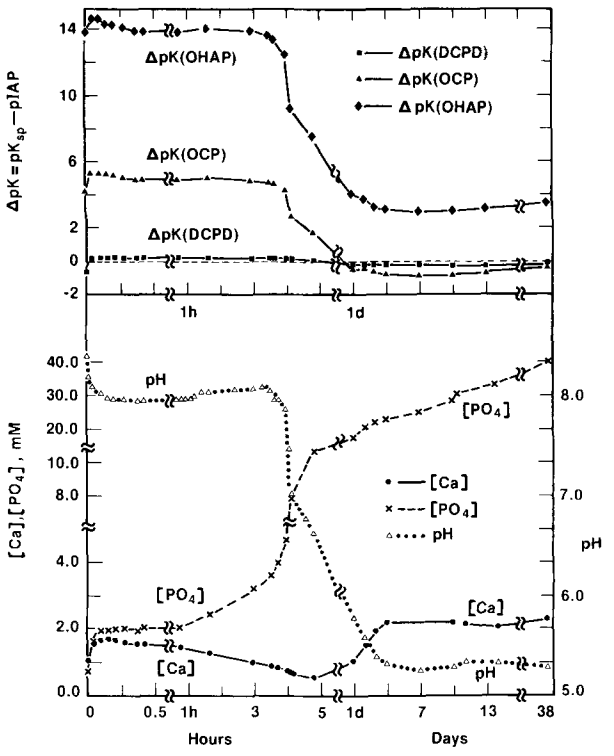

Fig. 1. Hydrolysis of DCPD (30 mg/ml) in u.f.s. in a closed system. Ca and PO_4 concentrations, pH, and ΔpK for relevant calcium phosphate phases are shown as functions of time.

dropped sharply (Fig. 1) from 8.0 to 7.0 in a 20 ± 3 minute interval (four experiments). This was accompanied by a rapid increase in the phosphate concentration and a slow decrease in the calcium concentration. In the next hour the pH continued to decrease from 7.0 to 6.6 and then to 5.73 ± .05 (Table 1) in about 1 day. This was accompanied by a concomitant increase in the phosphate concentration and a slow increase in the calcium concentration (Fig. 1 and Table 1). During this period of changes, the pIAP(OCP) increased to 48.6 ± 0.2 (four experiments), which is in the range of quoted pK_{sp}(OCP) values (Tung, Eidelman, Brown, in preparation) and of pIAP(OCP) in u.f.s. [23]. The presence of OCP in the precipitate was confirmed by its peaks in the X-ray diffraction pattern along with those of DCPD. The pH continued to decrease slowly to about 5.5 (2 days), calcium increased slowly to 1.6 mM, and phosphate concentration to 20–28 mM. During the latter period of change, the ion activity products for β-TCP and OCP decreased to values below their respective solubility products. Hydrolysis of the remaining DCPD and OCP continued slowly as the solution proceeded towards greater undersaturation with DCPD, β-TCP, and OCP and approached but never reached the value as low as the solubility product of OHAp, pK_{sp}(OHAp).

The pH stabilized in the range of 5.3–5.4 after 3 days of the process and remained constant (5.33 ± 0.06, n = 13 samples from three experiments) for periods as long as 38 days (Table 1). The pIAP(OHAp) stabilized at 55.2 ± 0.7 (n = 13 samples taken in the interval 3–38 days from three experiments). This value is about three units below the pK_{sp} for pure OHAp (58.6) [18, 24] but in the range of values reported for biological minerals such as enamel and bone powder [25–27] and similar to those found earlier in equilibration of OHAp with u.f.s. [23]. The combined solid phases collected after 10, 11, and 14 days of the process yielded an X-ray powder diffraction pattern showing the presence of OHAp peaks in addition to those of residual DCPD and OCP. Comparison of the X-ray pattern of the solid phases harvested after 38 days to that of the solid after 1 day showed clearly that the OCP peak at 2θ = 4.75° had almost disappeared and those at 2θ = 25.9° and 31.8° of OHAp were present in addition to those of DCPD and other small peaks of OHAp. This is strong evidence that the transition of OCP to a more basic apatitic product occurs in u.f.s. and that under the present experimental conditions the process was relatively slow.

In control experiments with u.f.s. without addition of solids, the pH and the calcium and phosphate concentrations were quite stable for a long time. As mentioned in the "ultrafiltration" section, part of the CO_2 escaped during the process and handling; consequently, in the closed system (without addition of CO_2) there were variable amounts of CO_2 but less than 5.5% (the physiological partial pressure). The main influence of carbonate comes through its effect on pH. The carbonate affects also the concentrations of the ion pairs $CaCO_3^0$ and $CaHCO_3^+$. The concentrations of these ion pairs *per se* were insignificant in the pH range 5.3–6.9 during the phase transformation. Even if a maximum of 5.5% CO_2 had been assumed in the calculations of the pIAPs, the value would have differed insignificantly from the one given here and would not have affected the levels and trends in supersaturation. Therefore, the calcium carbonate complexes were not taken into account in the calculations of the pIAP in the closed system.

Similar experiments were also conducted under 5.5% CO_2 atmosphere. On exposure of u.f.s. samples to 5.5% CO_2, the pH dropped almost instantly to 7.5 (i.e., close to physiological pH). This pH did not change on addition of DCPD crystals (Fig. 2, representative experiment). Both calcium

Table 1. Results of experiments with 30 mg/ml DCPD in u.f.s. (closed system)

Time of process	No. of exp.	No. of samples	pH	Ca (mM)	PO_4 (mM)	Solid phases by XRD
6 min–3½ h	7	32	8.05 ± 0.09	1.7–0.8	2–4.5	DCPD
4–5 h	4	8	7–6.6	0.6 ± 0.04	8–17	DCPD + OCP
1 d	5	5	5.73 ± 0.05	1.1 ± 0.1	17–27	DCPD + OCP
2 d	2	2	5.47–5.57	1.6	20–28	DCPD + OCP
3–38 d	3	13	5.33 ± 0.06	2.0 ± 0.4	22–40	DCPD + OCP + apatitic phase

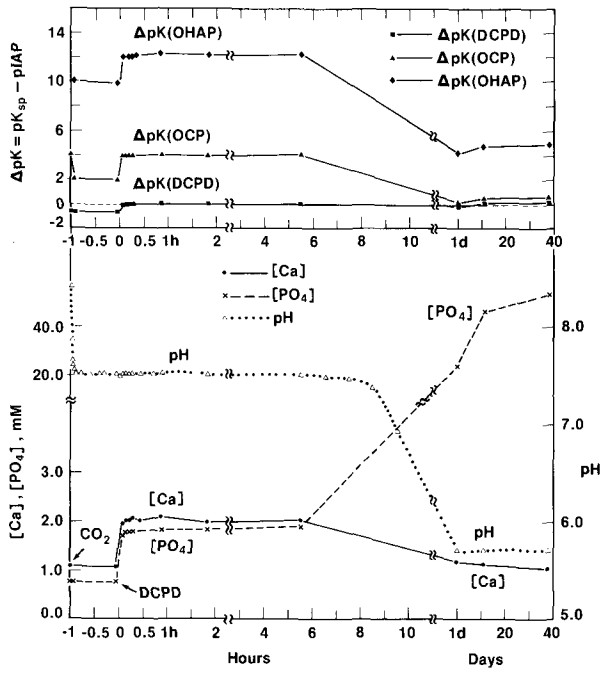

Fig. 2. Hydrolysis of DCPD (30 mg/ml) in u.f.s. under 5.5% CO_2 atmosphere. Ca and PO_4 concentrations, pH, and ΔpK are shown as functions of time. 5.5% CO_2 atmosphere was introduced at time = −1 hour; DCPD crystals were introduced at time = 0.

and phosphate concentrations increased within 5 minutes to around 2 mM. The pH, calcium and phosphate concentrations remained constant for more than 5 hours. Thereafter, the decreases in calcium concentration and pH, the increase in phosphate concentration, and the precipitation of OCP took place in ways similar to those in systems without CO_2 but at considerably lower rates (4 hours from pH 7.5–7.0 instead of 20 minutes in a closed system, Figs. 1, 2). The OCP peaks in the X-ray diffraction patterns appeared later than in experiments without CO_2, an additional indication that CO_2 slowed the DCPD → OCP transformation. It appears that CO_2 also slowed the OCP → OHAp transition as can be seen from comparison of parallel experiments using u.f.s. from the same source with and without CO_2. After 38 days the OCP peak at $2\theta = 4.75°$ in the experiment with CO_2 was stronger than in the experiment without addition of CO_2. In control experiments of u.f.s. under 5.5% CO_2 without addition of solids, the pH, calcium, and phosphate concentrations were stable for long periods of time.

In the experiments carried out with a lower DCPD-to-solution ratio (10 instead of 30 mg/ml) in a closed system, the phenomena were about the same as for the higher solid-to-solution experiments. However, the saturation with DCPD was reached more slowly (Fig. 3a), the sharp decrease of pH from 8.3 to pH 7 began after 9 hours instead of 3.5 hours and was complete in 36 minutes, and OCP precipitation was detected sooner than in experiments with the higher solid-to-solution ratio. The most notable difference was that the solid phase after 1 day in the low solid-to-solution ratio experiment was well-crystallized OCP without detectable DCPD (Table 2).

Under CO_2 (Fig. 4), the saturation with DCPD in the experiment with a low solid-to-solution ratio was achieved almost immediately; the pH started to decrease after 9 hours also, but it took 6 hours to go from 7.5–7.0; some of the DCPD remained even after 2 days (Table 2).

As a consequence of the observation that the DCPD appeared to have hydrolyzed completely to OCP after 1 day in the low solid-to-solution experiment without addition of CO_2, a second, longer-term experiment was carried out to determine if the OCP that was formed in u.f.s. would hydrolyze to OHAp as it does in dilute H_3PO_4 solutions. Samples were taken after 4, 8, 20, 40, and 150 days (Fig. 3b, Table 2). The solid phases sampled between 4 and 20 days were found to be OCP by X-ray diffraction analysis. This confirmed that the DCPD hydrolysis was complete. The long-term rise in the pH (Fig. 3b) is probably caused by the slow but continuous escape of CO_2. Since the hydrolysis of DCPD would drive the pH down when this is the dominant process, the effect of gradual loss of CO_2 on pH would be negligible. However, when the hydrolysis reaction is near completion, the escape of CO_2 can cause the pH to rise. Partial hydrolysis of

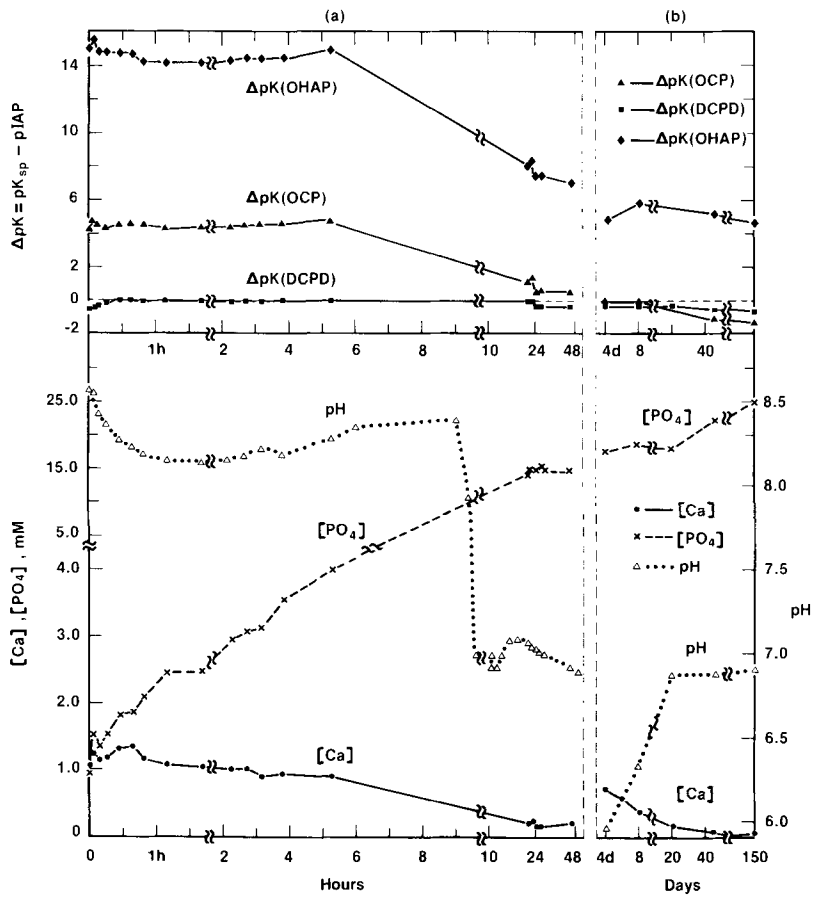

Fig. 3. Hydrolysis of DCPD (10 mg/ml) in u.f.s. in a closed system. Ca and PO_4 concentrations, pH, and ΔpK are shown as functions of time. **(a)** Short-term experiment (0–48 hours). **(b)** Long-term experiment (0–150 days).

Table 2. Results of experiments with 10 mg/ml DCPD in u.f.s. with and without CO_2

Time	CO_2	pH	Solid phases by XRD
3 min	—	8.5	DCPD + OCP
5 h	—	8.2	DCPD + OCP
1 d	—	7.0	OCP
4 d	—	6.0[a]	OCP
8 d	—	6.3	OCP
20 d	—	6.9	OCP
46 d	—	6.9	OCP + apatitic phase
5 mo	—	6.9	Apatitic phase
6 min	+	7.5	DCPD + OCP
1 d	+	6.3	DCPD + OCP
2 d	+	5.9	DCPD + OCP

[a] Different experiment (see text and Fig. 3)

OCP to OHAp was detected after 20 days by an increase in phosphate concentration, decreases in the calcium concentration, in the intensity of the OCP peak in the XRD, and in the values of ΔpK(OCP). The hydrolysis appeared to continue for about 5 months when the main OCP peak almost disappeared, but the pIAP(OHAp) was 54.0, considerably smaller than pK_{sp}(OHAp). This is consistent with the observations [23, 25, 26] that the apatitic phases that form in biological systems either have higher solubilities than OHAp, or their formation from supersaturated solutions is greatly retarded by the presence of natural crystal-growth inhibitors in u.f.s. [11]. This is also further evidence that OCP → OHAp is a relatively slow process even when the pH is higher (6–7 in comparison to 5.3 in the high solid-to-solution ratio experiment).

In experiments carried out at 25°C instead of 37°C, the phenomena were about the same except that they occurred at slower rates.

In preliminary experiments with whole serum, the DCPD hydrolyzed completely to OCP in the lower solid-to-solution ratio experiment (10 mg/ml) after about 1 day. This phase hydrolyzed completely to an apatitic product after about 13 days (Table 3). In the higher solid-to-solution ratio experiments (Table 3), the final product (32 days) was DCPD plus an apatitic phase. Qualitative comparison between results obtained in u.f.s. and in whole serum showed that in the low ratio experiments the rate of DCPD → OCP transformation is quite the same, but the OCP → OHAp hydrolysis is faster in whole serum (compare Table 2 and Table 3). Thus,

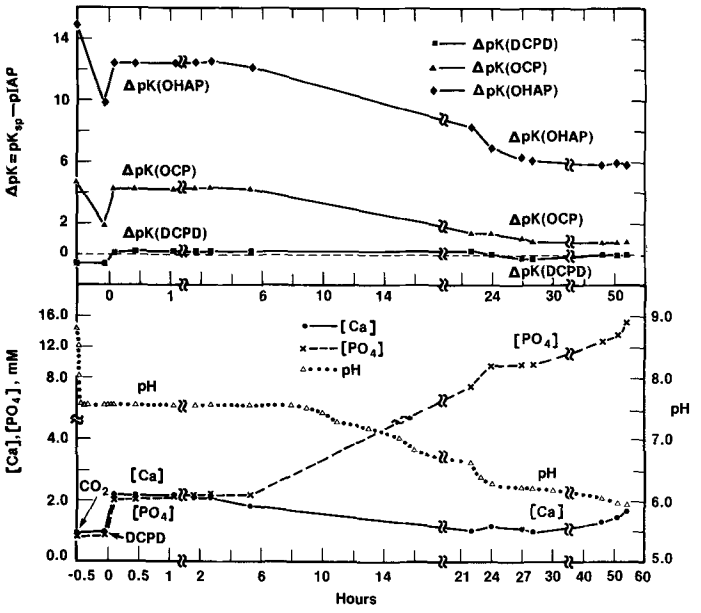

Fig. 4. Hydrolysis of DCPD (10 mg/ml) in u.f.s. under 5.5% CO_2 atmosphere. Ca and PO_4 concentrations, pH, and ΔpK are shown as functions of time. 5.5% CO_2 atmosphere was introduced at time = −.5 hours; DCPD crystals were introduced at time = 0.

whole serum may contain constituents with molecular weight above 50,000 (the cut-off of the ultrafiltration membrane) that facilitate hydrolysis of OCP to an apatitic product. This warrants further investigation.

Discussion

Brown et al. [28] were the first to report the transformation DCPD → OCP → OHAp in dental calculus. Later they described the *in situ* hydrolysis of OCP to an apatitic product as seen with a petrographic microscope [29]. It is well known that at physiological pH a solution saturated with DCPD is supersaturated with OCP and OHAp, and that a solution saturated with OCP is supersaturated with OHAp. Thus, the transition DCPD → OCP → OHAp observed in all of the above experiments is consistent with thermodynamic expectations. In addition to the transformation sequence DCPD → OCP → OHAp, there is an important distinction in the mechanisms of crystal formation. The transformation DCPD → OCP appears to take place almost always by dissolution of the DCPD crystals, followed by reprecipitation of the Ca and PO_4 ions as OCP. In contrast, the mechanism preferred in the OCP → OHAp transition is hydrolysis *in situ* to form nonstoichiometric, carbonate-containing, apatitic crystals that are pseudomorphs of the original OCP crystals [30].

Why is OCP an intermediary in the transition from DCPD to OHAp instead of direct formation of OHAp? The answer consists of two parts. First, as a general principle, hydrated crystals appear to crystallize more rapidly than anhydrous crystals; examples of this are the general observations that DCPD and OCP crystallize much more rapidly than the anhydrous compounds, anhydrous dicalcium phosphate (DCPA), β-TCP, and OHAp. And secondly, as a general observation the crystal growth rates of hydrated crystals are affected less by growth poisons than are those of anhydrous crystals. Thus, since OCP is the precursor in many simple *in vitro* systems, it is even more likely to be the precursor in systems such as serum which contain a variety of ionic substances that are known to impede crystal growth [11].

The simple *in vitro* observations on serum reported here have the implication that OCP is a precursor to formation of the apatitic phase in other passive biological calcifications. Furthermore, it is unlikely that DCPD can normally be a precursor in calcifications in serum because serum is usually undersaturated with DCPD [23]. In this connection, it is of interest that the two known examples where passive calcification can take place and which can also produce DCPD are dental calculus and renal stones. Both saliva and renal fluid can be supersaturated with DCPD, yet, in both of these it is known that OCP can act as a precursor to the formation of OHAp [28, 31].

A possible precursor for OCP in biomineralization might be amorphous calcium phosphate (ACP), which appears initially in acitve calcifications such as in model systems: liposomes [32], in Pressman cells [33], or in cartilage [34]. However, ACP is more soluble than OCP at physiological pH [35, 36]

Table 3. Results of DCPD hydrolysis in whole serum in 37°C, closed system

Time of process	Solid-to-solution ratio	pH	Solid phases by XRD
27 min	30 mg/ml	8.23	DCPD
3.5 h	30 mg/ml	8.04	DCPD
6 d	30 mg/ml	5.73	DCPD
32 d	30 mg/ml	5.27	DCPD + apatite
70 min	10 mg/ml	8.30	DCPD
1 d	10 mg/ml	6.24	OCP + small quantity of DCPD
6 d	10 mg/ml	5.72	OCP
13 d	10 mg/ml	5.60	Apatite
32 d	10 mg/ml		Apatite

and converts spontaneously to OCP through a dissolution-precipitation process. Thus, ACP would behave in the same way as DCPD and would not preclude OCP's role as precursor.

As noted above, when OCP hydrolyzes to OHAp, the process is mostly a solid-state transition in which the OCP crystals either incorporate calcium ions from the surroundings or release phosphate ions, as can be seen from the following two idealized limiting representations of the hydrolysis process:

$$Ca_8H_2(PO_4)_6 \cdot 5H_2O + 2Ca(OH)_2 = 2Ca_5(PO_4)_3OH + 7H_2O$$

$$Ca_8H_2(PO_4)_6 \cdot 5H_2O = 8/5\ Ca_5(PO_4)_3OH + 6/5\ H_3PO_4 + 17/5\ H_2O$$

The above equations explain the observations that the calcium ion concentration decreased, the phosphate ion concentration increased, and the pH decreased when OCP hydrolyzed to a more apatitic form. Similar changes in Ca, PO_4, and pH apply to the hydrolysis of DCPD to OCP except that this is not a solid-state process. The *in situ* hydrolysis of OCP to OHAp stems directly from the structural relationship between these two compounds [6]. It has been suggested [37] that the highly irreversible, solid-state nature of the hydrolysis process is the reason why biological apatites tend to be nonstoichiometric and contain relatively large amounts of impurities.

The observation that CO_2 in the atmosphere slowed the hydrolysis process has two explanations. In the first place, CO_2 lowers the pH so that the degree of saturation of OCP relative to that of DCPD is reduced. The second reason is that carbonate may interact with the OCP surface, slowing the hydrolysis [10] as well as incorporating carbonate into the resulting apatitic phase.

The relative rates of the various processes, dissolution of the DCPD, precipitation of OCP, and hydrolysis of OCP, have important bearing on the observations. A plausible description of the rates of the processes that took place is as follows: Immediately after the DCPD crystals were added, the dissolution of DCPD was more rapid than any other process and the solution became saturated with DCPD. However, after OCP crystals formed, the rate of their growth increased with the increase of their surface area and became the dominant process, at which time the ion activity product in solution approached K_{sp}(OCP). When a low DCPD solid-to-solution ratio was used, this provided a low surface area of DCPD crystals so that the OCP precipitation reaction became the dominant process earlier than when the high solid-to-solution ratio was used. Eventually IAP(OCP) decreased below the K_{sp}(OCP), and the changes in the solution composition revealed that OCP was hydrolyzed to a more basic product. On the other hand, in the presence of CO_2 the OCP precipitation process slowed down so that the DCPD dissolution process could dominate for a longer period of time.

Although the DCPD → OCP → OHAp transformation observed in the present study took place under experimental conditions that do not mimic exactly the *in vivo* situation, the key findings described above appear to be valid over a wide range of conditions. For example, direct precipitation of OHAp was not detected in u.f.s. in a wide range of pH (8.5–5.3) and pIAP(OHAp) (44–54). Also, the same DCPD → OCP → OHAp transformation process was observed in the closed system, in which the pH fluctuated considerably, and under the 5.5% CO_2 atmosphere where the pH change was much smaller. Four major conclusions from the present study have important biological implications:

1. The direct formation of well-formed OHAp in the presence of serum is a very slow process. This is the most plausible explanation of why blood can be highly supersaturated with OHAp. This would mandate formation of the apatitic product by irreversible hydrolysis of OCP. Such a product may contain impurities and defects that can make it more soluble than OHAp. As a consequence, this apatitic material is more compatible with serum that is supersaturated with OHAp. In contrast to the extremely slow rate of formation of OHAp in serum, the direct formation of OHAp is relatively rapid in reagent grade solutions that are free of crystal growth poisons.

2. Since OCP appears to be a mandatory precursor for OHAp formation, the degree of supersaturation of serum with OCP may be the dominant variable controlling the rates of pathological calcifications. This suggests the possibility that the rate of

mineral formation can be reduced by lowering the ion activity product of OCP in serum by artificial means.

3. If OCP is a mandatory precursor, and since serum is slightly supersaturated with OCP [23], then this could provide an answer to that old question, "Why don't most tissues calcify under normal physiological conditions even though they are bathed with solutions supersaturated with OHAp?" If it is true that direct formation of OHAp is relatively slow, as the results given above suggest, then precipitation of OHAp would not occur in any tissue. The alternative route—formation of OCP followed by its hydrolysis—must take place with relatively low levels of supersaturation. Thus, such tissues need be only slightly inhospitable to calcification (e.g., lower pH or Ca and PO_4 concentrations) and OCP would not precipitate.

4. The apatitic mineral in calcified deposits appears to be formed by hydrolysis of OCP *in situ* [38]. The mechanism of this highly irreversible process determine to a large degree the physicochemical properties of the mineral that is formed. An important factor is the rate of formation of OCP relative to the rate of its hydrolysis. For example, if the rate of formation of OCP is more rapid than the rate of hydrolysis, the product will be mostly OCP. In reverse situations, the product will be mostly the poorly formed apatitic phase. The relative rates of these two processes may be subject to control by the constituents in serum that are either naturally present or introduced artificially.

Most of the experiments on OCP have been carried out in a pure system [6, 10, 29] and, therefore, one might question the relevance to biomineralization. The compatibility between the results obtained there and in a serum medium makes the former biologically more relevant.

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