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The involvement of calcium phosphates in biological mineralization and demineralization processes

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Abstract - The formation, remineralization and dissolution of hard tissues are complex processes that involve numerous calcium phosphate phases. Many biological mineralization processes, may therefore involve the formation of metastable intermediates which may subsequently transform into thermodynamically more stable phases. Physical chemical constant composition methods are discussed that enable kinetics studies to be made of the simultaneous growth and/or dissolution of multiple calcium phosphate phases. These methods have been used to investigate the participation of precursor phases such as dicalcium phosphate dihydrate and octacalcium phosphate in the formation of the thermodynamically more stable hydroxyapatite. Ions other than those of the crystal lattices as well as other molecules in the solution while not significantly incorporated into the precipitated crystallite phases may markedly influence the rates of mineralization and demineralization. This is especially true in supersaturated biological fluids such as serum and saliva in which many macromolecules, while behaving as precipitation inhibitors when present in the solution phases, may actually nucleate calcium phosphate phases when immobilized on surfaces.

INTRODUCTION

Many biological minerals contain calcium phosphate as a major component. Since many phase formation and transformation reactions may be involved in biomineralization, there is considerable interest in the elucidation of the mechanisms of crystallization and dissolution of these salts. This presents a considerable challenge to the surface physical chemist not only because of the numerous solid phases that may be involved but also because of the presence of multiple components in the solution media. It is therefore essential to make speciation calculations during the reactions. The presence of macromolecules as well as simple ionic species, may markedly influence the course of the mineralization reactions. In biological systems, calcium phosphate mineralization has frequently been suggested as proceeding through precursor phases such as amorphous calcium phosphate (ACP) or octacalcium phosphate (OCP) before transformation to the thermodynamically more stable hydroxyapatite (HAP) (1,2). Empirical formulae of these and other calcium phosphate phases that may be involved in the mineralization reactions are summarized in Table 1.

From Fig. 1, showing the solubility isotherms of the most important phases as a function of pH it can be seen in that numerous calcium phosphate phases may form and subsequently dissolve as spontaneous precipitation reactions proceed in solutions at relatively high initial supersaturation. Similarities in crystal structure, for instance, have led to the suggestion that OCP may be a precursor to the formation of HAP during a precipitation reaction (3). The thermodynamically less stable OCP can then undergo transformation through nonstoichiometric apatites to HAP.

Table 1. Calcium phosphate phases.

		Empirical formula	Molar Ca/P ratio
Dicalcium Phosphate Dihydrate	DCPD	$CaHPO_4 2H_2O$	1.00
Dicalcium Phosphate	DCPA	$CaHPO_4$	1.00
Octacalcium Phosphate	OCP	$Ca_8H_2(PO_4)_6 5H_2O$	1.33
b-Tricalcium Phosphate	TCP	$Ca_3(PO_4)_2$	1.50
Whitlockite	Mg-TCP	$Ca_{3-v}Mg_v(PO_4)_2$ $0 \leq v \leq 2$	3-v/2
Amorphous Calcium Phosphate	ACP	$Ca_9(PO_4)_6 xH_2O$	1.50
Hydroxyapatite	HAP	$Ca_{10}(PO_4)_6(OH)_2$	1.67
Defect Apatites		$Ca_{10-y}(HPO_4)_{6-y}(OH)_{2-y}$ $0 \leq y \leq 2$	10-y/6
Fluoroapatite	FAP	$Ca_{10}(PO_4)_6F_2$	1.67
Fluorohydroxyapatite	FHAP	$Ca_{10}(PO_4)_6F_z(OH)_{2-z}$ $0 \leq z \leq 2$	1.67

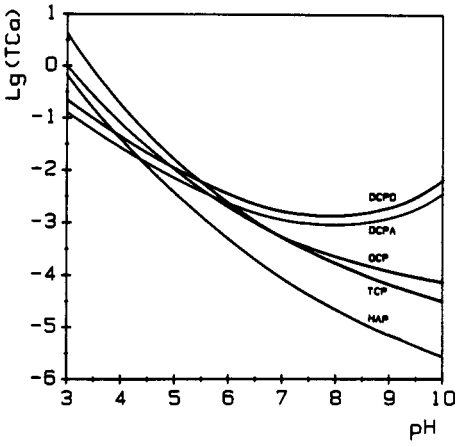

Fig. 1 Solubility isotherms of calcium phosphate phases in the ternary system $Ca(OH)_2$-H_3PO_4-KNO_3-H_2O at 37°C. The curves have been calculated for solutions in which $T_{Ca}=T_p$ and the ionic strength is 0.1 mol L^{-1}.

The nature of the phases that form depends upon the pH and, *in vivo*, whether normal or pathological mineralization is occurring. Thus *in vivo*, there is strong evidence that the formation of HAP is preceded by the precipitation of ACP having approximate TCP stoichiometry. For pathological mineralization and *in vitro* systems, all three precursor phases, TCP (4), OCP (5) and DCPD (6) have been proposed. A major difficulty in these studies is that different workers have used very different experimental conditions in the preparation of the calcium phosphate phases and in studying the course of the precipitation reactions. Although considerations based upon relative solubility data (Fig. 1) and thermodynamic driving force for growth or dissolution may be important, it is now quite well established that kinetic factors may have a considerably greater effect in determining the nature of the calcium phosphate solid phases present during a precipitation reaction. Moreover, different calcium phosphate phases may be stabilized or destabilized by the presence of various cations and anions which may not be significantly incorporated into the crystal lattices but may markedly influence the nucleation and subsequent growth processes.

Another complicating factor in the elucidation of the mechanism of calcium phosphate crystallization is that mixed solid phases may form by the growth of one phase upon another in the metastable supersaturated solutions. Although in most cases, it is not possible to unequivocally

confirm true epitaxial relationships in the growth of these mixed phases, the presence of the substrate allows nucleation and growth of the depositing phase at supersaturations considerably lower than those required for homogeneous precipitation. In the formation of these solid phases in vivo, the process must be mediated not only by biological restraint but also by factors, familiar to the crystal growth scientist, which control the formation of nuclei and their subsequent crystallization.

Usually, the rates of growth and dissolution, R_g and R_d, respectively are represented in terms of an empirical rate equation (1)

$$R_{g,d} = k_{g,d} \, (m/m_0)^{\pm p} \, \sigma_{g,d}^{\,n} \qquad (1)$$

in which k_g and k_d are the corresponding rate constants, σ_g and σ_d the relative super- and under-saturations, n the order of reaction and m_o and m are the masses of solid phase initially and at time t; positive and negative signs refer to growth and dissolution. The relative super and undersaturations may be expressed in terms of equation 2 in which IP is the ionic activity product of the phase undergoing reaction and ν the number of ions in the formula unit.

$$\sigma_{g,d} = \pm [(IP/K_{so})^{1/\nu} - 1] \qquad (2)$$

CONSTANT COMPOSITION METHODS

As discussed above, conventional free-drift crystal growth and dissolution kinetics methods suffer from two significant disadvantages: (i) a changing ion speciation and thermodynamic driving force during the reactions making it difficult to deduce rate data and (ii) the inability to prepare sufficiently large amounts of new material for physical chemical characterization. In the constant composition (CC) method, maintenance of solution composition is made possible by the use of suitable techniques such as conductimetry or potentiometry involving ion selective electrodes. For growth experiments, following the preparation of metastable supersaturated solutions, the reactions are initiated either by allowing spontaneous crystallization to set in or by introducing well characterized seed crystals. During the reaction, the changes in activity in crystal lattice ions under investigation are sensed by the electrode system which triggers the addition of titrants from multiple burets to restore the solution composition to its initial value. The rates of reaction may be calculated from the titrant addition data with a precision impossible to achieve in free-drift growth and dissolution studies. A significant advantage of the CC method is that relatively large extents of crystallization and dissolution can be achieved even at very low driving forces, enabling newly precipitated solid phases to be characterized by physical chemical methods (7).

Another advantage of the CC method is the ability to select particular points on the solubility isotherms in Fig. 1 in order to limit the number of precursor phases that can form following inoculation of the supersaturated solutions with seed crystals. Thus, in the region between the DCPD and OCP solubility curves, it has been possible to grow, exclusively, OCP in the pH range 6.0 to 7.0 upon adding OCP seed crystals (8). Representation of the experimental data by eqn. 1 yields a value $n \sim 2$ suggesting a spiral growth mechanism at low supersaturation and a polynucleation mechanism with $n \sim 4$ at higher driving forces. The formation of highly crystalline platelets during the reaction led to marked decreases in specific surface area consistent with the predicted morphology based on the Hartman-Perdock theory (9). The ability to obtain reliable growth rates at very low supersaturation enables studies of the growth of HAP in solutions supersaturated only with respect to this phase. Careful analysis of the kinetics data revealed the formation of defect apatites ranging in composition from OCP to HAP with values of y ranging $0 < y \leq 1.1$. HAP seeded growth experiments resulted in the formation of defect apatites with calcium/phosphate molar ratios ranging from 1.49 to 1.65, as the pH changed from 6.0 to 9.0 (10).

Simultaneous processes occurring in crystal suspensions are not rare. Although the CC method has found wide application in kinetic studies of the nucleation and growth of a number of sparingly soluble salts, it is limited to studies of single kinetics processes. When more than one reaction occurs simultaneously, the solution composition change reflects all the processes taking place. However if each of these reactions can be maintained at a constant driving force the composition of the solution as a whole can be kept constant. Solution mediated transformation such as that described above are usually regarded as proceeding through a combination of dissolution of the original phase and growth of the new phase. In principle therefore, two CC devices may be used to control both processes simultaneously. Such considerations, led to the development of the Dual Constant Composition (DCC) technique.

In the absence of a common ion, the growth of phases such as BA and DC can be investigated using two ion specific electrodes reversible with respect to B or A and D or C ions, respectively, since the two reactions do not influence each other in the constancy of solution composition. However, when crystals such as BA and BC having a common ion, B, are studied, interference between the two reactions might be important. The DCC method was developed for such processes (11). It has been used to study a number of systems including the simultaneous growth of DCPD and OCP in solutions supersaturated with respect to these phases (Fig. 1). In the dual titrant systems, the first titrant, controlled by means of a calcium electrode was designed for DCPD growth while the second set of titrants having OCP stoichiometry was controlled by means of a pH electrode. Fig. 2 shows typical plots of titrant volumes as a function of time following the addition of mixtures of OCP and DCPD seed crystals. The rates of each of the reactions could be calculated from the volumes of titrants added. Moreover, the rates of the individual growth reactions shown in Fig. 2 could be duplicated in CC experiments using single phase seed crystals.

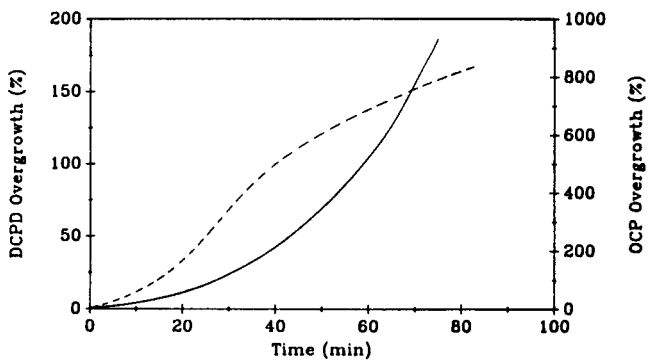

Fig. 2 Concurrent DCPD (- - - -) and OCP (———) overgrowth seeded with DCPD and OCP at pH = 6.00, TCa = TP = 5.00 mM and ionic strength = 0.150 M (NaCl).

DISSOLUTION MECHANISMS

It is now generally accepted that the biological precipitation of calcium phosphates may involve not only the deposition of lattice ions but also the concomitant dissolution of precursor phases. Such dissolution rates can be expressed in terms of empirical equations such as eqn. 1 and a CC study of the rate of HAP dissolution in the pH range 5-7.2 in 30-90% undersaturated solution showed that the rate was less than that calculated on the basis of pure mass transport by a factor of almost 10^4 (12) and followed a polynucleation mechanism. It was also shown that hydrogen ions played an important role in the dissolution process not only as a complexing agent for hydroxyl ions in solution but also as a catalyst for the exchange of phosphate ions between the crystal surface and the solution (13).

CC studies, in which the rates of dissolution can be measured even at very low driving forces, have also revealed changes in mechanism. For OCP dissolution, the effective order of reaction (n in eqn. 1) with respect to relative undersaturation increased as equilibrium was approached. Such changes in dissolution mechanism as the undersaturation is reduced are of considerable importance since the surface mediated reactions at lower driving forces are more sensitive to the influence of both inhibitors and promoters than are mass transport processes. In the presence of inhibitors, the dissolution of both DCPD and OCP also follow these trends (14). In CC experiments, only the overall dissolution rates of crystal suspension can be calculated directly from the recorded titrant consumption as a function of time. Despite the fact that the undersaturation remained constant during the experiments, the dissolution rates for DCPD, OCP and HAP crystals were invariably found to decrease with the extent of reaction. The observed retardation of dissolution suggests the participation of the second term on the right hand side of eqn. 1 with p reflecting the shape factors of the crystals being 0, 1/2 and 2/3 for one, two and three dimensional dissolution, respectively (15). Since the dissolution of DCPD and OCP follows spiral dislocation mechanisms, it is tempting to propose that the decrease in the number of dislocations emerging at a crystal surface might be responsible for the observed rate reductions (16). A similar mechanism was proposed to explain the cessation of whisker growth (17), with screw dislocations, especially those with large edge components, driven out of the whiskers by thermal energy. Some evidence to support this mechanism was the appearance of flat bottomed etch pits on the (010) faces of dissolving DCPD crystals as revealed by scanning electron microscopy, indicating that screw dislocations which had initiated the etch pits were removed during dissolution (18).

THE ROLE OF ADDITIVES

In addition to the alkali and alkaline earth cations, the calcium phosphates may take up many other metal ions and extensive studies have been made of the stoichiometry of substituted apatites. Of the anions, the substitution of carbonate is probably the most important. Carbonate is known to be present in biological apatites and a great deal of work has been done in an attempt to locate its position in the molecule (19). CC studies in carbon dioxide atmospheres showed that carbonate ion decreased the rate of growth of HAP crystals (20). Comparative dissolution kinetics CC studies of the dissolution of carbonated apatite, HAP and human tooth enamel, also showed that carbonated apatite may be a better model for enamel than near stoichiometric synthetic HAP (21).

Although simple metal ions may dramatically change the mineralization and demineralization reactions by being incorporated into the crystal lattices, different calcium phosphate phases may be stabilized or destabilized by the presence of other ions and molecules in solution which, though not significantly incorporated into the crystal lattices, markedly influence the rates of reaction. This is especially true in supersaturated biological fluids such as serum and saliva in which many macromolecules, while behaving as precipitation inhibitors when present in solution phases may actually nucleate calcium phosphate phases when immobilized on surfaces (22). In saliva, the molecules most important for maintaining a balance between re- and demineralization processes include two groups of negatively charged proteins, statherin and the proline-rich proteins. All these proteins have been shown to bind at HAP surfaces and are able to coordinate ions in solution that can participate in the formation of calcium phosphate nuclei. Proteins such as human serum albumin, HSA, and osteocalcin are also involved in bone formation and remodeling. HSA, when adsorbed at certain concentrations on HAP surfaces actually increases the growth rate of calcium oxalate monohydrate at the surfaces (23).

A recent study of the adsorption and growth inhibition activities of statherin and synthesized fragments of the molecule suggest that the N-terminal part of statherin is most responsible for binding at HAP

surfaces. Moreover the adsorption affinities could be expressed in terms of a simple Langmuir isotherm. The inhibiting influence on HAP crystal growth, however, was shown to be also due to the C-terminal fragment. The high affinity of the N-termini for HAP may anchor the statherin molecule at the surface allowing both ends of the molecule to cover growth sites thereby preventing incorporation of growth units. The relatively small influence of the middle section of the statherin molecule (having few acidic amino acid residues) suggests that it is of minor importance in inhibiting HAP crystallization (24). A recent CC investigation of mineralization at immobilized layers of statherin incorporated in a flow-through cell indicated that salivary proteins while inhibiting mineralization when present in solution, may induce calcium phosphate formation when adsorbed at surfaces. In CC seeded growth experiments where the protein was preadsorbed onto HAP crystals, there was an inhibition of growth as compared with that in the absence of protein. However at higher protein surface concentrations, this inhibition was reduced suggesting changes in conformation of protein at the surface depending upon the concentration (24). It is possible that the proteins, when adsorbed either at foreign substrate surfaces such as germanium or onto HAP seed crystals, may actually induce the formation of mineral. This may also occur at biological surfaces and this observed dual role of proteins and other macromolecules may explain the exquisite control of mineralization and demineralization observed *in vivo*.

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REFERENCES

1. J. Christoffersen, M.R. Christoffersen, W. Kiblczyc, F.A. Andersen, *J. Cryst. Growth* **94**, 767 (1989).
2. B.B. Tomazic, T.S. Tung, T.M. Gregory, W.E. Brown, *Scanning Mic.* **3**, 119 (1989).
3. W.E. Brown, *Nature* **196**, 1048-1050 (1962).
4. E.D. Eanes, A.S. Posner, *Calcif. Tiss. Res.*, **2**, 38 (1968).
5. H. Newesley, *Arch. Oral Biol.* Spec. Suppl.. **6**, 174 (1961).
6. M.D. Francis, N.C. Webb, *Calcif. Tiss. Res.*, **6**, 335 (1971).
7. P.G. Koutsoukos, Z. Amjad, M.B. Tomson, G.H. Nancollas, *J. Amer. Chem. Soc.* **102** 1553 (1980).
8. J.C. Heughebaert, G.H. Nancollas, *J. Phys. Chem.* **88** 2478 (1984).
9. R.A. Terpstra, P. Bennema *J. Crystal Growth* **82** 416 (1987).
10. S.J. Zawacki, J.C. Heughebaert, G.H. Nancollas, to be published.
11. A. Ebrahimpour, J. Zhang, G.H. Nancollas, *J. Cryst. Growth* in press.
12. J. Christoffersen, M.R. Christoffersen, *J. Cryst. Growth* **43** 501 (1978).
13. J. Christoffersen, M.R. Christoffersen, *J. Cryst. Growth* **87** 51 (1988).
14. J. Zhang, G.H. Nancollas, to be published.
15. J.P. Barone, G.H. Nancollas, Y. Yoshikawa, *J. Cryst. Growth* **63** 91 (1983).
16. J. Zhang, Ph.D. thesis, State University of New York at Buffalo (1990).
17. J.P. Hirth, F.C. Frank, *Phil. Mag.* **3** 1110 (1958).
18. J. Zhang, G.H. Nancollas, Mullin Symposium, Butterworth, in press (1991).
19. R.Z. LeGeros, J.P. LeGeros, O.R. Trautz, W.P. Shirra, *Adv. X-ray Anal.* **14** 57 (1971).
20. A.A. Campbell, M.LoRe, G.H. Nancollas, *Colloids & Surfaces,* **54**, 25 (1991).
21. J.A. Budz, M. LoRe, G.H. Nancollas, *Adv. Dent. Res.* **1(2)** 314-321 (1987).
22. A.A. Campbell, G.H. Nancollas, *Colloids & Surfaces* **54** 33 (1991).
23. A. Ebrahimpour, L. Perez, G.H. Nancollas, *Langmuir* **7**,577 (1991).
24. M. Johnsson, G.H. Nancollas, unpublished results.