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Formation of hydroxyapatite in serum

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The kinetics of the formation of calcium-deficient and carbonated hydroxyapatite at 38 °C were investigated by isothermal calorimetry. Hydroxyapatite (HAp) was formed by reaction of the particulate calcium phosphates $CaHPO_4$ and $Ca_4(PO_4)_2O$. Compared with its rate of formation in DI water, the formation of calcium-deficient HAp is significantly inhibited in serum. When serum is diluted with DI water, the extent of inhibition varies with the extent of dilution. When collagen or HAp seeds are present the extent of inhibition in serum is reduced. The kinetics of HAp formation were also examined in various concentrations of albumin to establish the extent to which inhibition is associated with the presence of this plasma protein. While HAp formation is inhibited in albumin, the extent of inhibition is not as great as in serum. The formation of carbonated HAp is also inhibited in serum and albumin. However, the extent of inhibition is significantly reduced. The variations in sodium and carbonate in solution during HAp formation indicate that these species are incorporated at different rates, with carbonate incorporation being more rapid. Elevated sodium concentrations in solution result in solution pH values near 12. The reduction in the inhibition of HAp formation may be associated with the reaction to carbonated HAp occurring at elevated pH or with the influence of pH on protein adsorption.

1. Introduction

Bone mineral is sometimes characterized by the formula $Ca_{10}(PO_4)_6(OH)_2$. Because of the fluid environment in which bone mineral forms [1], a variety of substitutions occur. By simulating this fluid environment, comparable substitutions can occur in the poorly crystalline apatite formed in aqueous solutions *in vitro*. In particular, reactions of the following type can be used to form such substituted apatites:

$$2CaHPO_4 + 2Ca_4(PO_4)_2O \Rightarrow Ca_{10}(PO_4)_6(OH)_2 \quad (1)$$

The calcium-to-phosphate ratio in hard tissue is near 1.67 but the average carbonate content is about 3.5% in enamel, 5.6% in dentin and 7.4% in bone [2]. The role of carbonates in determining the structures of tooth apatite, in particular, have been analysed by transmission electron microscopy TEM [3]. The densities of atomic defects in carbonated apatites is much greater than in stoichiometric hydroxyapatite. Nelson directly observed deformation by twinning in stoichiometric hydroxyapatite and domain structures approximately 3000 nm in diameter in 4.1 wt % carbonated hydroxyapatite. Hydroxyapatite with 9.6 wt % carbonate had smaller domain structures, approximately 800 nm in diameter. Nelson also observed single dislocations in domain structures and strained and disordered regions between domain structures. The rate of dissolution of 6.5 wt % carbonated hydroxyapatite according to [4] is approximately twice that of pure stoichiometric hydroxyapatite with apparent activation energies of 11.5 kJ mol^{-1} and 18 kJ mol^{-1}, respectively. Carbonate principally replaces phosphate in apatite formed at low temperature. Charge compensation requires more than a simple 1-to-1 substitution [5, 6]. This may include the protonation of phosphate, paired substitution of sodium for calcium, and formation of vacancies on a calcium site and on an hydroxyl site. However, structural differences between pure and substituted hydroxyapatite formed at low temperature are difficult to distinguish by X-ray diffraction analysis when the extent of substitution is less than about 6 wt % [2].

Biological fluids have significant concentrations of low and high molecular weight substances that inhibit the formation of hydroxyapatite. The major constituents of serum are listed in Table I [7, 8].

Investigations of the roles of these substances during formation of HAp may explain the inhibition of natural biomineralization of skeletal tissue or elucidate their effects on the *in vivo* formation of HAp or HAp-based composites. Meyer and Fleisch [9] report the transformation of ACP to HAp at normal physiological concentrations is most strongly inhibited by albumin followed by magnesium, pyrophosphate and citrate. They used the Langmuir adsorption model to describe the inhibition of hydroxyapatite formation. They also noted citrate, magnesium, and albumin increases the apparent solubility product of brushite while pyrophosphate causes it to decrease. However, when corrections are made for complexation of calcium in the case of citrate and phosphate in the case of magnesium, the solubility product of brushite, $pK = 6.59$, is constant [9]. Apparently the brushite crystal structure does not allow the incorporation of

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TABLE I The principal inorganic species and selected organic components of serum

Component	MW	$g\,l^{-1}$	$\mu mol\,l^{-1}$
Ca^{2+}	40.08	0.1	2500
Na^+	22.99	3.31	144000
K^+	39.1	0.196	5000
Mg^{2+}	24.31	0.036	1500
Cl^-	35.45	3.79	107000
HCO_3^-	60.0	1.62	27000
HPO_4^{2-}, $H_2PO_4^-$	94.97 (PO_4)	0.190	2000
SO_2^{2-}	64.06	0.032	500
Urea	60.04	0.24	4000
Albumin	66000	40	600
IgG	150000	8–17	53–113
Glucose	180	1	5600
Lactate	90	0.11	1200
Other proteins		10–20	600

these species to an appreciable extent. Each of these species acts differently. Magnesium interferes with hydroxyapatite formation by poisoning nucleation and causing $(Ca, Mg)_3(PO_4)_2$ to form [10, 11]. Magnesium concentration increases substantially in the pre-stages of biomineralization of turkey tibia tendon [12]. However, alkaline phosphatase activity also increases during biomineralization and magnesium is an important cofactor and a strong activator of alkaline phosphatase. Therefore, the inhibitory activity of inorganic magnesium may be reduced by its role as a cofactor. Pyrophosphate is also a strong HAp nucleating poison which causes nuclei to be of subcritical size for growth [13]. Pyrophosphoric acid has also been observed to bè a strong inhibitor of hydroxyapatite dissolution [14]. Citrate chelates calcium ions and rapidly imbalances the calcium-to-phosphate ratio [15]. Albumin rapidly coats HAp surfaces forming a proteineous layer. Muhr, *et al.* [16] found that long-term interfacial residence time did not influence desorption characteristics. However, the degree of surface coverage slows the kinetics of desorption in that interfacial molecular "cross-linking" increases with increased surface coverage.

A reaction of the type summarized in Equation 1 is of interest in that it may be used to form hydroxyapatite *in vivo*. Under such conditions contact with biological fluids during the period of reaction is inevitable. As a consequence inhibitory substances in serum, which may interact with an implant comprised of reacting HAp precursors, may preclude HAp formation in clinically relevant periods of time. The objective of the present study was to investigate the effects of serum and its constituents on the formation of hydroxyapatite by an acid–base reaction of calcium phosphate precursors.

2. Materials and methods

$Ca_4(PO_4)_2O$ (TetCP) prepared in our laboratory from reagent grade $CaCO_3$ (Fisher) and $CaHPO_4$ (DCP) (Fisher) were used in all experiments in this investigation. Bovine calf serum (Sigma) and collagen (Sigma) were used in all experiments requiring serum or collagen. Calcium-deficient hydroxyapatite can be made by complete reaction of $CaHPO_4$ and $Ca_4(PO_4)_2O$ in a molar ratio of 2:1 according to:

$$6CaHPO_4 + 3Ca_4(PO_4)_2O \Rightarrow 3Ca_9(HPO_4)(PO_4)_5(OH) \quad (2)$$

TetCP and DCP were reacted in an isothermal calorimeter at 38 °C using solutions having various serum-to-water ratios as the reaction media. All calorimetric experiments were conducted at a liquid-to-solids ratio of 1. Effects of the presence of collagen and of 5 wt % of HAp seeds on the kinetics of reactions in undiluted serum were determined. The kinetics of the reaction were also investigated in solutions whose albumin concentrations ranged from 0 to 600 μM.

The effects of paired substitutions on the kinetics of HAp formation and on the solution chemistry were determined. The substitutions investigated were Na for Ca and CO_3 for PO_4 according to the following reaction:

$$5/8NaHCO_3 + 2CaHPO_4 + 2Ca_4(PO_4)_2O \Rightarrow HAp \quad (3)$$

These proportions were selected to achieve a CO_3/PO_4 molar ratio of about 0.10. This assemblage was reacted in DI water and in serum at 38 °C, also at a liquid-to-solids ratio of 1, and the heat evolution measured by isothermal calorimetry. The experiments involving $NaHCO_3$ were carried out at a liquid-to-solids ratio of 5 for solution chemistry determinations. An Orion 920 pH meter was connected to a computer for monitoring pH. The concentrations of calcium, sodium, phosphate and carbonate in the supernatant solutions were determined as a function of time using DC plasma atomic emission spectroscopy. Solutions were separated from the solids by filtration through 0.22 μm disposable filters. The compositions of the solids in contact with these liquids were determined by X-ray diffraction analysis.

3. Results and discussion

3.1. Kinetics

3.1.1. Calcium-deficient HAp formation in serum

Compared with its formation in DI water, formation of calcium deficient HAp is considerably inhibited when $CaHPO_4$ and $Ca_4(PO_4)_2O$ react in bovine serum at 38 °C. Fig. 1a shows the heat evolution over 24 h in media composed of DI water, serum, and DI water and serum mixed in volume ratios of 0.5, 0.25, and 0.125. Reactions in undiluted serum and in 50 vol % serum are incomplete after 24 h. However, X-ray diffraction analyses show these reactions reach completion in about three days. When formation of HAp occurs in the more dilute serum solutions (0.25 and 0.125 volume fraction), it progresses more rapidly and the kinetics observed in DI water are approached.

Two heat evolution peaks occur in the formation of HAp. The first of these is typically referred to as the

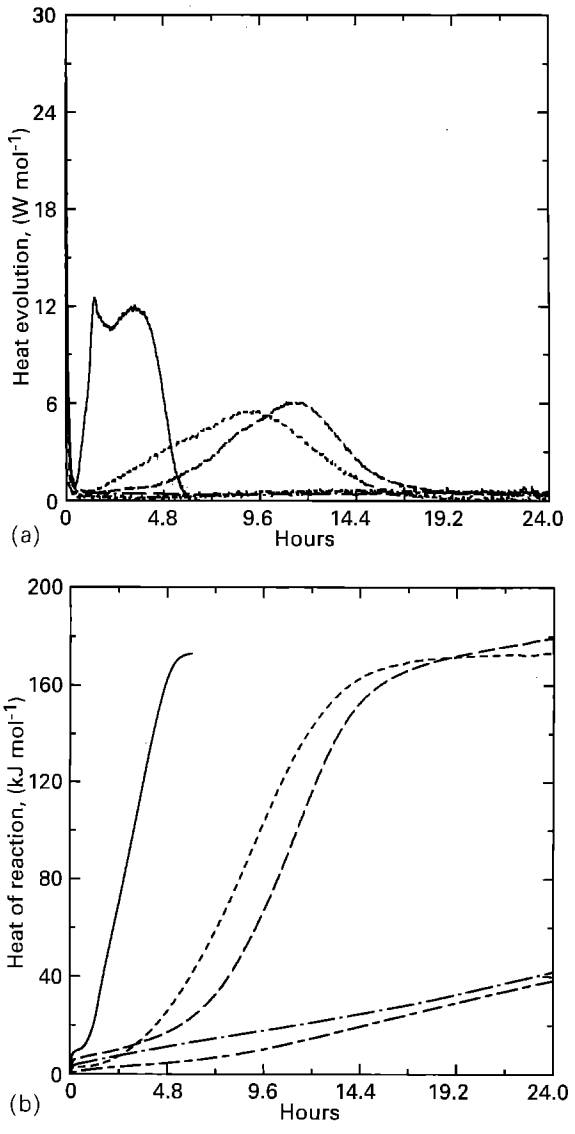

Figure 1 (a) The rates of heat evolution (dQ/dt) during the formation of $Ca_9HPO_4(PO_4)_5OH$ in serum and DI water at 38 °C; –·– DI water; – – – 12 vol % serum; - - - - 25 vol % serum; ····· 50 vol % serum; —— serum. (b) The heat evolution curves (ΔH_r) for $Ca_9HPO_4(PO_4)_5OH$ formation in serum and DI water at 38 °C; - - - serum; –·– 50 vol %; – – 25 vol %; - - - 12 vol %; —— DI water.

mixing peak. Mixing peaks are the result of the heat of wetting and of the heat evolved during initial dissolution of reactants. The latter is a function of reactant surface area and temperature; both are constant in this study. The mixing peaks in Fig. 1a overlay each other. Heat evolution during mixing increases with the increasing volume fraction of serum. This is consistent with the adsorption of serum proteins onto the HAp; precursor phases may be the cause of this trend in the heats of wetting.

The heats of reaction, ΔH_r, for the different volume ratios are shown in Fig. 1b. The reactions that are complete in 24 h show the total heat of reaction ΔH_r to be approximately 175 kJ per mole of TetCP consumed. The heat evolution curves share features in common in that there is an initial period of slow reaction, an induction period, which is followed by a period of rapid reaction. Although there is a kinetic difference between the reactions in water and in 0.125 and 0.25 volume fraction serum, this difference is principally related to the lengths of the induction periods. The rates of heat evolution, as determined by the slopes of the heat evolution curves, are nominally the same in water and in 0.125 and 0.25 volume fraction of serum. When the volume fraction of serum is 0.5 or when reaction occurs in undiluted serum, the period of slow reaction extends for more than 24 h.

There are two major factors that may cause the inhibition of HAp formation in serum. These are the presence of albumin and those of the other major species found in serum. Albumin is a negatively charged protein and has been reported to be the dominant inhibitor of hydroxyapatite formation *in vivo* [9]. The kinetics of albumin desorption from HAp surfaces has been characterized [16]. The extent of surface coverage increases with increasing albumin concentration. Increased coverage facilitates intermolecular "cross-linking" on the surfaces of the crystallites and this slows desorption. Thus, two mechanisms may contribute to the retardation of HAp formation in serum: the absorption of albumin on the surfaces of the reactants as well as on the growth surfaces of the HAp.

To further establish the mechanism of inhibition of $Ca_9HPO_4(PO_4)_5OH$ formation in serum, the effect of seeding the reaction with 5 wt % $Ca_9HPO_4(PO_4)_5OH$ and the effect of the presence of collagen were evaluated. In one experiment, 5 wt % of HAp seeds were mixed with the particulate precursors. In a second, the calcium phosphate precursors were embedded in a collagen matrix prior to reaction. The volume ratio of collagen-to-calcium phosphate precursors used was 1:8. If blood contacts the assemblage, it is crucial that HAp formation not be excessively inhibited. Fig. 2a compares the effects of the presence of collagen and HAp seeds on heat evolution in serum to that which occurs in their absence. Inhibition is overcome by seeding and by collagen addition; however, it is likely that the mechanisms are different. Fig. 2b shows the total heat evolution curves. Although inhibition is reduced in both circumstances, it is not eliminated. The presence of HAp seeds increases the heat evolved at 24 h from about 38 kJ mol^{-1} of TetCP to about 170 kJ mol^{-1} while collagen increases it to nearly 242 kJ mol^{-1} of TetCP. Compared to the heat evolution data shown in Fig. 1b, the presence of the HAp seeds allows complete reaction in about 24 h. The evolution of approximately 240 kJ mol^{-1} of TetCP when collagen is present remains an unexplained phenomenon. However, a similar circumstance was observed when calcium sulphate hemihydrate formed the dihydrate in the presence of gelatin. The total amounts of heat evolved for complete reaction to gypsum varied significantly depending on the solution pH [17]. Whether there is a conformational aspect to these hydration reactions involving polypeptides and the inorganic constituents, which has a significant influence on heat evolution, remains speculative.

3.1.2. Carbonated HAp formation in serum

To introduce carbonate into hydroxyapatite, solid $NaHCO_3$ was blended with the calcium phosphate precursors which had been mixed at a calcium-to-

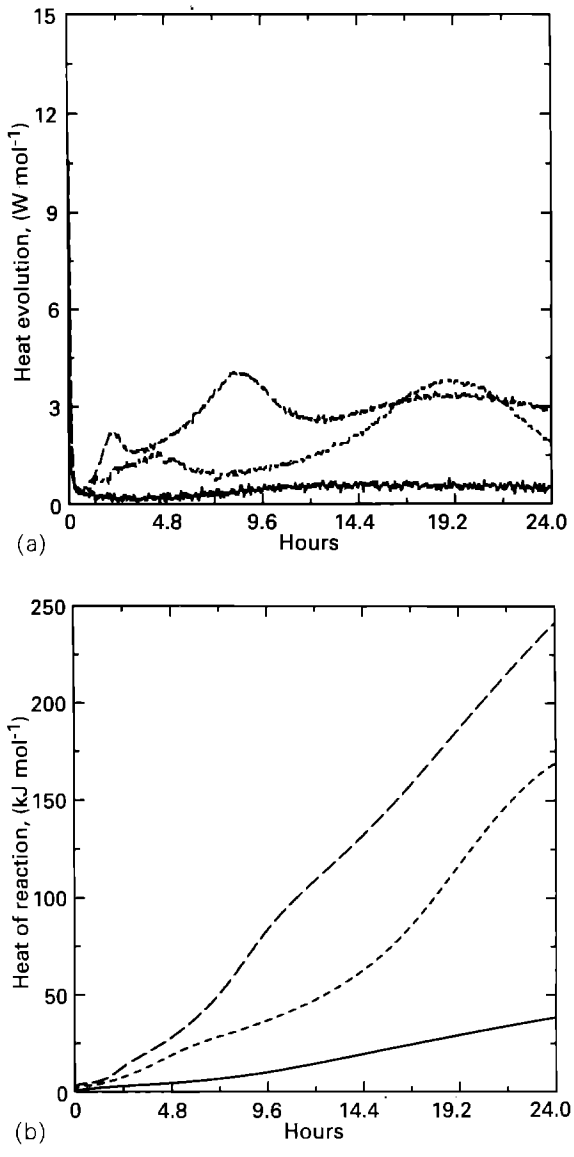

Figure 2 (a) A comparison of the rates of heat evolution during $Ca_9HPO_4(PO_4)_5OH$ formation in serum when reaction is non-seeded, is seeded with 5 wt % $Ca_9HPO_4(PO_4)_5OH$, or occurs in the presence of collagen; --- 8 vol % collagen; –·– 5 wt % HAp; —— unseeded. (b) The total heat evolution curves; –– 8 vol % collagen; --- 5 wt % HAp; —— unseeded.

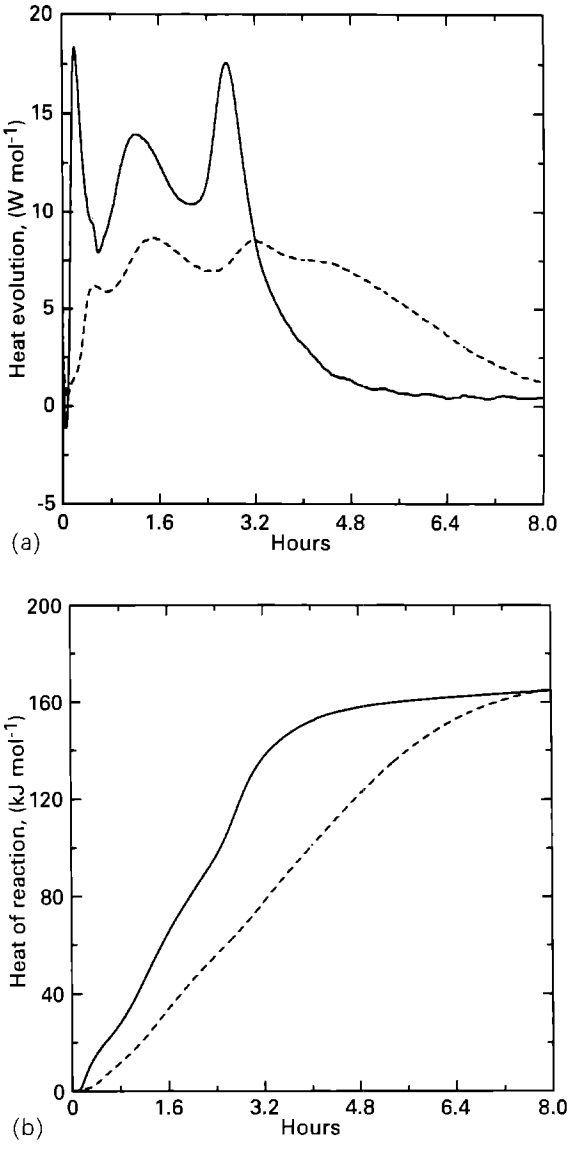

Figure 3 (a) The rates of heat evolution during carbonated HAp formation in DI water and in serum at 38 °C; --- serum; —— DI water. (b) The total heat evolution curves; --- serum; —— DI water.

phosphate ratio of 1.67. As will be discussed, this reaction involves the paired substitutions of Na for Ca and carbonate for phosphate. DI water or serum were the reaction media and reactions were carried out at 38 °C at a liquid-to-solids ratio of 1. Fig. 3a compares the rates of heat evolution from reactions involving $NaHCO_3$ in DI water and serum. Inhibition is evident for the reaction in serum. Reactions involving $NaHCO_3$ result in three major heat evolution peaks. In serum, the peaks are more spread over time and more convoluted. No induction period occurs in either medium. A slight endotherm occurs at the onset of the reactions when $NaHCO_3$ is present. Hydroxyapatite is not detectable by X-ray diffraction until the appearance of third peak at 2.4 h in DI water and 2.7 h in serum. Fig. 3b compares the total heats of reaction ΔH_r, in serum and in DI water. Reaction in DI water reaches completion approximately 3 h before that in serum. Compared with the formation of $Ca_9HPO_4(PO_4)_5OH$ in serum, which requires 3 days, carbonated hydroxyapatite formation in serum is significantly more rapid reaching completion in about 7 h. When serum is not present, reaction is nominally complete in about 4 h. As shown in Fig. 1b, a similar length of time is required to form calcium deficient HAp. However, comparison of the rate data suggests the presence of $NaHCO_3$ may alter the mechanistic path taken. The total heat evolved after 8 h is about 165 kJ mol^{-1} of TetCP regardless of the reaction medium. This value is close to those observed for the other reactions.

3.1.3. HAp formation in albumin

The effects of albumin concentration on the formation of $Ca_9HPO_4(PO_4)_5OH$ and on carbonated hydroxyapatite were also studied by isothermal calorimetry at 38 °C. The heat evolution curves for reactions in DI water and in albumin concentrations of 30.3, 121, and 600 μM are shown in Fig. 4. Fig. 4a shows the heat

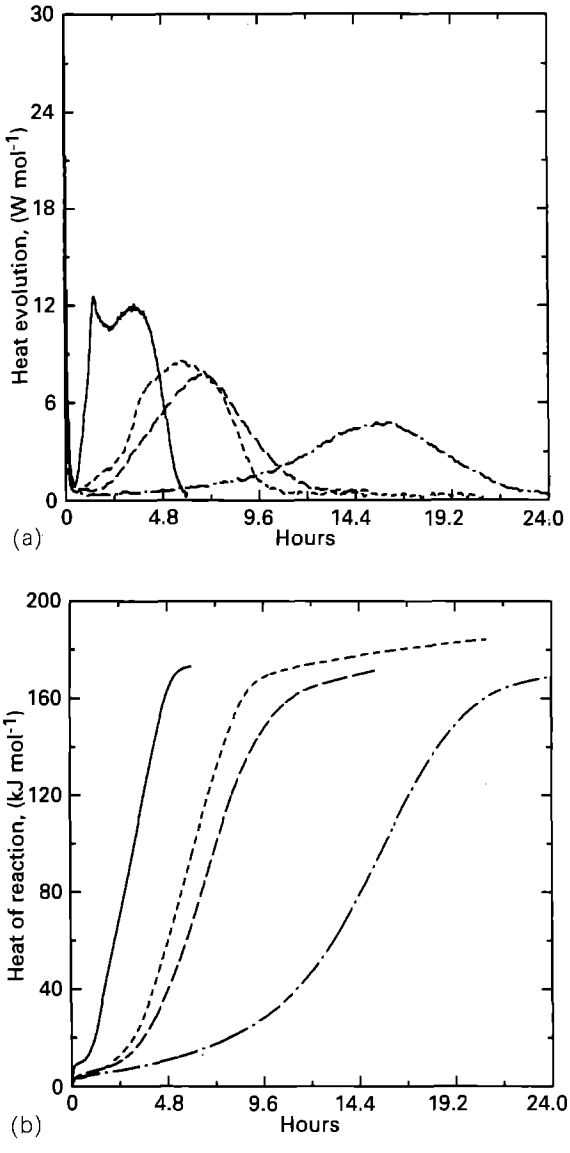

Figure 4 (a) The rates of heat evolution during the formation of $Ca_9HPO_4(PO_4)_5OH$ in various concentrations of albumin at 38 °C; –·–· 600 μM; – – – 121 μM; - - - - 30.3 μM; —— DI water. (b) The total heat evolution curves; –·–· 600 μM; – – – 121 μM; - - - - 30.3 μM; —— DI water.

evolution during $Ca_9HPO_4(PO_4)_5OH$ formation. The two heat evolution peaks, which occur when $Ca_9HPO_4(PO_4)_5OH$ forms in DI water, become a single peak when albumin is present. Increasing albumin concentrations increasingly inhibit the progress of the reaction. When the albumin concentration is 600 μmol l^{-1}, the human physiological concentration, complete reaction requires approximately 22 h. However, the inhibition is not as great as when serum is used as the reaction medium, Fig. 1a. Therefore, albumin is not solely responsible for the inhibition of hydroxyapatite formation in serum. Albumin constitutes 50–60% of the plasma proteins so the cumulative effects of the remaining proteins and the electrolytes present may cause the greater inhibition in serum. The ability of albumin to inhibit HAp formation may be a function of the extent of saturation with proteins and fatty acids [18]. Substances in plasma, such as fatty acids and bilirubin, bind to albumin which may effect the absorption of albumin on HAp crystals. Although the rate reaction is slowed, the total heats evolved are approximately 175 kJ mol^{-1} of TetCP, Fig. 4b; again, this is consistent with the other values obtained for complete reaction.

The manner in which heat is evolved during the formation of carbonated hydroxyapatite in various albumin concentrations is complex as is shown in Fig. 5a. These curves also show initial endotherms followed by a complex series of heat evolution peaks. Both the magnitudes of these peaks and the times at which they occur vary with albumin concentration. As the albumin concentration increases the magnitudes of the heat evolution peaks decrease and they tend to occur at longer reaction times. When reaction occurs in the highest albumin concentration, a third peak cannot be discerned. The total heat evolution curves are shown in Fig. 5b and the extent of heat evolution at any given time decreases with increasing albumin concentration.

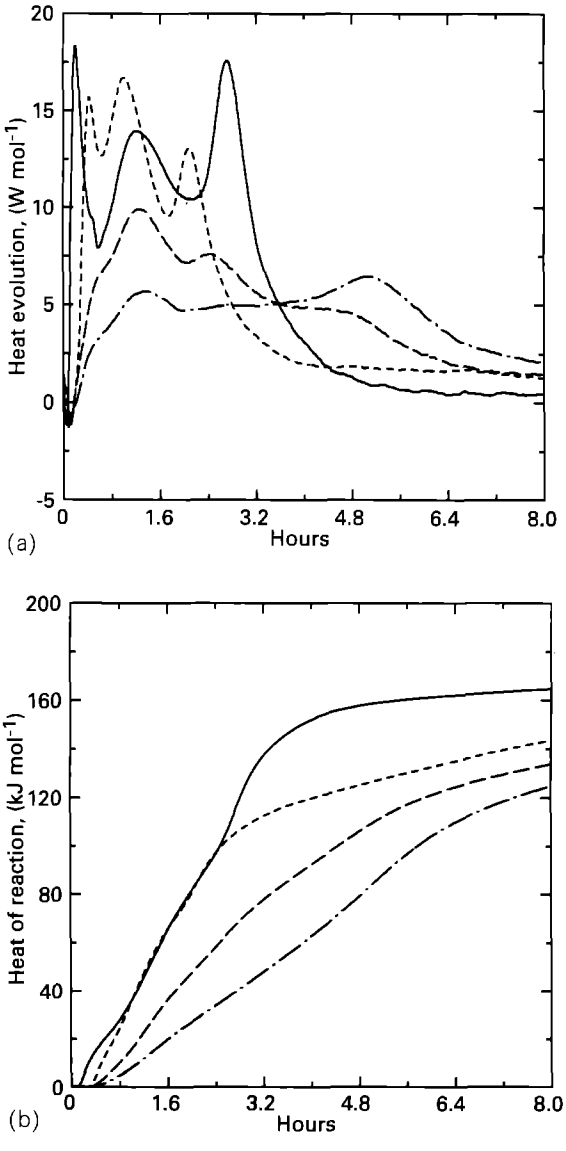

Figure 5 (a) The rates of heat evolution during the formation of carbonated HAp in albumin and DI water at 38 °C; –·–· 600 μM; – – – 121 μM; - - - - 30.3 μM; —— DI water. (b) The total heat evolution curves; –·–· 600 μM; – – – 121 μM; - - - - 30.3 μM; —— DI water.

3.2. Solution chemistry

The variations in the concentrations of Ca, Na, PO_4, and CO_3 in solution during the reactions involving $NaHCO_3$ in DI water at 38 °C were determined and are plotted in Fig. 6. Approximately one minute after mixing, which is the time of the first measurement, the calcium concentration is 0.25 mM. This concentration drops continuously throughout the course of reaction, reaching about 0.025 mM after 6 h. These values are substantially below those observed when HAp formation occurs in water [19]. The variation in calcium does not indicate the formation of intermediates as might be indicated by the peaks in the calorimetric rate curves. Two peaks are observed in the variations in the phosphate concentration. These occur at about 0.5 and 4 h. Phosphate rises from 6.5 mM to 12.75 mM in the first half-hour. Its concentration decreases to 8 mM at 1.8 h and then at approximately 2 h begins to rise in correspondence to the third hydration peak in Fig. 3a. The highest concentration of phosphate is 21 mM and occurs after about 4 h of reaction. This maxima occurs near the time when heat evolution is nearly complete. Subsequent to this the phosphate concentration continuously decreases.

The variations in the sodium and carbonate concentrations are of particular interest because their variations indicate the extent of their incorporation into hydroxyapatite. Dissolution of the available $NaHCO_3$ in the absence of sodium or carbonate incorporation into HAp would result in Na and HCO_3 concentrations of 122 mM for the liquid-to-solids ratio used in these experiments. The concentrations observed did not approach 122 mM; the maximum concentration of sodium observed was 67 mM, while that from carbonate was 42 mM. After reaching maxima after a few minutes, both Na and HCO_3 declined continuously reaching values of a few millimoles after several hours. However, the Na and carbonate concentrations did not decrease at the same rate. Rather, the carbonate concentration was always smaller than that of Na suggesting the HAp formed during this period may not be of uniform composition. No other phases other than HAp could be identified by X-ray diffraction analysis. While the average Na and CO_3 contents are 1.37 and 3.66 wt %, respectively, it is likely that the first-formed material contains higher concentrations than these. The actual average composition of the HAp formed is unknown. However, based on the molar ratios of the reactants used the following idealized, limiting reaction can be represented:

$$\tfrac{5}{8}NaHCO_3 + 2CaHPO_4 + 2Ca_4(PO_4)_2O \Rightarrow 1.067Ca_{9.375}Na_{0.586}(PO_4)_{5.375}(CO_3)_{0.586}(OH)_{1.961} + \sim \tfrac{5}{16}H_2O \quad (4)$$

for the case where a "stoichiometric" substituted apatite forms. Alternatively, if it is assumed that one of the phosphates is protonated, charge compensation could occur by the formation of an hydroxyl vacancy.

The changes in pH during the formation of carbonated hydroxyapatite in DI water, in serum, and in 600 μM albumin solution and those during $Ca_9HPO_4(PO_4)_5OH$ formation in DI water and serum are plotted in Fig. 7. The variations in pH during calcium deficient HAp in DI water are typical of those previously observed [20]. Formation of calcium deficient HAp in serum shows similar behaviour to that in DI water with the exception that the reduction in the rate of reaction results in steady state pH near 8.6 persisting for more than 12 h.

The effects of serum and albumin on the variations in pH during carbonated HAp formation are complex and correlate with the peaks observed when the rate of heat evolution is plotted against time, Figs 3a and 5a. These data suggest that albumin in serum plays a major role in affecting hydroxyapatite formation and this effect influences both heat evolution and solution chemistry. Carbonated HAp formation in DI water reaches a steady state at a pH near 12. This would be

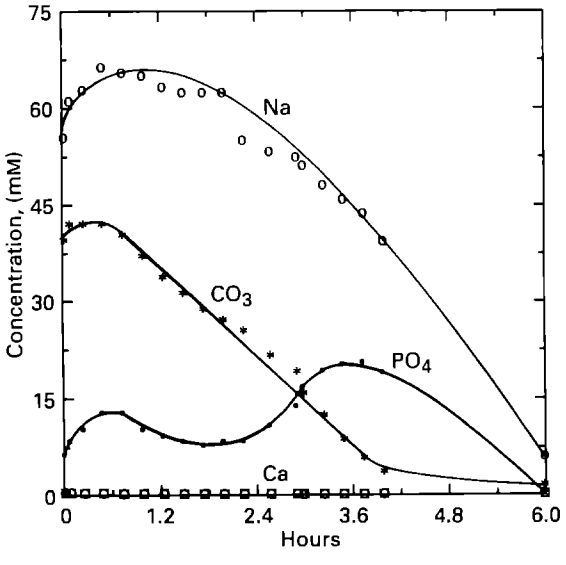

Figure 6 The calcium, sodium, carbonate and phosphate concentrations during carbonated HAp formation in DI water at 38 °C

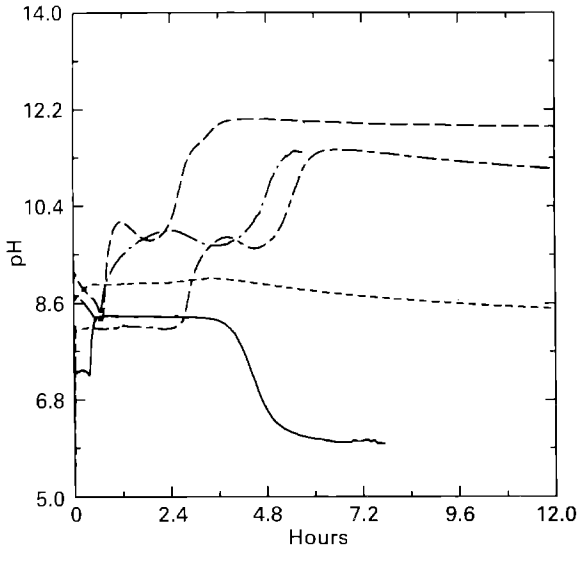

Figure 7 The variations in pH during the formation of calcium deficient and carbonated HAp in DI water, serum, and albumin at 38 °C; —— carbonated HAp in DI water; —·— carbonated HAp in 600 μM albumin; —- - — carbonated HAp in serum; - - - Cal. Def. HAp in serum; —— Cal. Def. HAp in DI water.

consistent with a Na concentration in solution of about 8 mM (Fig. 6), and suggests that the incorporation of Na is incomplete in 12 h. The initial steady state pH observed in serum is close to that observed in the formation of calcium deficient HAp. However after about 3 h the pH rises to a value near 11.3 and remains high thereafter. An initial steady state pH is not observed for reaction in albumin; with this exception the variation in pH mimics that in serum and it seems likely that the elevation in pH is the result of sodium in all three instances.

HAp formation in the presence of sodium bicarbonate reduces the inhibitory effects of serum and albumin. According to Andersson [18] a conformational change in the albumin structure initiates at about pH 10.3. Apparently, this change reduces the inhibitory effect of albumin on hydroxyapatite formation. This reduction in inhibition correlates to the end of the third heat evolution peak in the curves in Fig. 5a and with the rise of the fourth peak with increasing albumin concentration. Hydroxyapatite is detectable by X-ray diffraction analysis starting at this time.

4. Summary

Formation of calcium deficient HAp is strongly inhibited by serum. Serum influences both the initial heat evolution peaks associated with reactant dissolution and the major heat evolution peak associated with HAp formation. This suggests that constituents of serum are adsorbing onto the available surfaces regardless of whether they are reactants or products. While inhibition can be partially overcome by reaction in the presence of HAp seeds or collagen, the mechanisms causing this remain to be established. Regardless of the presence of serum and its concentration, the amount of heat evolved at complete reaction is constant at approximately 175 $kJ\,mol^{-1}$ of $Ca_4(PO_4)_2O$ undergoing reaction. Albumin has a retarding effect similar to that of serum, but when reaction is carried out at the albumin concentration present in serum, inhibition is not as great. This indicated that other serum constituents also exhibit an inhibitory effect on HAp formation. The rate of formation of carbonated HAp is less inhibited than that of calcium-deficient HAp. This appears to be related to the formation of carbonated HAp occurring at a pH above which albumin undergoes a conformational change rendering it less effective as an inhibitor.

Acknowledgements

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