A Chemical Study of Apatites Prepared by Hydrolysis of Amorphous Calcium Phosphates in Carbonate-containing Aqueous Solutions

D. J. Greenfield, J. D. Termine, and E. D. Eanes

Molecular Structure Section, Laboratory of Biological Structure,
National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland

The Ca/P molar ratio, % CO_3^{2-}, and % HPO_4^{2-} was determined for a number of apatites prepared by the hydrolysis of amorphous calcium phosphate in the presence of solution carbonate. The Ca/P molar ratio for each apatite was significantly higher than for the corresponding amorphous precursor. The change in carbonate content was slight but the HPO_4^{2-} level in these apatites, as determined by the standard pyrolytic technique, was greatly reduced, sometimes to a near zero value. Infrared analysis, on the other hand, clearly showed an appreciable level of HPO_4^{2-}. Evidence obtained indicated that the carbonate interferes with the pyrolytic assay for HPO_4^{2-} by either blocking condensation to $P_2O_7^{4-}$ or by breaking down already formed $P_2O_7^{4-}$. It is suggested that the pyrolytic method be used with great caution in assaying for HPO_4^{2-} in calcified tissue.

Key words: Acid phosphate — Apatite — Carbonate — Pyrophosphate

Le rapport molaire Ca/P, % CO_3^{2-} et % HPO_4^{2-} sont déterminés pour un certain nombre d'apatites préparés par hydrolyse de phosphate de calcium amorphe en présence de solution de carbonate. Le rapport molaire Ca/P pour chaque apatite est significativement plus élevé que celui du précurseur amorphe correspondant. Le changement en contenu de carbonate est faible, mais la concentration en HPO_4^{2-} de ces apatites, déterminée selon la technique pyrolytique habituelle, est nettement réduite, et atteint souvent une valeur nulle. L'analyse infra-rouge montre, d'autre part, une concentration appréciable de HPO_4^{2-}. Il semble que le carbonate réagit au cours de la pyrolyse avec HPO_4^{2-} soit en bloquant une condensation de $P_2O_7^{4-}$ ou en dégradant du $P_2O_7^{4-}$ déjà formé. Il apparait qu'il faut utiliser la méthode de pyrolyse avec prudence pour déterminer la présence de HPO_4^{2-} dans les tissus calcifiés.

Das molare Ca/P-Verhältnis sowie die prozentualen Anteile von CO_3^{2-} und HPO_4^{2-} wurden bei einer Anzahl von Apatiten bestimmt, welche durch Hydrolyse von amorphem Calcium-phosphat, bei Anwesenheit von Karbonat in der Lösung, erhalten wurden. Das molare Ca/P-Verhältnis war bei jedem Apatit bedeutend höher als bei der entsprechenden amorphen Vor-stufe. Das Karbonat veränderte sich wenig, jedoch war der HPO_4^{2-}-Gehalt dieser Apatite, mittels der pyrolytischen Standard-Technik bestimmt, stark reduziert und lag manchmal nahe beim Nullwert. Andererseits zeigte die Infrarot-Analyse eindeutig einen beträchtlichen HPO_4^{2-}-Gehalt. Somit wird bewiesen, daß Karbonat die pyrolytische Bestimmung von HPO_4^{2-} stört, entweder indem es die Kondensation zu $P_2O_7^{4-}$ blockiert oder schon gebildetes $P_2O_7^{4-}$ abbaut. Es wird vorgeschlagen, daß die pyrolytische Methode zur Bestimmung von HPO_4^{2-} in verkalktem Gewebe nur mit großer Vorsicht anzuwenden ist.

Introduction

A recent chemical study (Greenfield and Eanes, 1972) demonstrated that amorphous calcium phosphates (ACP) prepared from carbonate-containing

solutions at physiological pH values are complex inorganic salts containing appreciable levels of both acid phosphate (HPO_4^{2-}) and carbonate (CO_3^{2-}). The exact extent to which these anions were incorporated into the ACP depended upon the conditions existing at the time of preparation, with low solution pH favoring HPO_4^{2-} incorporation and high pH together with elevated solution carbonate facilitating the inclusion of CO_3^{2-}. The HPO_4^{2-} level in ACP, however, appeared to be a function of pH alone since it was not altered appreciably by the presence of carbonate in the precipitating medium.

It has also been found that in carbonate-free preparations the HPO_4^{2-} level in the initially precipitated ACP phase was not significantly altered by hydrolysis to crystalline apatite (CA) (Termine and Eanes, 1972). The present paper describes the changes occurring in the constituents, HPO_4^{2-} and CO_3^{2-}, when ACP hydrolyses into CA in the presence of solution carbonate under conditions of constant pH.

Methods

The amorphous calcium phosphates used in this study were synthesized by spontaneous precipitation from freshly mixed aqueous solutions of $Ca(NO_3)_2$ and Na_2HPO_4. All preparations were carried out in solutions buffered with 0.1 M tris (hydroxymethyl) aminomethane and adjusted to the desired pH by titration with HCl. In the pH range 7.4–7.8, 10 mM calcium solutions were mixed rapidly with equal volumes of solutions 10 mM in total phosphate and either 0, 60, or 180 mM in $NaHCO_3$. For preparations made at pH 7.2, the calcium, phosphate, and carbonate concentrations before mixing were 14, 14, and 28 mM, respectively. After 5 min, portions of the resultant suspensions were millipore filtered (100 mμ pore size), washed thrice with distilled water and then lyophilized. The remaining suspensions were allowed to stand until the solid phase became crystalline (3–72 h) before filtering and washing. Even though apatite crystal maturation in solution continues indefinitely, it was found that the major changes in crystal ripening occurs within the first few days following conversion. Consequently, suspensions were not sampled beyond 72 h. Some apatitic preparations were rinsed with several (3–7) changes of one of the following solutions instead of distilled water: 0.1 M tris, 0.1 M tris—5 mM $NaHPO_4$, 0.1 M tris—30 mM $NaHCO_3$. All apatitic preparations were dried by lyophilization.

The amorphous or apatitic nature of the precipitates was established by X-ray powder diffraction examination. Diffraction photographs were taken with a 57.3 mm Debye-Scherrer camera using Ni-filtered copper radiation ($\lambda = 1.54$ Å) at 35 kV and 20 mA.

All samples were analyzed for calcium by atomic absorption spectrophotometry and for orthophosphate by the method of Gee and Deitz (1953). Carbonate was determined by a modification of the Conway microdiffusion technique (Hirschman and Sobel, 1965). Acid phosphate (HPO_4^{2-}) was determined indirectly by the heat-induced pyrophosphate method of Gee and Deitz (1955). Samples were generally ignited either in air or *in vacuo* at 450–475° for periods ranging from 0.5 to 24 h. The heating temperatures employed are the minimum required for optimum $P_2O_7^{4-}$ production in CO_3-free apatites (Termine and Eanes, 1972). Additional samples were heated at 250°, 300°, 325°, and 500° for periods ranging from 0.5 to 84 h. In some experiments the samples were mixed with measured quantities of either $CaCO_3$, Na_2CO_3, CaO, or $CaSO_4$ before pyrolysis.

Infrared (IR) spectra of the freeze-dried precipitates were obtained by the use of KBr pellets (0.6 mg sample per 300 mg KBr) with a dry-, CO_2-free, air purged Perkin Elmer 621 infrared spectrophotometer in the linear absorbance mode. IR assessment of the approximate amount of HPO_4^{2-} present in these CO_3^{2-}-containing apatites was made by comparing their HPO_4^{2-} integrated band intensities with those from a chemically compatible series of CO_3^{2-}-free synthetic apatites (Termine and Eanes, 1972) similar in crystal size but ranging in HPO_4^{2-} content from 2–22%. In these assessments, curve fitting procedures were used to approximate the ν_2 CO_3^{2-} contribution to the P-(OH) intensity in the 865 cm^{-1} region. HPO_4^{2-}-free apatitic specimens were used as baseline samples in assessing the intensity of the 1105 and 525 cm^{-1} HPO_4^{2-} shoulders in these spectra.

Results

The analytical data reported in Table 1 clearly show that the Ca/P molar ratio of the CA is higher than that of the corresponding ACP precursor. In those preparations where the initial solution CO_3/P molar ratio was 6, the carbonate content of the converted apatite was higher than that of its precursor. On the other hand, in the preparation where the solution CO_3/P was 18, the carbonate level in the apatite dropped. The ACP in this case, however, had approximately twice the amount of carbonate as did the other amorphous preparations listed in Table 1.

The most striking chemical change upon conversion of ACP to CA was the precipitous drop in acid phosphate (HPO_4^{2-}) to near zero values as measured by the pyrolytic production of $P_2O_7^{4-}$ (Table 1). The % HPO_4^{2-} data given in this table are for samples pyrolyzed at 450°. Essentially similar results (0–3%) were obtained from CA specimens pyrolyzed at 250° (1, 2, 22 h), 300° (0.5, 1, 2 h), 325° (2, 6, 24, 49, 84 h), and 500° (2 h).

The mechanical mixing of CO_3-free apatite rich in HPO_4^{2-} with $CaCO_3$ also resulted in considerably less $P_2O_7^{4-}$ than expected upon pyrolysis (Table 2). Mixtures containing one mole $CaCO_3$ for every 5.6 moles of apatitic Ca (the equivalent of 9.0% anionic CO_3^{2-} per total sample weight) measured a 40–65% reduction in $P_2O_7^{4-}$. Mixing the apatite with one mole of $CaCO_3$ for every mole of apatitic Ca (equivalent to 30% CO_3^{2-} by weight) resulted in a maximum production upon pyrolysis of only 21–28% of the expected value after 1–2 h. Upon further heating the content of pyrophosphate was drastically reduced to almost non-detectable levels. Similarly, mixing CO_3-free apatite with either Na_2CO_3 in the CO_3/P molar ratio of 1.0 or with one mole CaO for every mole of apatitic Ca resulted in no detectable $P_2O_7^{4-}$ upon pyrolysis. Comparable levels of $CaSO_4$, on the other hand, were relatively ineffective in reducing net $P_2O_7^{4-}$ production in these apatites (Table 2).

Pyrophosphate production in HPO_4^{2-}-containing apatites was also diminished by simply washing the apatites with carbonate-containing tris-buffered solutions before pyrolysis. The reduction in % HPO_4^{2-}, assessed by the amount of $P_2O_7^{4-}$ formed upon pyrolysis, was quite marked; the HPO_4^{2-} levels averaged 1.0%

Table 1. Comparison of the Ca/P molar ratio, acid phosphate, and carbonate contents of crystalline apatite (CA) with its amorphous precursors (ACP)

	ACP	CA	ACP	CA	ACP	CA	ACP	CA
Preparation pH	7.4		7.6		7.6		7.8	
Preparation CO_3/PO_4 [a]	6		6		18		6	
Preparation age	5 min	72 h	5 min	3 h	5 min	7 h	5 min	3 h
Ca/P (molar)	1.46	1.65	1.51	1.62	1.66	1.75	1.54	1.62
% HPO_4 [b]	17.4	0.8	11.2	0.5	10.4	0	12.1	2.1
weight % CO_3	2.97	3.48	2.51	3.00	6.48	5.34	3.39	3.75

[a] "Preparation CO_3/PO_4" refers to the "total carbonate/total phosphate" molar ratio existing in the reaction system.

[b] % HPO_4 value based on the amount of $P_2O_7^{4-}$ formed upon pyrolysis of the sample at 450° for 24 h *in vacuo*.

Table 2. The effect of various admixed inorganic salts on the pyrolytic formation of $P_2O_7^{4-}$ in CO_3-free apatite. Results given as % orthophosphate converted to $P_2O_7^{4-}$

Time (hours)	Apatite control		$CaCO_3$/Apatite[a]			$CaSO_4$/ apatite[a]
	450° (air)	450° (vac)	1.0 450° (air)	1.0 450° (vac)	0.18 450° (vac)	0.74 450° (air)
0.5	15.2	13.5	1.5	1.0	7.2	14.0
1.0	14.9	14.1	3.1	—	7.6	14.9
2.0	15.0	13.5	—	3.8	8.1	14.7
3.0	—	16.2	1.6	2.2	—	—
14.0	—	16.0	0.5	—	7.5	—
24.0	15.9	16.5	0	1.8	5.7	15.9

[a] Mole added salt/Mole apatitic Ca.

Table 3. The effect on $P_2O_7^{4-}$ production (475°-vacuum) of rinsing three times a CO_3-free apatite rich in HPO_4^{2-} with a CO_3-containing solution

Heating time (hours)	Rinse solution	
	Control[a]	Carbonate[b]
1	7.1	1.7
2	8.0	0.7
3.5	7.9	1.2
22	9.4	0.6

[a] Control rinse: 0.1 M tris, pH 7.4.
[b] Carbonate rinse: 0.1 M tris+30 mM $NaHCO_3$, pH 7.4.

Table 4. The effect on $P_2O_7^{4-}$ production (475°-vacuum) of rinsing a CO_3-apatite several times (7) with a buffered-solution containing 5 mM Na_2HPO_4

Heating time (hours)	Rinse solution	
	Control[a]	Phosphate[b]
0.25	2.4	8.7
4	1.4	6.4
24	—	4.9

[a] Control rinse: 0.1 M tris, pH 7.4.
[b] Phosphate rinse: 0.1 M tris, 5 mM Na_2HPO_4, pH 7.4

compared to 8.1% for control samples washed with CO_2-free buffer (Table 3). Reversing the procedure, i.e., rinsing a CO_3-apatite with a HPO_4^{2-} solution, elevated the $P_2O_7^{4-}$ content upon subsequent heating (Table 4). It is interesting to note, however, that $P_2O_7^{4-}$ production in this case decreased with increasing heating time (Table 4). Normally, $P_2O_7^{4-}$ production level was relatively constant under the time-temperature conditions employed in this study (see Table 3).

Acid phosphate (HPO_4^{2-}) absorption bands at 1105 cm^{-1}, 865 cm^{-1}, 525 cm^{-1} and 425 cm^{-1} (Fowler *et al.*, 1966; Termine and Eanes, 1972) were present in the
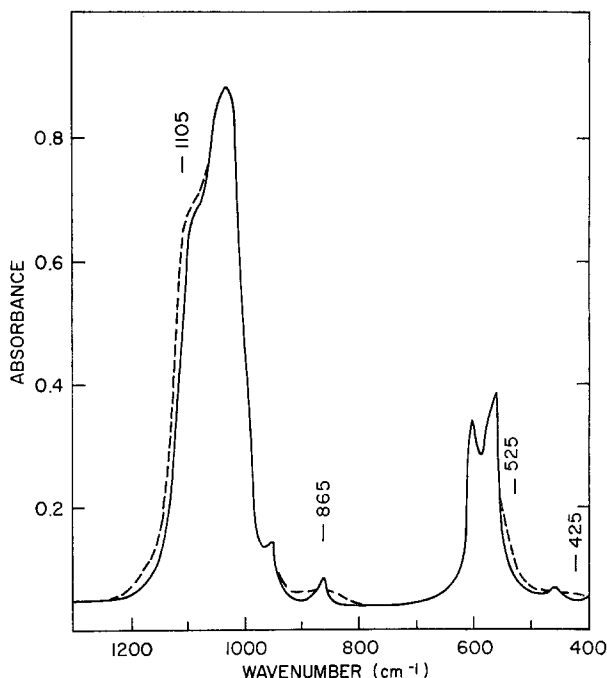
Fig. 1. Infrared spectra in the 1300–400 cm^{-1} region of a carbonate apatite containing no HPO$_4^{2-}$ (solid trace) and of an unheated apatite of the same crystalline texture and carbonate content as the first and having no detectable P$_2$O$_7^{4-}$ upon subsequent pyrolysis (dashed trace)

infrared spectra of lyophilized CO$_3$-apatites having no detectable amounts of P$_2$O$_7^{4-}$ upon subsequent pyrolysis (Fig. 1). From the intensity of the IR bands from the apatite sample illustrated in Fig. 1, it could be approximated that from 5 to 10% of the total phosphate in this specimen was in the form of HPO$_4^{2-}$. None of this HPO$_4^{2-}$ could be chemically assayed as P$_2$O$_7^{4-}$. Similarly, the IR assessment of HPO$_4^{2-}$ levels in CO$_3$-apatites containing chemically detectable HPO$_4^{2-}$ were always considerably higher than the corresponding pyrolytic value. For example, the HPO$_4^{2-}$ content of a CO$_3$-apatite prepared from a pH 7.25 solution with an initial CO$_3$/P ratio of 2 was 8% as determined by pyrolysis whereas the IR estimate was approximately twice this figure.

Discussion

The present study shows that the conversion of amorphous calcium phosphate to crystalline apatite in carbonate solutions is accompanied by significant increases in Ca/P molar ratios and by small, yet measurable, changes in carbonate content. The most striking finding, however, was an apparent dramatic decrease in HPO$_4^{2-}$ upon crystallization of carbonate-ACP into carbonate-apatite. This decrease is apparently related to the presence of carbonate in the system. The presence of occluded and/or incorporated Na$^+$ and Cl$^-$ ions appears to be minimal. Previously published data showed that washed CA preparations contains only
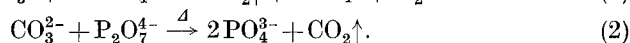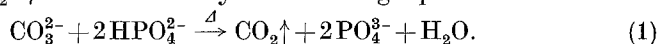
0.2% Na^+ and 0.02% Cl^- by weight (Termine and Eanes, 1972). In preparations where carbonate was excluded, the HPO_4^{2-} content of the CA's remained elevated (see Table 2) in agreement with previous data (Termine and Eanes, 1972). The two most probable explanations for this CO_3^{2-}-HPO_4^{2-} effect are: (1) the carbonate ion interferes with HPO_4^{2-} incorporation into the apatite during conversion, (2) the carbonate interferes with the assaying of HPO_4^{2-} by the pyrolytic method of Gee and Deitz (1955). Several lines of evidence favors this latter explanation as being the most plausible.

Quinaux (1964) found that the percentage of $P_2O_7^{4-}$ formed upon heating apatite-like tricalcium phosphate hydrate (TCPH) between 300° and 600° was significantly reduced in the presence of $CaCO_3$. The results obtained from mixing $CaCO_3$ and Na_2CO_3 with our apatites (Table 2) confirm her findings. These data demonstrate that the mechanical juxtaposition of a metal carbonate compound with HPO_4^{2-}-rich apatite can significantly suppress net $P_2O_7^{4-}$ formation in the latter substance.

The low to negligible levels of $P_2O_7^{4-}$ production seen in apatites made from CO_3-containing amorphous preparations and in HPO_4^{2-}-rich apatites mechanically mixed with carbonate salts were also achieved by simply washing apatites rich in HPO_4^{2-} with aqueous carbonate solutions prior to pyrolysis (Table 3). Conversely it was also possible to elevate $P_2O_7^{4-}$ production in CO_3-containing apatites by washing with aqueous HPO_4^{2-} solutions (Table 4). However, prolonged pyrolysis of these apatites resulted in a subsequent decrease in $P_2O_7^{4-}$. These latter two findings suggest that rinsing a CO_3-containing apatite with an HPO_4^{2-} solution results in an initial exchange and subsequent separation of these two ionic species, i.e., HPO_4^{2-} becomes adsorbed to the surface of the apatite crystals (with a concomitant reduction in surface carbonate) and the remaining carbonate is most probably internally located. This temporary separation allows some $P_2O_7^{4-}$ to form during initial pyrolysis but prolonged heating re-establishes carbonate interference probably by breaking down already formed $P_2O_7^{4-}$.
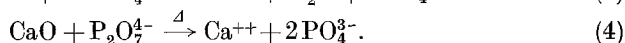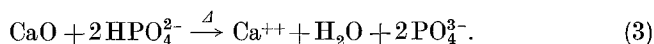
The infrared data provide more direct evidence that carbonate is interfering with the chemical detection of HPO_4^{2-}. As illustrated in Fig. 1, the infrared patterns of CO_3-containing apatites prepared at physiological pH's consistently showed the presence of HPO_4^{2-} even though only negligible $P_2O_7^{4-}$ was formed upon subsequent pyrolysis. Even in those apatites where some $P_2O_7^{4-}$ could be produced, the level of HPO_4^{2-} in the unheated samples, as estimated by IR measurements, was in considerable excess of that needed to account for the $P_2O_7^{4-}$ produced during pyrolysis.

Several reaction mechanisms can be advanced to account for the suppressing effect of carbonate on the pyrolytic assay for HPO_4^{2-}. Two possibilities involve the direct reaction of the carbonate anion with either unconsumed HPO_4^{2-} or with already formed $P_2O_7^{4-}$ as described by the following equations:

$$CO_3^{2-}+2\,HPO_4^{2-} \xrightarrow{\Delta} CO_2\uparrow+2\,PO_4^{3-}+H_2O. \tag{1}$$

$$CO_3^{2-}+P_2O_7^{4-} \xrightarrow{\Delta} 2\,PO_4^{3-}+CO_2\uparrow. \tag{2}$$

The data presented in this papers suggest that the carbonate ion does not necessarily have to react directly with either the HPO_4^{2-} or $P_2O_7^{4-}$ ions. The effectiveness of CaO (this paper) and $Ca(OH)_2$ (Quinaux, 1964), but not $CaSO_4$, in suppressing

net $P_2O_7^{4-}$ formation indicates that the metal oxide could also be an active agent. Here the reaction sequence would require a decomposition of the carbonate to the oxide and gaseous CO_2. This breakdown would then be followed by a neutralization of the oxide with either HPO_4^{2-} and/or $P_2O_7^{4-}$ as described by the following equations:

$$CaO + 2\,HPO_4^{2-} \xrightarrow{\Delta} Ca^{++} + H_2O + 2\,PO_4^{3-}. \tag{3}$$

$$CaO + P_2O_7^{4-} \xrightarrow{\Delta} Ca^{++} + 2\,PO_4^{3-}. \tag{4}$$

In Eqs. (1) and (3), a classical Bronsted-Lowry acid-base reaction (Moeller, 1952), the carbonate or the oxide effectively blocks the $P_2O_7^{4-}$ condensation reaction from occurring by providing an alternative mechanism for binding the hydrogen of the HPO_4^{2-} ion with oxygen to produce H_2O. Eqs. (2) and (4) describes the breakdown of already formed $P_2O_7^{4-}$ by either the carbonate or the oxide via a Lewis neutralization mechanism (Lewis, 1923). The $CaSO_4$ is ineffective as a $P_2O_7^{4-}$ inhibitor because the SO_4^{2-} ion is stable under the pyrolytic conditions used in this study, i.e., the $CaSO_4$ is not in itself a Lewis base and it does not decompose into the oxide and gaseous SO_2 (Moeller, 1952).

It is not possible from the data obtained in this study to establish unequivocally the relative importance of each of the above mechanisms in accounting for the poor $P_2O_7^{4-}$ yields from pyrolyzed CO_3-apatites. In special situations, such as during the initial pyrolysis of the mechanical mixtures and for the HPO_4^{2-}-washed CO_3-apatites, mechanisms (2) and (4) can be clearly delineated, but this is not to be taken as evidence for $CaCO_3/CaO$-induced $P_2O_7^{4-}$ breakdown as the major operative in reducing net $P_2O_7^{4-}$ in all cases. Mechanisms (2) and (4) are operative in the mixtures and the HPO_4^{2-}-washed apatites probably because $P_2O_7^{4-}$ had an opportunity to form in a transient CO_3-free environment.

It was noted earlier (Termine and Eanes, 1972) that the pyrolytic method was not a reliable assay for HPO_4^{2-} in apatites. The data presented in this paper demonstrates that this is particularly appropos to the CO_3 containing-apatites. On the other hand carbonate does not appear to seriously interfere with the HPO_4^{2-} assay in ACP's under equivalent conditions. It is not completely clear why HPO_4^{2-} pyrolysis in ACP should be considerably less affected by carbonate than in apatite, but it may be related in part to the temperature used for pyrolysis. It has been observed previously that $P_2O_7^{4-}$ yields from the pyrolysis of carbonate-containing amorphous calcium phosphates do not decrease appreciably from their maximum values until temperatures above 500°C are reached (Greenfield and Eanes, 1972). Even for ACP preparations containing 9% carbonate by weight, where the effect was most serious, the reduction in % HPO_4^{2-} from maximum value was less than 25% under the temperature conditions used in the present study (i.e., 450°). At 525°, however, the reduction in HPO_4^{2-} was 72%.

Finally, bone mineral is a complex mixture of carbonate-containing amorphous and apatitic calcium phosphates (Eanes and Posner, 1970; Termine, 1972). Based on the data obtained in this study, it is suggested that the pyrolytic method for HPO_4^{2-} be used in this tissue only with great caution and definitely not as an assay for total HPO_4^{2-} content. As an example, Pellegrino and Biltz (1972) report an inverse relationship between HPO_4^{2-} and CO_3^{2-} during maturation of embryonic chick bone which they ascribe to a CO_3^{2-} for HPO_4^{2-} substitution during the for-

mation of a CO_3-apatite. These results, obtained by pyrolysis, are consistent with the interpretation that increasing bone carbonate level accompanying maturity progressively interferes with the analytical detection of bone HPO_4^{2-}.

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