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Chemical and Structural Study of the Mineral Phase Associated with a Human Subcutaneous Ectopic Calcification

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A crystallographic, spectroscopic and chemical analysis of the inorganic subcutaneous deposits associated with a rare case of interstitial calcinosis has been carried out.

Two distinct types of calcium phosphate deposits have been demonstrated in the tissue by X-ray diffraction analysis. The main crystalline inorganic phase was hydroxyapatite, while β-tricalcium phosphate with a partial replacement of calcium with magnesium was present just in small amounts.

In order to determine the degree of substitution of magnesium ion to calcium in the β-tricalcium phosphate, this compound has been synthetized with a Ca/Mg molar ratio ranging from 1.9 to 37.6.

The low Ca/P molar ratio of 1.31 determined in the inorganic deposits is consistent with the presence of other ions, mainly carbonates, such as revealed by infrared analysis in the mineral phase.

It is suggested that the increased magnesium concentration in the tissue plays an important role in the development of the interstitial calcinosis.

Introduction

Ectopic calcifications of dystrophic type are known to be due to some local tissue abnormalities such as a diminution of mucopolysaccharides capable of protecting from mineralization reactive sites on collagen fibers.

We report here the crystallographic, spectroscopic and chemical analysis of the inorganic deposits associated with an exceptionally rare case of progressive interstitial calcinosis which had developed in a 48-year-old woman since her childhood. This calcinosis was characterized by salt deposition in the only subcutaneous and by uneven stiffening of the subcutaneous in the form of wrinkles and grooves, alternated with pale bronze-coloured spots.

The death of the patient occurred owing to respiratory failure due to progressive chest wall stiffening.

Experimental

Samples of calcified tissue were removed autoptically from the hardest regions of the patient's body. Such material was stored in formic aldehyde solution at –30 °C until used. The specimens were prepared after drying for 72 h at 110 °C to constant weight and then were heated at 700 °C for 12 h to eliminate the organic matrix.

The per cent content of the inorganic phase was determined by the difference between the weight of the tissue before and after heat-treatment.

Materials used for comparison were synthetic whitlockite, calcium magnesium phosphate and hydroxyapatite. Synthetic whitlockite with an increasing replacement of calcium with magnesium was prepared according to Le Geros [1] by addition of calcium and magnesium acetate (Ca/Mg molar ratio ranging from 97/3 to 3/97) into a phosphate solution. Hydroxyapatite was synthetized by dropwise addition of phosphoric acid into a calcium hydroxide solution.

Chemical Analysis

An atomic absorption spectrophotometer Perkin Elmer 373 was used to determine calcium and magnesium content in ashed tissue diluted to an appropriate volume with 10% lanthanum in 50% HCl.

Phosphorus was determined spectrophotometrically as molybdovanadophosphoric acid [2].

Carbonate content was determined by infrared analysis. About 1 mg of powdered sample was intimately mixed with 300 mg of KBr (infrared grade) and pelletized under vacuum. The pellets were analyzed using a Perkin Elmer 397 spectrophotometer, range 4000 to 400 cm^{-1}, normal slit, scanning speed 10 min (50 min for slow scan). The quantitative determination of the carbonate ion present in the inorganic phase of the tissue was obtained by analysis of the absorption bands of PO_4^{3-} and CO_3^{2-} at 960 and 870 cm^{-1} respectively. The ratio of the absorbances of CO_3^{2-} and PO_4^{3-} was correlated to the carbonate content in the sample through the comparison with the ratio of CO_3^{2-} and PO_4^{3-} absorbances of synthetic mixtures of hydroxyapatite containing from 0% to 100% of $CaCO_3$.

X-ray Diffraction Analysis

X-ray diffraction studies were carried out on samples of the tissue air-dried at room temperature by a flat diffraction camera (film to sample distance of 4 cm) and on dried powdered samples of tissue heat-treated at 700 °C and 1000 °C by means of a Debye–Scherrer camera (radius 57.3 mm) and an X-ray powdered diffractometer unit (Philips Norelco). The 2θ diffraction range covered ranged from 10° to 65° at a scanning speed of 1/2 degree/min. Patterns were interpreted by comparison with the American Society of Testing Materials (A.S.T.M.) index cards. All the scattering patterns were obtained with a nickel-filtered CuK radiation.

Results

Chemical Composition

The chemical analysis carried out on the samples of pathologic tissue after heat-treatment at 700 °C reveals constant calcium, magnesium, phosphorus and carbonate contents regardless of the area where the samples have been dissected. The average values obtained for the calcium and phosphorus contents expressed as moles per gram of ashed tissue are respectively 6.67×10^{-3} and 5.10×10^{-3}. The Ca/P molar ratio calculated from these values is 1.31. The magnesium content in the samples is too low to be determined by atomic absorption analysis.

Infrared Absorption Analysis

The infrared spectrum of the calcified samples heat-treated at 700 °C gives absorption peaks corresponding to vibration modes of the OH^-, PO_4^{3-} and CO_3^{2-} groups: these peaks reveal the contemporary presence of apatite and carbonate in this material (Fig. 1).

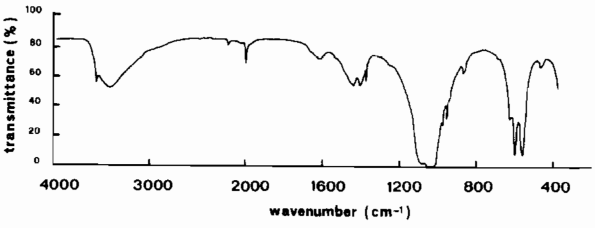

Fig. 1. Infrared absorption spectrum of the samples heat-treated at 700 °C.

The quantitative determination of CO_3^{2-} content obtained by the analysis of the absorption bands of PO_4^{3-} and CO_3^{2-} has revealed the presence of 9.17×10^{-4} moles of this ion for gram of ashed tissue corresponding to a CO_3^{2-} content of about 5.5% in the inorganic phase.

Crystallographic Analysis

Figure 2 shows the X-ray diffraction pattern of the powdered heat-treated samples. Most of the X-ray diffraction peaks are coincident with those exhibited from hydroxyapatite while the diffraction maxima marked by W can be attributed to the β-tricalcium phosphate with a partial replacement of calcium with magnesium.

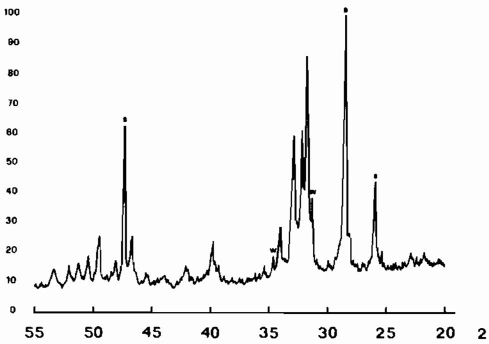

Fig. 2. X-ray diffraction pattern of the powdered sample after heat-treatment at 700 °C. W: diffraction maxima due to β-TCMP. S: standard reference (Si).

The lattice constants of the hydroxyapatite present in the mineral phase of the pathologic calcified tissue are coincident with those reported by A.S.T.M. data. The lattice constants of the β-tricalcium phosphate copresent with hydroxyapatite are a = 10.384(6) Å and c = 37.16(3) Å. The values of a and c parameters are smaller than those reported by A.S.T.M. data for β-tricalcium phosphate (β-TCP) (a = 10.429, c = 37.38) and β-tricalcium phosphate with a partial substitution of magnesium to calcium (a = 10.37, c = 37.19).

In order to determine the degree of substitution of magnesium for calcium ion in the whitlockite crystal structure present in the inorganic phase of the biological samples, numerous synthetic β-tricalcium phosphate specimens with a Ca/Mg molar ratio ranging from 1.9 to 37.6 were prepared. The lattice constants of these synthetic materials calculated by X-ray diffraction analysis are reported in Table I as a function of their Ca/Mg molar ratio. When the values of a and c parameters are very close to those measured for biological samples, the Ca/Mg molar ratio is about 11.4.

TABLE I.

Ca/Mg	*a*	*c*
46.4	10.442(6)	37.35(3)
29.3	10.406(6)	37.27(2)
16.4	10.370(4)	37.20(2)
11.4	10.342(1)	37.15(5)
4.4	10.332(2)	37.11(1)
3.6	10.334(3)	37.09(2)
2.8	10.335(8)	37.09(5)
2.1	10.336(3)	37.08(2)
2.0	10.337(2)	37.09(2)
1.9	10.337(4)	37.10(2)

Thermochemical Properties

The X-ray diffraction patterns of the calcified samples tested at room temperature by a flat camera do not reveal the presence of inorganic compounds but only some diffraction maxima corresponding to the molecular spacings of collagen fibers.

The X-ray diffraction patterns of the powdered samples, not heat-treated, obtained by a diffractometer do not show any diffraction maxima resolved from the background. This effect may be due either to the poor quantity of the inorganic crystalline salts in the fresh tissue respect to the organic matrix, or to the fact that these salts become crystalline only at high temperature.

The X-ray diffractometric patterns obtained on the samples after heat-treatment at 1000 °C are very close to that recorded for the same samples heat-treated at only 700 °C except for the increase of crystallinity as demonstrated by the further sharpening of the diffraction peaks.

Discussion

The X-ray diffraction analysis demonstrates the contemporary presence of two distinct types of calcium phosphates in the mineral phase of the pathologic tissue heat-treated at 700 °C: hydroxyapatite and β-tricalcium phosphate with a partial replacement of calcium with magnesium corresponding to a Ca/Mg molar ratio of 11.4. The X-ray diffraction pattern of the biological samples reported in Fig. 2 shows that the main crystalline inorganic phase is hydroxyapatite.

The chemical analysis revealed a Ca/P molar ratio of 1.31, which is lower than the 1.67 and 1.5 values exhibited by hydroxyapatite and β-tricalcium phosphate respectively. This small Ca/P molar ratio is consistent with the presence of other ions in the mineral phase. Diffractometric analysis shows that these ions are not probably present as substituted to calcium and phosphorus in the crystalline hydroxyapatite and tricalcium phosphate, but it is possible to suggest the presence of other salts in the inorganic phase. Among the ions which can substitute phosphate or hydroxyl ions in the hydroxyapatite crystal lattice, carbonate plays an important role in the biological apatites. In fact the presence of carbonate ions can be related to the low Ca/P ratio of some biological apatites. Furthermore hydroxyapatite formed in presence of carbonate tends to be smaller in crystal size than carbonate-free hydroxyapatite and shows lattice distorsion.

X-ray diffraction analysis can be used to determine the replacement of carbonate to phosphate or to hydroxyl from the evaluation of the *a* axis dimension which changes from $a = 9.42$ to $a = 9.11$ and $a = 9.54$ Å respectively.

In order to determine the carbonate content and its effect on the inorganic phase present in the sample, it is interesting to investigate the results obtained from the infrared spectrophotometric analysis. The asymmetric stretching bands at 1450–1410 cm^{-1} and the deformation out of plane at 880–850 cm^{-1} (Fig. 1) confirmed the presence of 5.5% wt carbonate which could not be detected by X-ray analysis. This result is in agreement with a lack of carbonate substitution to the hydroxyl or phosphate ions in the hydroxyapatite crystal lattice demonstrated by the X-ray and infrared patterns.

The impossibility of appreciating the presence of magnesium by atomic absorption spectrophotometric analysis is in agreement with the low content of β-TCMP and with the high content of hydroxyapatite in the inorganic phase of the samples. In fact according to Le Geros *et al.* [3] the presence of magnesium is a hindrance to the formation of crystalline hydroxyapatite.

The degree of replacement of magnesium to calcium in the β-TCP crystal lattice was obtained from the evaluation of the lattice constants and through the synthesis of β-TCP with varying ratios of substitution of magnesium to calcium, since it was impossible to determine the magnesium content by chemical analysis. These results have confirmed a substitution of magnesium to calcium corresponding to a Ca/Mg molar ratio of about 11.4.

The same apatite and whitlockite phases, although not in the same apatite/whitlockite ratios, are obtained when human enamel and dentina are heat-treated at 600 °C and 700 °C respectively [4, 5]. Human bone requires an ignition temperature of 900 °C for the appearance of small amounts of whitlockite. The different amounts of β-TCP produced under heat-treatment of bone and dentine are consistent with the higher magnesium content in dentine compared to that of bone.

Synthetic amorphous calcium magnesium phosphate is transformed mostly to whitlockite upon ignition. Since the samples were heat-treated at 700 °C the whitlockite phase, present together with apatite which represents the main product of calcification, may be induced by ignition.

Since the X-ray diffraction patterns obtained for the samples at 1000 °C show the contemporary presence of β-TCMP and hydroxyapatite also after this heat-treatment, while synthetic β-TCP is transformed to apatite either upon hydrolysis or upon ignition at 900 °C, we can suppose that β-TCMP resists to the transformation into apatite. This suggests that the partial substitution of magnesium to calcium in the whitlockite structure stabilizes this structure. Since the solid product reflects that of the solution from which it is formed, it is conceivable to assume that local factors are important in giving rise to apatitic or amorphous calcium phosphate compounds. It appears that the excess total body magnesium and increased magnesium concentration play an important role in the development of pathologic calcification [6]. It could be suggested that the calcium magnesium phosphate deposits formed in this ectopic calcification are either microcrystallites of magnesium whitlockite or a precursor of it and that its magnesium content stabilizes its amorphous state. Local factors such as the concentration of calcium, magnesium phosphate and carbonate will determine the type of calcium phosphate which will deposit in the tissue. Thus a higher Ca/Mg in the solution will favour the deposition of an apatitic compound while a higher Mg/Ca will favour the deposition of whitlockite or amorphous calcium magnesium phosphate compound.

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