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STRUCTURAL AND CHEMICAL CHARACTERIZATION OF A CUTANEOUS CALCIFICATION

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Dynamic thermogravimetry, X-ray diffraction, spectroscopic and chemical analyses have been carried out on the cutaneous calcifications present in a case of Porphyria cutanea tarda.

The inorganic deposits are constituted by a poor crystalline B carbonated apatite characterized by an almost stoichiometric Ca/P molar ratio, a low magnesium relative content and a high thermal stability.

The inorganic crystallites grow in the cutaneous calcification without any preferential orientation in the tissue where the collagen fibrils are isotropically distributed.

The results reveal that the cutaneous calcifications display a close similarity with the inorganic deposits isolated from atheromatous plaques of aorta and calcified mitral valves.

Keywords: apatite phase, biochemical studies, cutaneous calcification, structural modification of collagen

Introduction

Calcium phosphate deposition in dermis is referred to as *calcinosis cutis*. Four different types are known [1, 2] metastatic calcinosis, in presence of ipercalcemia and iperfosfatemia; dystrophic calcinosis, when calcium is laid down on an already damaged tissue (Scleroderma, Porphyria Cutanea tarda (PTC), etc.); idiopathic calcinosis, which is close to dystrophic calcinosis, but does not exhibit the copresence of other diseases and cutaneous calculi.

Numerous morphological, biochemical and ultrastructural studies have been carried out on calcinosis cutis in order to investigate the structural modifications of collagen, elastin and other components of the connective tissue and their relationship with calcium deposits [3]. On the other hand, only a few studies have been addressed to chemical and structural characterization of the inorganic

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deposits. The inorganic deposits have been identified as hydroxyapatite in several dystrophic calcifications [4]. Baldet *et al.* [5] and Legros *et al.* [6] provided more detailed informations of the inorganic phase deposited in the cutaneous calcifications showing that the deposits consisted of apatite with a partial substitution of carbonate to phosphate groups (B carbonated-apatite). Cutaneous calcification rarely occurs in PCT.

Preliminary results obtained on bioptic material from a patient affected by sclerodermic PCT revealed the presence of inorganic deposits constituted by poor crystalline hydroxyapatite [7].

Herein we report the results of thermogravimetric, X-ray diffraction, spectroscopic and chemical investigation carried out on the inorganic deposits isolated from the subcutaneous calcifications present in scleroderma like areas in a case of PCT. The results reveal that the apatite phase which constitutes these deposits is quite similar to the apatitic phase of the atheromatous plaques of aorta and mitral valve.

Materials and methods

Bioptic specimens were isolated from the cutaneous calcifications localized in the scleroderma like areas of a 65-year-old man who had been affected by PCT for sixteen years. The calcified areas ranged from 1 to 3 cm in diameter.

For structural and chemical analyses samples dissected from the calcified areas were washed with distilled water and air dried. Some of the samples were submitted to decalcification treatment with EDTA at $pH = 7$ [8].

Some samples of health cutis have been examined for comparison. Some samples have been submitted to heat treatment in oven at different temperatures from 100° to 1000°C for 15 hours.

Powder X-ray diffraction analysis was carried out by means of a Philips diffractometer using Ni-filtered CuK radiation. The 2θ range was from 10° to 65°C at a scanning speed of 0.5 deg·min^{-1}. The lattice constants were determined by least square refinements. High angle X-ray diffraction patterns were recorded using a flat camera with a sample to film distance of 40 mm. The patterns recorded using Nifiltered CuK radiation were collected on 3 M type *S* films.

For IR absorption analysis, 1 mg of the powdered samples was carefully mixed with 300 mg or KBr (infrared grade) and pelletized under vacuum. The pellets were analyzed using a Perkin-Elmer 380 IR Grating Spectrophotometer, range 4000–400 cm^{-1}, normal slit and scanning speed of 72 cm^{-1}·min^{-1}.

Calcium and magnesium content were determined using an atomic absorption spectrophotometer (Perkin Elmer 373); ashed tissues were diluted to an appropriate volume with 10% lantanum in 50% HCl.

Phosphorus content was determined spectrophotometrically as molibdovanadophosphoric acid [9].

Dynamic thermogravimetry was carried out using a Perkin Elemer TG-7 equipped with a P. E. 3700 Data Station. Heating was performed in a platinum crucible up to 900°C under air flow (20 cm^3/min) with a scanning rate of 5 deg·min^{-1}. The weight of the samples was 5–15 mg.

DTA was carried out with a Setaram TAG24 apparatus. Heating was performed in air in an alumina crucible using a rate of 10 deg·min^{-1} up to 1150°C. Each run was carried out on 30 mg of material.

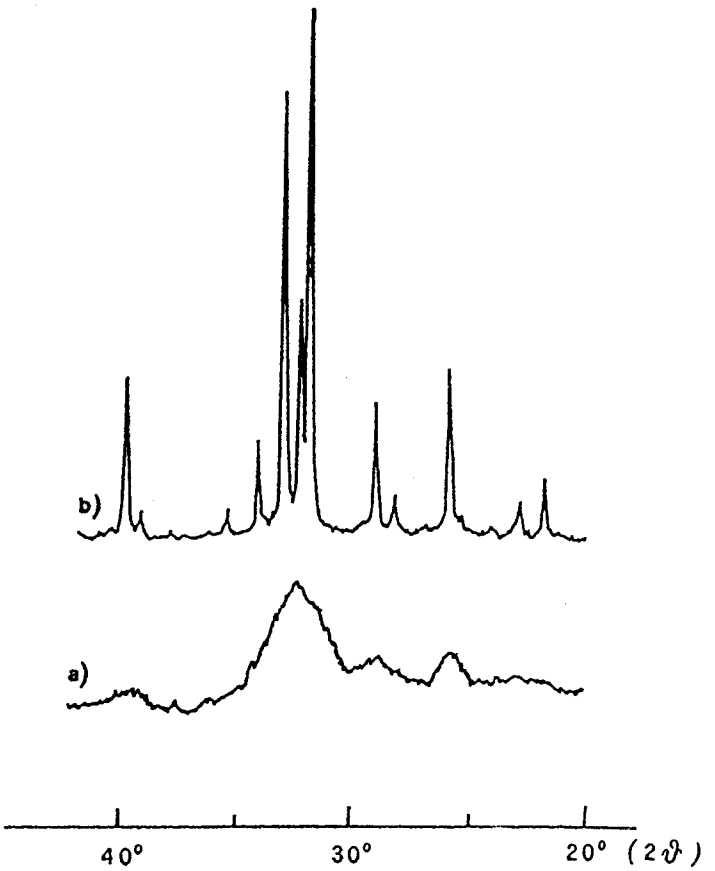

Fig. 1 Powder X-ray diffraction patterns from a sample of subcutaneous calcification at room temperature (a) and after heat tratment at 1000°C (b)

Results

The powder X-ray diffraction patterns recorded from samples of calcified tissue (Fig. 1a) reveal the presence of diffraction maxima characteristic of a poor crystalline apatitic phase. No appreciable differences have been observed among the sample isolated from the different calcified areas.

The powder X-ray diffraction patterns recorded from samples dissected from the same calcified area and submitted to heat treatment for 15 h at different temperature from 100° to 1000°C reveal an increase of the degree of crystallinity of the apatite phase with the increase of the heat treatment temperature. The pattern reported in Fig. 1b, which has been obtained from a sample heat treated at 1000°C, is that characteristic of crystalline hydroxyapatite.

The lattice constants calculated from the pattern reported in Fig. 1b are $a = 9.420(2)$ Å and $c = 6.881(1)$ Å.

In order to verify the orientation of the apatitic crystallites in the tissue, some fragments of prismatic shape, measuring about $3\times 1\times 1$ mm^3, where submitted to high angle X-ray diffraction analysis using a flat camera. The patterns reveal an isotropic intensity distribution of the diffraction reflections of hydroxyapatite, in agreement with an isotropic distribution of the crystallites inside the samples (Fig. 2a). No diffraction effect due to collagen fibers can be appreciated in these patterns due to the high relative amount of the inorganic phase in the samples. However, after inorganic phase remotion by decalcification with EDTA, the X-ray patterns show the diffraction maxima at 0.29 nm and 1.16 nm, characteristic of collagen molecular structure. The intensity distributions of these diffraction maxima appear isotropic (Fig. 2b).

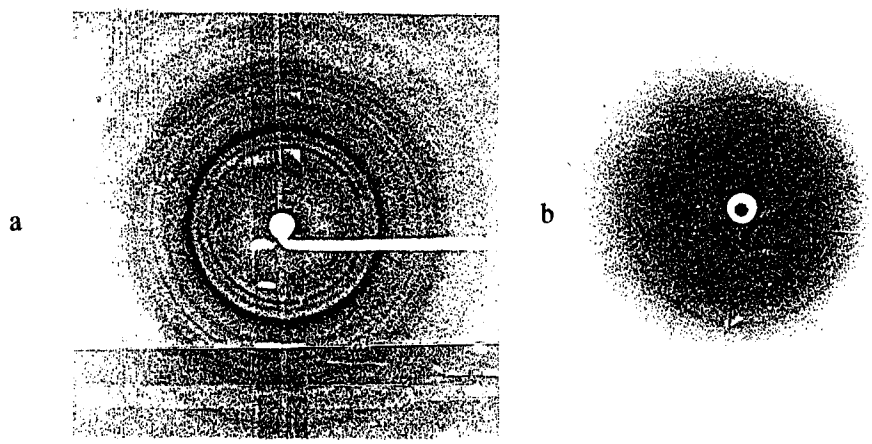

Fig. 2 High angle X-ray diffraction pattern from an air dried calcified sample before (a) and after (b) decalcification with EDTA. Sample to film distance of 40 mm

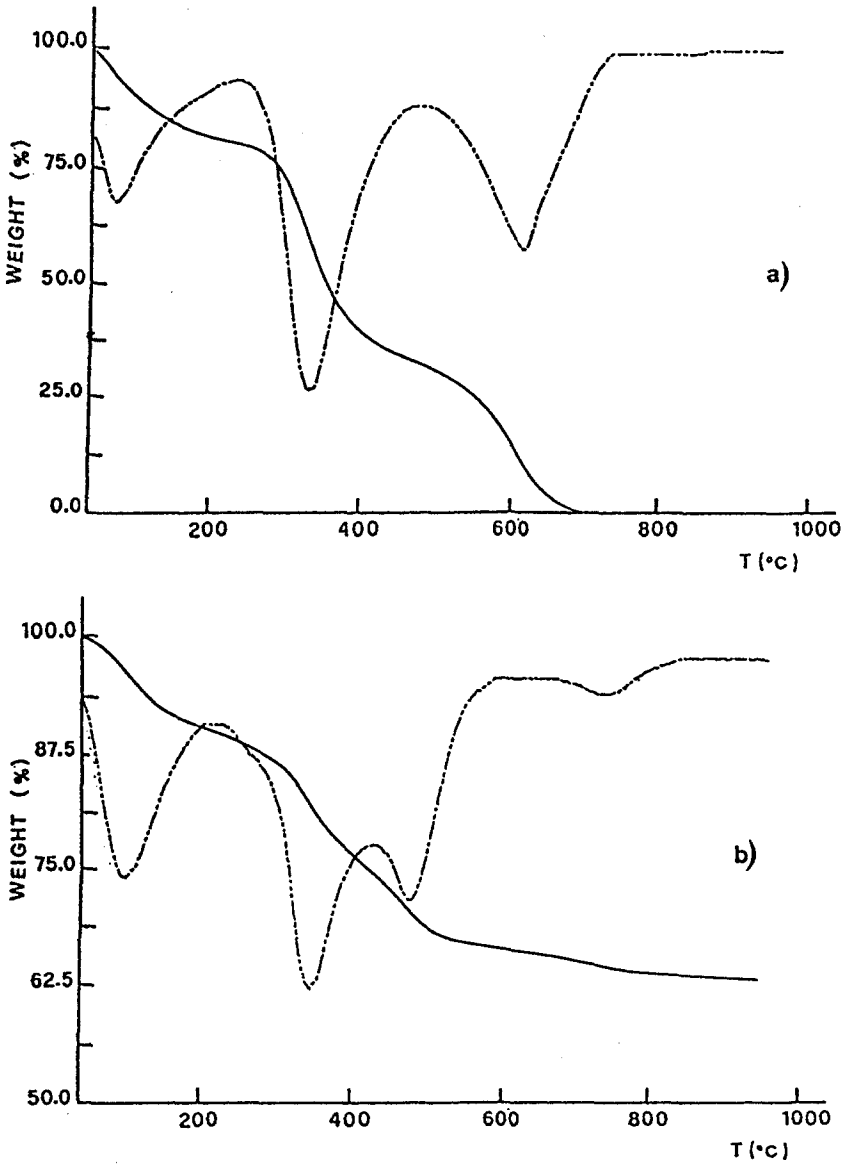

Fig. 3 TG-DTG curves of a calcified sample after (a) and before (b) decalcification with EDTA. DTG ordinate are in arbitrary units

Figure 3a displays the TG-DTG curves of decalcified cutis. The DTG curve shows three weight losses: a first one occurring in the range 40°–230°C, a second one at 230°–480°C and a third one at 480°–747°C, which, by comparison with the DTG curve of uncalcified turkey tendon [10], can be attributed to water release,

collagen decomposition and combustion of the residual organic components, respectively.

No appreciable difference has been observed in the TG-DTG curve of uncalcified cutis and of healthy cutis which has been examined for comparison.

The DTA plots of decalcified samples display a small endothermic process between 40° and about 200°C followed by exothermic broadened process up to 750°C.

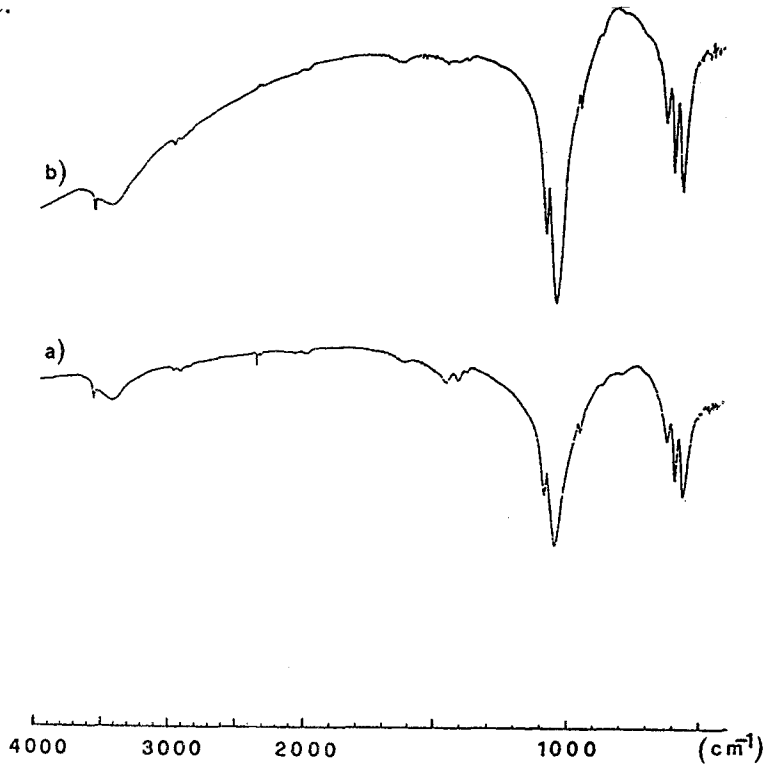

Fig. 4 Infrared absorption spectra of a calcified sample after heat treatment at 700°C (a) and 900°C (b)

The TG-DGT curves (Fig. 3b) obtained from calcified samples display four weight losses: the first one between 40° and about 228°C, the second one between 228° and about 449°C, the third one between 449° and 656°C and the fourth one between 700° and 950°C. The weight losses (W_i%) up to each minimum inflection point and the peak temperatures (T_i) are reported in Table 1 together with those obtained from TG-DTG curves of uncalcified and decalcified samples.

The DTA plots of calcified samples exhibit a small endothermic process between 40° and about 200°C, an exothermic broadened process between 200° and 700°C, after which the process becomes endothermic.

Table 1 Peak temperature (T_i) and weight losses (W_i) associated to the thermal processes detected in the TG-DTG curves of calcified, decalcified and uncalcified samples. (Each value is the mean ± s.d. on 5 determinations)

	$Peak_1$		$Peak_2$		$Peak_3$		$Peak_4$		Residue
Sample	T_1 / °C	W_1 / %	T_2 / °C	W_2 / %	T_3 / °C	W_3 / %	T_4 / °C	W_4 / %	W_R / %
Calcified	101±2	9.7±1.0	369±1	15.0±1.0	499±5	7.9±1.5	750±10	2.5±0.5	64.9±1.0
Decalcif.	70±6	15.8±2.0	335±1	49.5±1.0	620±7	34.7±5.0	–	–	
Uncalcif.	74±6	13.9±2.0	331±1	46.9±1.0	602±5	39.2±5.0	–	–	

The infrared absorption spectra of untreated samples show numerous absorption bands due to the organic matrix which partially hinders the analysis of the absorption bands characteristic of the inorganic phase. The infrared absorption spectra obtained from samples heat treated at 700°C, after complete remotion of the organic matrix, display the absorption bands characteristic of hydroxyapatite together with the absorption bands at 1455–1430 and 870 cm^{-1} characteristic of carbonate (Fig. 4a). These last bands are barely appreciable in the IR spectra obtained from samples heat treated at temperatures higher than 900°C (Fig. 4b).

The average Mg content of the ashes obtained after elimination of the organic matrix by heating at 700°C is 1.4±0.5 atom % (where calcium atoms + magnesium atoms = 100%) and the Ca/P molar ratio is 1.68±0.06.

Discussion

In the cutaneous calcifications present in PCT the inorganic crystallites, as well as the collagen fibrils, appear randomly distributed. The inorganic deposits are constituted of a poor crystalline apatitic phase that does not convert into any other phase by heat treatment up to 1000°C and exhibits cell parameters very close to those of stoichiometric hydroxyapatite. A similar behaviour to heat treatment was previously observed for the inorganic deposits isolated from highly calcified mitral valves, atherosclerotic plaques and bioprosthetic calcifications [11–13]. The low magnesium relative amount and the Ca/P molar ratio close to the stoichiometric value of these inorganic deposits are probably responsible of the high thermal stability of the apatitic phase. In fact, the inorganic deposits containing high magnesium relative amount and/or a Ca/P molar ratio significantly different from the stoichiometric value of 1.67 exhibit a partial conversion of the apatitic phase into β-tricalcium phosphate (β-TCP) by heat treatment [13–14].

The presence of the absorption bands at 1455–1430 and 870 cm^{-1} in the IR spectra of these deposits indicates a partial substitution of CO_3^{2-} for PO_4^{3-} ions in the crystal lattice of hydroxyapatite (type B apatite) [15].

The results of the infrared absorption investigation carried out on samples submitted to thermogravimetric analysis indicate that the fourth thermal process which takes place between 700° and 950°C is due to carbonate remotion from the samples, in agreement with the endothermic process observed in the DTA plot in this range of temperature. Carbonate ions account for about 3.7% wt of the mineral phase.

The thermal decomposition of the cutis obtained after decalcification of the tissue by EDTA treatment is very close to that of uncalcified cutis revealing that the decalcification treatment does not affect appreciably the cutis thermal stability. The DTG curve exibited by cutis is very close to that of tendon collagen, in spite of their different composition [16]. Keeping in mind that the thermal

decomposition of tendon collagen takes place at about 332°C [10], it can be hypothesized that the second peak at 335°C in the DTG curve of cutis is due mainly to the collagenous component. On the same basis, the third peak at about 620°C can be ascribed to combustion of the residual organic components, in agreement with the broad exothermic process observed in the DTA plot. Cutaneous calcification during PCT induces an increase of the temperature of collagen decomposition up to 369°C in agreement with a higher thermal stability of collagen fibrils in calcified areas. On the other hand, the peak temperature of the third process is lower than that of uncalcified cutis. This could be explained by the fact that the increase of the temperature of collagen decomposition in calcified areas produces less stable residual organic components which, therefore, decompose at a lower temperature.

The TG-DTG curves indicate that the thermal decomposition of calcified cutis is very similar to that reported for human femoral bone [17]. However, the thermal stability of the apatitic phase of cutaneous calcification, which does not appreciably convert into β-TCP by heat treatment up to 1000°C, is higher than that of bone apatite which partially converts int β-TCP already at 900°C [14]. This finding, as well as the values of magnesium and carbonate relative content, Ca/P molar ratio and lattice constants indicate that these inorganic deposits resemble those observed in atherosclerotic plaques and highly calcified mitral valves [11–13]. In these pathological calcifications, as well as in PCT, the inorganic deposits are laid down without any evident structural relationship with collagen fibrils.

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Zusammenfassung — Mittels DTG, Röntgendiffraktion, spektroskopischen und chemisch analytischen Methoden wurden Kalkablagerungen der Haut, wie sie bei Porphyria cutanea tarda auftreten, untersucht.
Die anorganischen Ablagerungen bestehen aus einem niederkristallinen B karbonisiertem Apatit, was durch ein fast stöchiometrisches Ca/P-Verhältnis, einen relativ niedrigen Magnesiumgehalt und eine hohe Wärmestabilität gekennzeichnet ist.
Die anorganischen Kristallite wachsen in der Kalkablagerung der Haut, im Gewebe, wo die Kollagenfasern isotrop verteilt sind, ohne jegliche Bevorzugung einer Orientierung.