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A COMPARATIVE STUDY OF BOVINE PERICARDIUM MINERALIZATION: A BASIC AND PRACTICAL APPROACH

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Abstract

The biomineralization of bovine pericardium (BP) heart valve bioprostheses was investigated by simulation of the process under *in vitro* and *in vivo* conditions. The nature and composition of calcific deposits that formed in human heart valve bioprostheses were compared with the mineral formed on BP discs immersed in a calcifying medium or subcutaneously implanted into Sprague-Dawley rats. The early stage of experimental biomineralization *in vitro* took place on the surface only, while *in vivo* deposition appeared to be intrinsic, as documented by histological cross sections. The chemical composition of the initial mineral phase formed under *in vitro* conditions had a mean molar Ca/P ratio = 1.255, standard deviation (S.D.) = 0.057, (n = 6). The composition of mineral formed under *in vivo* conditions had a low initial Ca/P ratio. From 4 days to three weeks the average *in vivo* ratio was 1.239, S.D. = 0.203 (n = 38). This ratio increased to 1.707, S.D. = 0.038 (n = 6) following seven weeks of implantation. The latter ratio is similar to that of biomineral formed in human bioprostheses: Ca/P = 1.645, S.D. = 0.021 (n = 6). The deproteinated biomineral showed a population of microparticles in the range of 50-200 nm with a microcrystalline apatitic X-ray diffraction pattern. The biomineral contained a significant amount of carbonate, and the solubility was markedly higher than the solubility of hydroxyapatite. Combined information from *in vitro*, *in vivo* and bioprosthetic mineralization supports the concept that octacalcium phosphate is a precursor that transforms into bioapatite and is implicated in the calcification of bioprosthetic heart valves.

Key words: Bioapatite, bovine pericardium, cardiovascular biomineralization, hydroxyapatite, octacalcium phosphate, solubility.

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Introduction

Surgical replacement of diseased natural heart valves with bioprosthetic heart valves is a very common and successful procedure in modern cardiology. Since 1960 when the first aortic mitral valve was replaced [12], more than 600,000 patients have received valve substitutes [26]. Glutaraldehyde-pretreated bovine pericardium (BP) is used to fabricate one type of heart valve bioprosthesis. Hilbert et al. [14] provided detailed information of BP to be compared with properties of other tissue derived biomaterials. Prolonged application of BP and other tissue-derived bioprostheses is challenged by degeneration, predominantly (>60%) caused by intrinsic calcification and occasionally accompanied by extrinsic calcification, the latter arising from the ulceration of intrinsic deposits. The sequence of events is well documented by Levy et al. [21] and recent publications [26,27] reporting and reviewing the problem of degeneration of bioprostheses. The process of calcification of tissue-derived biomaterials appears to be very complex. At present there is no general mechanism that takes into account all biomineralization growth factors. Even the nature and identity of calcific deposits (CD) are not well known. The general consensus that CD is hydroxyapatite (HAP) appears to be a gross oversimplication [30]. Nelson proposed a theory for formation of calcified deposits in tissue based on *in vivo* mineralization kinetic data [24] with special emphasis on inhibitors. The task of inhibition of bioprosthetic calcification is very important, and has been attempted with surfactants [4], magnesium chloride [4], 1-hydroxy-ethylidene-1,1-bisphosphonic acid (HEDP) [20] and phosphocitrate [34]. The administration of inhibitors via a local controlled-release system was more successful and advantageous compared to injection therapy [21]. Calcification can be controlled and effectively suppressed, but it may recur if the administration of the inhibitor is discontinued or if the inhibitor is depleted from the controlled-release device. The controlled-release device has good potential but apparently has a distinct time limitation. Therefore, the development of new agents, especially formulations with longer performance times, is desirable. Studies of the

prevention of calcification are more likely to facilitate this development, if the studies include investigations on inhibition of the precursor phase formation and its transformation to the final CD. There is good evidence that octacalcium phosphate (OCP) is a precursor in biomineral formation [2]. The aim of the present work is to provide qualitative information concerning this biomineralization process by analysis of CD formed under different conditions. The proper identification of precursor(s) and the inhibition of their formation and transformation may be major steps to successful prevention of cardiovascular biomineralization.

Materials and Methods

Materials.[1]

All solutions used in the present study were prepared by dissolving reagent grade chemicals in distilled water, followed by filtration of stock solutions through a 0.8 μm Millipore membrane. Bovine pericardium (BP) pretreated with and stored in 0.625 vol % glutaraldehyde - 0.02 M KH_2PO_4 solution, pH = 7.40, was kindly provided by Baxter Corporation, Irvine CA. The surgically explanted bioprosthetic heart valves were preserved by immersion in a formaldehyde medium.

In vitro mineralization.

The mineralization of glutaraldehyde-treated BP *in vitro* was studied by immersing precut discs with surface areas of 6.6 cm^2, fixed on a glass rod frame, in a metastable solution containing calcium chloride (2.3 - 2.5 mM), potassium hydrogen phosphate (1.35 - 1.5 mM) and 0.15 M sodium chloride. The pH of the solution was adjusted to 7.40 by the addition of 0.01 M potassium hydroxide. The mineralization systems were kept in double-walled, thermostated vessels (t=37 ^{0}C) and purged with water-saturated nitrogen gas to prevent carbonate contamination. The solutions also contained 0.1% sodium azide to prevent bacterial growth during prolonged mineralization of BP. During the mineralization, the pH was held constant by pH-stat addition of 0.01 M potassium hydroxide, with a system consisting of a METROHM 632 pH meter, a 614 Impulsomat and a 655 Dosimat (Brinkmann Instruments). The progression of the mineralization was followed by periodic sampling and chemical analyses of the mineralizing solution which was filtered through 0.22 μm Millipore membrane. Calcium and phosphate concentrations were determined by atomic absorption (AA) and molybdate vanadate spectrophotometric [9] methods, respectively. BP discs were retrieved for chemical analysis of the calcium phosphate deposition and for microscopic examination.

[1]Certain commercial materials and equipment are identified in this paper to specify the experimental procedure. In no instance does such identification imply recommendation or endorsement by the National Institute of Standards and Technology or the ADA Health Foundation or that the material or equipment identified is necessarily the best available for the purpose.

In vivo mineralization.

The mineralization *in vivo* was accomplished by subcutaneous implantation of two 1x1 cm^2 segments of glutaraldehyde-treated BP into the dorsal walls of Sprague-Dowley rats (40 adult, 7-week-old male rats, 200-225 g), which were kept on a normal Purina diet. The progression of biomineralization was assayed at selected times, ranging from one day to 7 weeks. After the sacrifice of the animals by overdose of pentobarbital, segments were retrieved and characterized by chemical and microscopic methods. Levi et al. [19] have shown that the implant calcification is very extensive in young animals (3 weeks opposed to 8 months old). The supplied 7-week-old rats were assumed to be at the proper level of maturity to produce considerable implant mineralization in the present study.

Characterization of the mineralizing media and the equilibrated solutions.

The mineralizing media were analyzed during *in vitro* BP mineralization experiments. The analytical data were used to calculate ionic speciation and ionic activity products (IAPs) of representative calcium phosphate phases, hydroxyapatite (HAP), octacalcium phosphate (OCP), β-tricalcium phosphate (TCP) and dicalcium phosphate dihydrate (DCPD) with a previously described computational procedure [29]. The pIAP for calcium phosphate phases are defined as:

$$pIAP(DCPD) = -\log (Ca^{2+})(HPO_4^{2-}) \quad (1)$$

$$pIAP(OCP) = -\log (Ca^{2+})^4(PO_4^{3-})^3(H^+) \quad (2)$$

$$pIAP(TCP) = -\log (Ca^{2+})^3(PO_4^{3-})^2 \quad (3)$$

$$pIAP(HAP) = -\log (Ca^{2+})^5(PO_4^{3-})^3(OH^-) \quad (4)$$

(The parentheses represent ionic activities). The results define saturation conditions of the mineralizing solution and the thermodynamic driving force - ΔG for formation of the particular calcium phosphate phase: $-\Delta G = RT \ln IAP(X)/K_{sp}(X)$, where X = DCPD, OCP, β-TCP or HAP, and K_{sp} is the thermodynamic solubility product of the calcium phosphate phase. The same calculation procedure was also used to estimate solubilities of deproteinated calcific deposits isolated from bioprosthetic heart valves after equilibration with diluted mineral acid as described elsewhere [30].

Chemical analyses of calcific deposits.

A. Experimental mineralization. The mineral formed on BP segments *in vitro* was dissolved by acid rinse. Individual segments were treated with 2 ml of 1 M HCl, 2 ml concentrated HCl or 2 ml concentrated HNO_3, respectively, as described in Table 1. The biomineral formed *in vivo* was recovered by dissolving the individual implant in 10 ml of 6 M HCl. Aliquots of solute were diluted with water to obtain 2 to 10 ppm Ca and P concentration range. Calcium and phosphate were determined by atomic absorption (AA) and visible spectroscopy [9], respectively.

B. Bioprosthetic heart valve deposit. The calcific deposit was carefully removed from

Table 1. Composition of mineral formed on BP discs. Six discs recovered, processed and analyzed after 16 hours mineralization *in vitro*.

Sample #	μM Ca/g deposited[x]	wt. of deposition (mg) Ca	PO_4	Ca+PO_4	Ca+ PO_4/cm^2	Ca/P molar ratio
D-1*	246	0.492	0.882	1.374	0.2082	1.32
D-2*	112	0.222	0.451	0.673	0.1020	1.17
D-1**	230	0.460	0.891	1.351	0.2047	1.23
D-2**	86	0.173	0.328	0.501	0.0760	1.25
D-1***	83	0.166	0.298	0.464	0.0703	1.32
D-2***	81	0.163	0.301	0.464	0.0703	1.24
Mean	139.7	0.279	0.525	0.804	0.1219	1.255
S.D.	77.2	0.154	0.285	0.438	0.0665	0.057

[x] = The μmoles Ca/g (g = dry weight of implant) and the molar Ca/P ratio of the deposits were calculated after the initial content of Ca and P in segments were subtracted from the measured values after dissolution of the BP implants. The average initial content was 120 μmoles Ca/g and 26 μmoles P/g (n=5).

* = mineral was dissolved in 1 M HCl (3 min "vibromix" shaking agitation); cc 95% of mineral was recovered.

** = mineral was dissolved in concentrated HCl, followed by evaporation and oxidative decomposition of protein residue with H_2O_2; cc 97% of mineral was recovered.

*** = mineral was dissolved in concentrated HNO_3, followed by evaporation and oxidative decomposition of protein residue with H_2O_2; cc 98 % of mineral was recovered.

explanted human bioprostheses and deproteinated by hypochlorite treatment [38]. Two mg samples of CD were dissolved by addition of 0.5 ml of 1 M HCl followed by water dilution to 10 ml. Aliquots of prepared solutes were additionally dilluted for determination of Ca, Na and Mg contents by AA and phosphate content by spectrophotometry [9]. The HPO_4^{2-} contents in the *in vitro* formed products and in native bioprosthetic deposits were determined by a modification of the pyrolytic method of Gee and Dietz [7]. The materials were pyrolyzed in a vacuum (0.1 torr) at 500°C over 24 h, and the fraction of HPO_4^{2-} was determined from the difference in absorbance between hydrolyzed and non-hydrolyzed samples. Carbonate content was determined by microtitration [5]. After deproteination, the inorganic fraction was routinely characterized by x-ray diffraction (XRD) and Fourier transform infrared (FTIR) spectroscopy. The mineral grown on BP segments *in vitro* was characterized by XRD for comparison with cardiovascular deposits. XRD patterns were recorded with a Phillips automated powder diffractometer, with CuK_α radiation operating at 40 kV and 25 mA. The powder patterns were compared with the JCPDS values for OCP and HAP [39].

Microscopic characterization.

The spatial character of *in vitro* vs *in vivo* experimental mineralization was demonstrated on cross sections of paraffin embedded BP segments. Location of mineralization was determined microscopically by examination of histologic sections of BP stained by von Kossa's method [22].

Morphology of mineral formed on BP segments was examined by scanning electron microscopy (SEM) (air dried, Au coated samples) with a JEOL JSM-5200 scanning electron microscope. Deproteinated bioprosthetic heart valve deposits were examined by SEM for comparison with non-deproteinated deposits.

The *in vitro* formed mineral and heart valve calcified deposits were characterized by polarizing microscopy. Measurements of average refraction indices (R.I.) of microcrystalline materials were performed by the immersion method with a Leitz orthoplane research microscope.

Results

In vitro BP mineralization.

Bovine pericardium segments fixed on a glass rod frame were placed in a metastable mineralizing solution supersaturated with respect to DCPD, OCP, TCP and HAP. The typical starting total calcium concentration, TCa, was 2.47 mM, Ca/P solution ratio 1.66. The calculated pIAP (-log IAP) values were 6.57, 44.50, 25.76 and 46.40, compared to pK_{sp} values 6.60 [10], 48.70 [35], 28.91 [11] and 58.55 [23] for corresponding calcium phosphate phases. The mineralization of BP started after an induction period ranging from 2-15 hours, depending on initial degree of supersaturation. Special care had to be taken to avoid spontaneous precipitation of calcium phosphate from the mineralizing media, which were highly supersaturated with three calcium phosphate phases. In the case of spontaneous precipitation, rapid pH-stat KOH uptake resulted and most of the precipitation occurred in

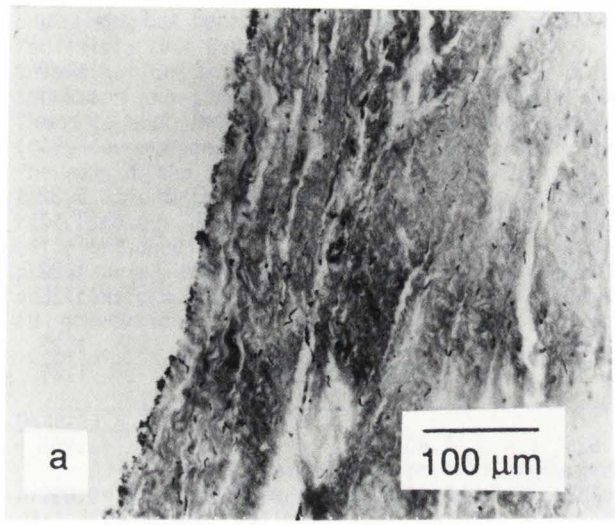

Fig. 1. Histologic section of mineralized BP; a) surface mineralization *in vitro*.

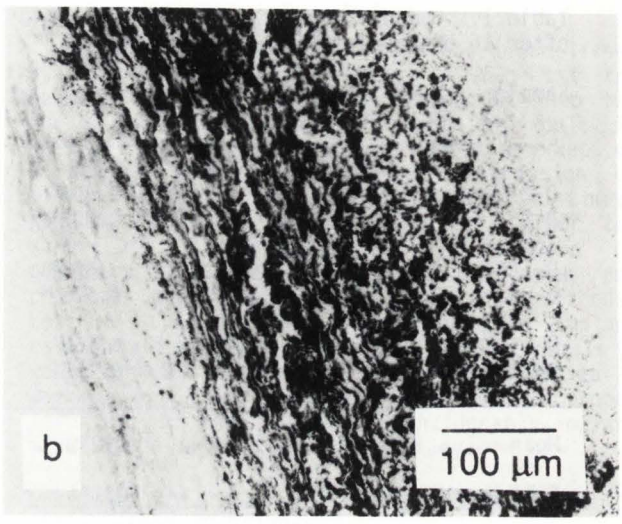

Fig. 1b. Subsurface biomineralization *in vivo*. Paraffin-embedded tissue, van Kossa stain.

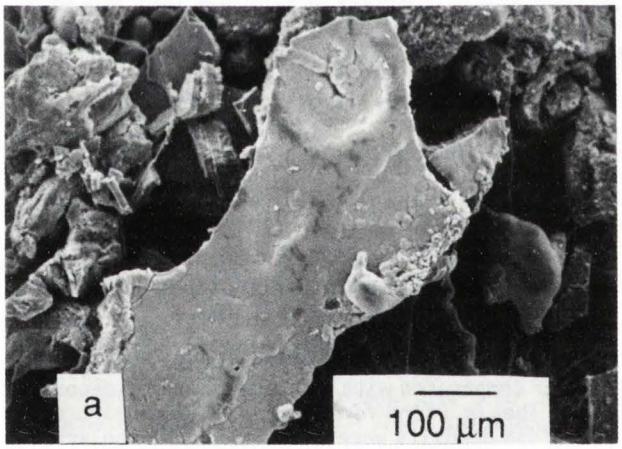

Fig. 2. Scanning electron micrograph of mineral formed *in vitro* on BP surface after 16 hours; a) large plate-like crystals (minor fraction).

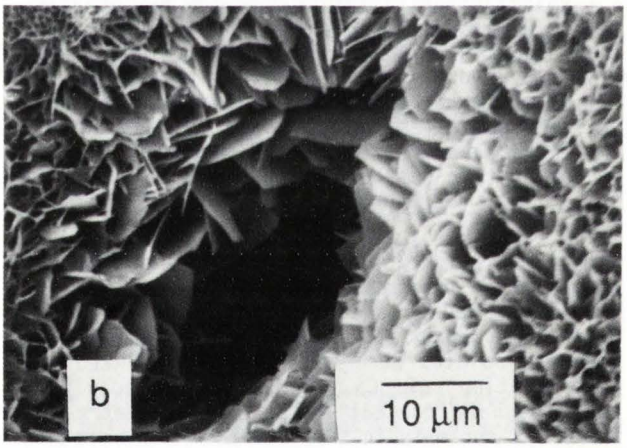

Fig. 2b. Rosette-like OCP crystals (major fraction).

solution. In this case the experiment was terminated, because this study was concerned only with mineralization associated with BP. The controllable mineralization of BP, without spontaneous precipitation of calcium phosphate, was characterized by a slow KOH uptake with a constant pH=7.40 being maintained. The mineralization was followed for up to five days. After a relatively fast start, mineralization gradually slowed and practically stopped after five days. Analyses of the solution during the controllable BP mineralization showed the same concentration changes with or without filtration, indicating that all calcium phosphate deposition was associated with the BP. Fig. 1a, a histologic cross section of van Kossa stained BP, indicates that the calcium phosphate deposition occurred only on the surface of the BP without penetration into the tissue. The chemical compositions of the deposits formed at 16 hours (Table 1) show a remarkable constancy and the Ca/P ratios were slightly lower than those for OCP (1.33). The extent of surface mineralization was highly variable. This variablity could have been a consequence of heterogeneity of the BP surfaces or uneven fluid dynamics during the mineralization process. At a later reaction time (56 hours), the Ca/P ratio increased to 1.42. In another experiment, performed at pH = 7.20, mineralization was slower and practically stopped after 3 days. The product grown in this case had a Ca/P ratio of 1.38 (76 hours). At this time, the pIAP values for DCPD, OCP, TCP and HAP were 6.83, 45.76, 26.77 and 48.17, indicating that the solution was undersaturated in respect to DCPD, moderately supersaturated in respect to OCP and TCP and markedly supersaturated in respect to HAP. Table 1 shows that the described acid rinses successfully removed 95-98% of the mineral content (the remaining 2-5% was determined by additional rinsing of BP with 1 M HCl or dissolving the yellow residue after oxidative evaporation with H_2O_2). The data shown in Table 1 represent important new

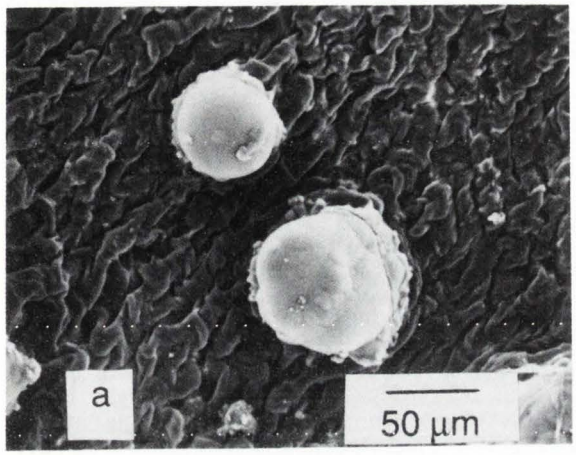

Fig. 3. Scanning electron micrographs of mineral formed *in vitro* on smooth serosal BP surface after 5 days.

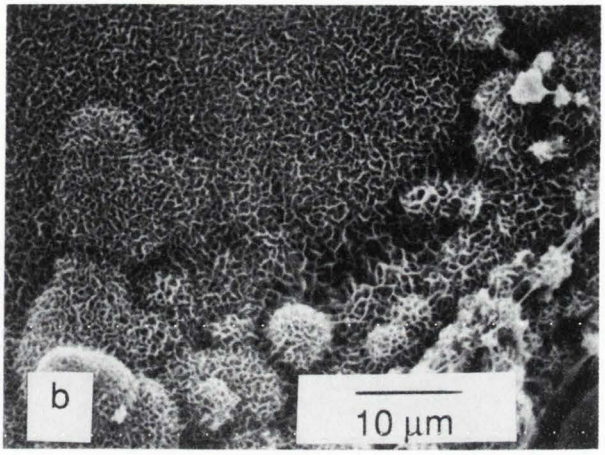

Fig. 3b. Detailed surface morphology of time-transformed product.

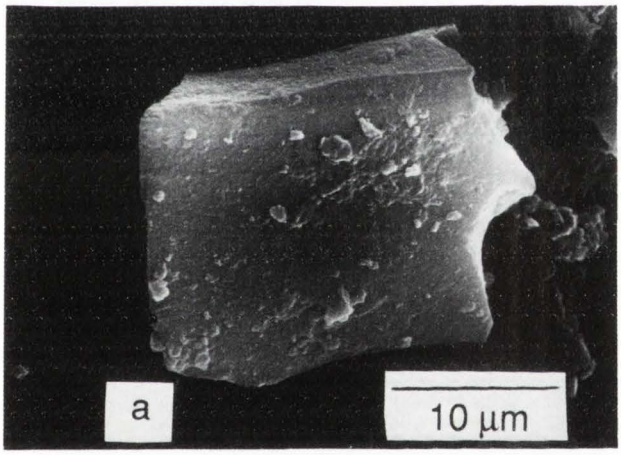

Fig. 4a. Scanning electron micrograph of native bioprosthetic calcific deposit.

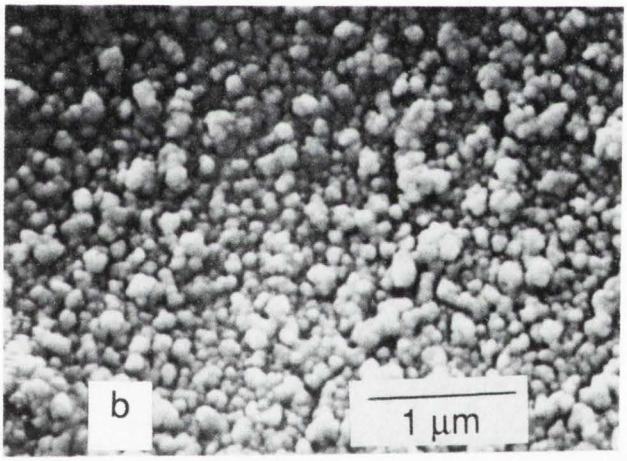

Fig. 4b. Bioprosthetic calcific product following hypochlorite deproteination.

information on the composition of early mineralization product, which may represent the precursor phase in cardiovascular calcification.

The SEM micrographs of *in vitro* grown mineral deposits are shown in Figs. 2 and 3. The mineralization took place on the smooth outflow (serosal) and rough inflow (epipericardial) surface of BP. These two host surfaces are strikingly different [14], but the present observations did not indicate that one surface was preferred to the other as a growth site. The early deposit appeared to be heterogeneous and showed a minor fraction of large plate-like crystals resembling DCPD (Fig. 2a) and a major fraction of smaller rosette-like crystals, presumably OCP (Fig. 2b). The OCP identification was made by comparison with a crystallized calcium phosphate of similar morphology, that has been positively identified as OCP [13]. At a later stage, the morphology of the surface mineral was markedly changed. As shown in Fig. 3a, the smooth serosal surface was covered with "smooth" spherical particles. These particles had the very fine "honey comb" surface structure shown on Fig. 3b, indicating the hydrolytic transformation of early deposits shown in Figs. 2a-b. Similar time-dependent morphological changes of calcium phosphate were observed in the course of pH-controlled mineralization of demineralized dentine [28].

The transient formation of OCP was documented by chemical analyses (Table 1) and XRD (Fig. 5A). The XRD spectrum is complex, but the presence of a weak 100 OCP reflection is in agreement with the finding that OCP once formed and then hydrolyzed into a more basic apatitic substrate OCPH [32]. Polarizing microscopy of *in vitro* grown materials provided the following information: The material is composed of several particle types.

Type 1: Isotropic, gel-like matrix with incipient crystallization, 0.15-15 µm needles showing preferred orientation and having R.I.: N_1 = 1.553 and N_3 = 1.560. Material is soluble in dilute HCl and by this description indicates DCPD. However, complete absence of DCPD XRD pattern rules out DCPD to be type 1 material.

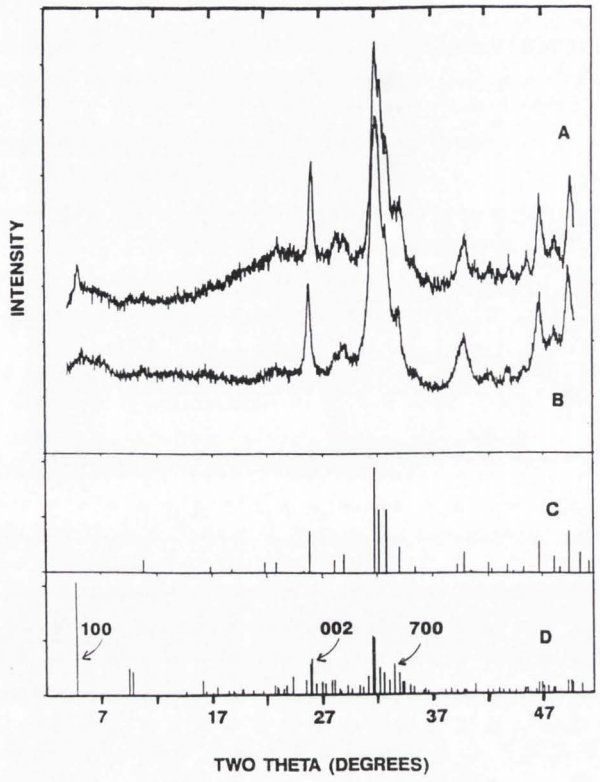

Fig. 5. X-ray diffraction patterns of: (A) *in vitro* formed products; (B) bioprosthetic calcific deposit; (C) HAP; and (D) OCP [39].

Type 2: Grainy particles of amorphous appearance with zonal overgrowth of colloform banding, having R.I. from 1.575 to 1.585, but predominantly 1.585, comparable to the R.I. values for OCP [1]. Apparently the grainy particles formed initially and then converted to a more stable form (the 1.585 veneers) with time, indicating transient formation of OCP.

Important new information was provided by studying the phosphate species present in the *in vitro* formed products and in native bioprosthetic deposits, DC. After pyrolysis, the determined fraction of HPO_4^{2-} in the *in vitro* grown product (Fig. 3b) and the CD (Fig. 4a) was 8% and 12%, respectively. These experimental data provided valid evidence of transient formation of HPO_4^{2-}-containing OCP followed by subsequent hydrolysis, which takes place under given experimental conditions [31] and presumably in maturing CD [2].

In vivo BP biomineralization.

The implants retrieved in the early stages of the experiment (up to four days) were swollen. The surrounding tissue appeared to be inflamed. At a later stage (after 7 days) the surgical wound had healed resulting in 100% survival of the animals prior to sacrifice. Histologic examination of 10 μm cross sections of BP implants retrieved after 7 weeks showed only subsurface calcification (Fig. 1b), in agreement with an earlier report [21]. The character of the progression of the biomineralization is shown in Table 2 and can be summarized as follows: a) biomineralization started within 24 hours and the initial rate was very slow; b) biomineralization was markedly accelerated after 4 days when the healing process had advanced; c) the

Table 2. *In vivo* biomineralization of subcutaneously implanted BP segments.

Residence time(days)	Number of implants	μmoles Ca/g deposited*	S.D.	Ca/P molar ratio	S.D.
1	10	34.8	1.9	0.65	0.05
2	10	66.4	32.0	0.92	0.21
4	10	381.2	124.9	1.20	0.07
7	10	1590.4	373.3	1.26	0.15
14	10	2111.8	468.3	1.26	0.20
21	8	**	-	1.24	0.35
35	8	**	-	1.86	0.27
49	6	**	-	1.71	0.04

* The μmoles Ca/g (g = dry weight of implant) and the molar Ca/P ratio of the deposits were calculated after the initial content of Ca and P in segments were subtracted from the measured values after dissolution of the BP implants. The average initial content was 120 μmoles Ca/g and 26 μmoles P/g (n=5).
** = not determined on dry weight of implant basis.
Vertical bars define times at which Ca/P ratios of formed deposits were not significantly different by Duncan's ranked multiple comparison procedure.

Table 3. Chemical composition and solubility data of deproteinated bioprosthetic heart valve deposits.

No.	G	A	BM	D	Ca	Na	Mg	PO_4	$\%CO_3$	pIAP(OCP)*	pIAP(HAP)*
	(case data)				(molar ratio)				(weight)		
1	F	42y	PV	15y	1.639	.0729	.0329	1.0	5.21	49.59	54.59
2	F	65y	PV	6y	1.647	.1175	.0297	1.0	5.85	49.58	54.63
3	F	21y	BP	2y	1.620	.1817	.0319	1.0	5.49	49.79	54.97
4	F	19y	PV	6y	1.672	.0713	.0381	1.0	6.25	49.20	54.14
5	F	33y	PV	10y	1.634	.1290	.0240	1.0	N.D.	49.90	54.87
6	M	68y	PV	13y	1.609	.0970	.0320	1.0	N.D.	50.01	55.07
				Aver.	1.637	.1115	.0314	1.0	5.70	49.68	54.71
				S.D.	0.022	.0415	.0046	-	.29	.29	.33
				n obs.	6	6	6	6	9	32	32

G = gender (Female or Male); A = age of recepient; BM = type of bioprosthetic material (PV or BP); D = duration of implant.
* = Average pIAP values calculated from analytical data from five or six independent equilibrations of deproteinated deposits with diluted solutions for phosphoric acid under 5% CO_2/95% N_2 stream; 48 hours, 37° C.

density of biomineralization varied greatly within the same time implantation group; d) the Ca/P ratios of formed mineral were found to be time dependent and significantly different ($p < 0.001$, ANOVA); Duncan's ranked multiple comparison procedure [37] showed that the Ca/P ratios were significantly different for implantation times shown in parentheses: (1), (2), (4,7,14,21) and (35,49) days at $p < 0.05$; e) Ca/P ratios were low for the early mineralization stage (from four days to three weeks the Ca/P ratio was approximately 1.25, i.e. close to the OCP ratio); f) the final Ca/P ratio = 1.71, S.D. = 0.04, close to aortic biomineral [30].

Bioprosthetic heart valve deposits.

The morphology of retrieved native calcific deposit is shown in Fig. 4a. After quantitative deproteination by sonication in hypochlorite, the morphology significantly changed from large "crystals" to submicron particles, ranging from 50-200 nm in size (Fig. 4b). XRD patterns of this material shown in Fig. 5b were of microcrystalline apatite and practically identical to aortic calcific deposits [30].

Chemical analyses of six bioprosthetic deposits (Table 3) showed that bioapatites contained significant fractions of sodium, magnesium and carbonate. Comparisons of FTIR spectra of deproteinated bioprosthetic heart valve calcific deposits with aortic and total artificial heart (TAH) deposits were practically identical, indicating that the carbonate/phosphate substitution occuring in B-type carbonate-containing bioapatite [29] had also taken place in the bioprosthetic mineral. Table 3 provides the solubility data for the listed deposits, expressed as pIAP values. These data clearly show that deposits were less soluble than OCP and markedly more soluble than HAP, since the average Δ pIAP(OCP) (i.e., pIAP(OCP)-pK_{sp}(OCP) = + 0.9 and corresponding Δ pIAP(HAP) = - 3.9. As a practical illustration of relative solubilities, computer simulation showed that if equilibrium was attained at pH 5.75, for a constant total phosphate level of 1.9 mM, the total calcium level would be 3.0, 1.27 and 0.2 mM for OCP, #4 PV deposit and HAP, respectively. If the dissolution of the three materials proceeded congruently, i.e., stoichiometrically as given by their chemical compositions, the solubilities under the above conditions would be 0.3348, 0.1355 and 0.0198 g/L, excluding water of hydration. These figures provide solubility ratio for OCP : #4 PV deposit : HAP of 16.91 : 6.82 : 1. In this way, the solubility of the deposit can be properly compared with two standards with known thermodynamic properties [23,35].

Discussion

Calcific degeneration of tissue-derived bioprostheses is a special case of biomineralization. By its complexity, the process goes beyond present concepts of deposit formation mechanisms and of solutions for practical

prevention. The application of present results to the understanding of the mineralization of bioprosthetic heart valves may lead to more rational solutions to severe heart insufficiency problems. The present contribution aims to couple basic information from *in vitro* and *in vivo* biomineralization studies with information gained directly from a physicochemical characterization of actual bioprosthetic heart valve deposits. *In vitro* mineralization of BP at constant pH (Table 1) shows that mineral with a low Ca/P ratio is formed at an early stage, indicative of OCP. This finding was also supported by SEM. The pH-stat controlled crystallization practically stopped after three days in spite of the fact that the solution medium was moderately supersaturated with respect to OCP and highly supersaturated to HAP. The fact that growth did not proceed for the next two days indicates stabilization of the precursor phase(s). The slight increase in the Ca/P ratio at a later reaction time perhaps resulted from slow hydrolytic transformation of OCP to the more basic product OCP-hydrolyzate (OCPH), in accordance with previous findings [31]. Since precursor phase(s) were kinetically favored, the present data do not indicate direct growth of HAP. Koutsoukos et al. [16] have shown that the direct precipitation of HAP on the surfaces of collagen appeared to take place under conditions where the growth medium was supersaturated only with respect to HAP. At higher supersaturation, OCP formation was indicated. These findings are in agreement with the present data despite the differences in the growth surfaces. In the present and another independent study [16], the onset of mineralization was preceded by an induction period whose length depended on the degree of OCP supersaturation. The previously observed adsorption of phosphate on collagen surfaces [15] may well take place on BP surfaces. This was not directly documented in the present study. The average Ca/P ratio of the mineral formed after 16 hours of growth, 1.26, is slightly lower than the ratio of 1.33 for OCP. This may indicate absorption of phosphate prior to surface nucleation of OCP. Another nucleation factor would be the surface of BP, which contains many surface defects capable of interacting with mineral-building ions. Mineralization under *in vitro* conditions proceeds only on the surface of BP, as shown on Fig. 1a. This is crucially different from *in vivo* biomineralization, which is subsurface (Fig. 1b). Consequently, additional experimental work with animals is justified to obtain realistic information about pathologic biomineralization. Mineralization of tissue-derived bioprostheses has already been reported. This phenomenon was discussed as being dependent on biomaterial pretreatment [8] and compressive mechanical deformations [3,33] that may affect transport of mineral-forming species.

Table 2 provides quantitative data on the progression of mineralization with special emphasis on Ca/P composition of the forming mineral to elucidate the nature of the precursor(s). Preimplantation processing results in the loss of the mesothelial cell layer [14] which may act as a protective layer in cardiovascular tissue. This may be a determining factor for rapid biomineralization of BP implants in particular, and calcific degeneration of BP bioprostheses in general. After implantation, a thickening of the implants was observed. The BP implants were swollen, which probably increased permeability and thus facilitating more active ion diffusion. The rates of diffusion of calcium and phosphate ions through BP are not known. It is possible that under these conditions, phosphate diffuses faster than calcium, explaining the very low initial Ca/P ratio (0.65). This does not necessarily mean that the earlier precursor had a similar composition because the formation of monocalcium phosphate (Ca/P = 0.5) cannot occur under physiological conditions. Another possible explanation for the low Ca/P ratio is that preferential absorption of phosphate on collagen was involved [15]. This could provide a favorable site for nucleation.

After the surgical wound healed, the consistency of the Ca/P ratio at 1.25 during three weeks supports the proposed mechanism involving OCP as the precursor in biomineral formation [2,30]. Remarkable agreement exists between the *in vitro* and *in vivo* models, regardless of deposition site and growth environment. Both processes took place in media highly supersaturated with respect to HAP, which did not form because OCP is kinetically favored and particularly in the presence of magnesium ion [25]. An explanation for the absence of pure HAP in cardiovascular biomineral [30] is the presence of natural inhibitors in the serum environment which apparently prevents direct HAP formation [6]. The final 7-week residence product has the ratio 1.71, suggesting that HAP had formed by this time. The product may be apatitic, but it more likely arose from the transformation of the OCP precursor, either *in situ* by solid state [1] or by dissolution/precipitation [31] mechanisms incorporating many different ions [32]. It should be emphasized that biomineral is very different from pure HAP in composition, structure, surface and physicochemical properties. The amount of aqueous phase in the tissue is limited; therefore, the transformation of a precursor probably proceeds *in situ* [2].

The basic considerations on the formation mechanism discussed above appear to be substantiated with specific information on the physicochemical properties of calcific deposits isolated from heart valve bioprostheses. As shown in Table 3, these materials can be represented as sodium, magnesium and carbonate-substituted or incorporated apatite. The thermodynamic solubilities of these materials are probably the most important indication that a biomineral is formed from a more soluble, presumably acidic precursor. The biomineral is significantly more soluble than HAP, ruling out this phase as a valid representative of calcific deposition. Another indirect proof of this hypothesis is the striking similarity in the physicochemical properties of natural biominerals (Table 3) with material prepared by hydrolysis of OCP in a sodium carbonate medium (Ca/P=1.71, Na/Ca=0.06 ratios, 6.4% carbonate content, pIAP(OCP)=49.99, pIAP(HAP)=54.96, and very similar XRD pattern and infrared (i.r.) spectra [32]).

At this point, we suggest that successful suppression of biomineralization may involve: 1) inhibition of the formation of precursors (i.e., inhibition of proliferation of calcified sites [27]), and 2) inhibition of transformation (i.e., inhibition of crystal augmentation and nodule confluence leading to clinical failure [27]). The above two processes may take place at different reaction rates which may be controlled with different inhibitors. It is well known that magnesium effectively inhibits hydrolytic transformation of OCP [36] and may be a valid candidate for controlling the second process. Results of an earlier study support this proposition [18]. Therefore, the development of new inhibitor formulations is a very important goal in the practical application of this information. In consideration of Mg as a practical inhibitor of cardiovascular biomineralization, factors such as Mg homeostasis and toxicity must be critically evaluated, and is a task for future studies.

Conclusions

The present data correlate information obtained from the investigation of mineralization of bovine pericardium segments, implants and bioprosthetic heart valves. The composition of the initial mineral indicates that octacalcium phosphate is a precursor in the genesis of cardiovascular biomineralization. The distinct difference between *in vitro* and *in vivo* processes appears to be the mineralization site, BP surface vs. BP subsurface mineralization. This finding supports the validity of the animal model as being the more realistic. Subsurface mineralization is probably facilitated by increased tissue permeability of swollen implants. The composition and physicochemical characteristics of bioprosthetic calcific deposits support the mechanism of formation via the OCP precursor. The evidence of transient formation of OCP in experimental calcification and during formation of bioprosthetic calcific deposits was corroborated by chemical, polarizing microscopy, structural and thermodynamic solubility data. Solubilities of deproteinated deposits are significantly higher than the solubility of HAP and comparable to hydrolyzed OCP. This also supports the new proposed mechanism of pathologic biomineralization.

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Discussion with Reviewers

R. J. Levy: Spontaneous precipitation is mentioned, and since this occurred, the frequency of this occurrence should be noted since this would pose an important experiment problem for anyone attempting to duplicate these experiments.

Authors: Spontaneous precipitation, which was unfortunately overlooked by some researchers, is one possible drawback of *in vitro* mineralization experiments. In answer to the question on frequency of this occurrence, to say 50% would be an oversimplification, and the question cannot be answered in such a manner. The experimental criteria for proving that only surface *in vitro* mineralization takes place has been described in detail sufficient to permit a careful experimentalist to distinguish the critical difference between the two types of processes.

R. J. Levy: The very low calcium phosphorous ratios noted at 24 and 48 hours are inconsistent with any mineral phase. In fact, the authors should be aware of previous publications by others demonstrating the onset of mineralization in this model system occurring after 48 hours. This should be discussed, especially since the authors feel that their data are different in terms of calcification actually occurring at 24 hours.

Authors: In case the determined blank value for calcium shown in Table 2 was too high (120 μmoles Ca/g), the corrected Ca/P ratio would be most affected in the early stage (1 and 2 days). This situation may be an explanation for the initial low ratios (0.65 and 0.92). Levy et al. [19] provided data for calcium and phosphorous accumulation in subcutaneously implanted glutaraldehyde-preserved porcine aortic valves over a three-week period. The authors concluded that the calcium phosphate ratios were consistent with the predominance of hydroxyapatite. Our calculations based on reported average

tissue calcium and phosphorous (μg/mg) levels [19] show the molar Ca/P ratios of 1.56, 1.31, 1.36 and 1.36 for duration of 3, 7, 14 and 21 days, respectively. This is in good agreement with our present data covering a 4 to 21-day duration period; both sets of data are consistent with the predominance of octacalcium phosphate rather than hydroxyapatite [19]. Only at a later stage, did the ratio increase, the increase being consistent with apatite formation by the proposed mechanism of hydrolytic transformation of an OCP precursor [2].

F. Bilge: The main problem with this paper is its inability to show the relationship between *in vitro* and *in vivo* calcification. The question, "Can *in vitro* calcification be used to model *in vivo* calcification?" is not answered.
Authors: The question is very appropriate, and in some respects it has been answered by stating that *in vivo* data are more realistic because the mineral deposition environment cannot be completely duplicated in the *in vitro* runs. The kinetic factors are very dependent on the actual reaction site and medium; therefore, the present data were not intended to be used to compare the rates of the two calcification processes. However, the relationship between the two processes can illuminate very basic and essential questions on the reaction mechanism and elucidation of precursor(s) in pathologic cardiovascular calcification. Structural, chemical, solubility and microscopic properties of materials formed *in vitro* and in bioprosthetic deposits are very comparable and therefore justify the propositions stated in this paper about the relationship between the two types of experimental calcifications.

F. Bilge: It is mentioned that calcific deposits on TAH are identical to deposits on bioprosthetic heart valves. Can this be interpreted to mean polymers calcify similarly to glutaraldehyde fixed tissue?
Authors: TAH and bioprosthetic deposits were practically identical based on FTIR spectroscopic structural evidence. It would be premature speculation to state that polymers, i.e., polyurethane, calcify similarly to glutaraldehyde fixed tissue. However, the precursor(s) apparently underwent a similar sequence of events to form bioapatite, which incorporated carbonate and metal ions, in accordance with the proposed mechanism of hydrolytic transformation of an octacalcium phosphate precursor [2, 32], additionally supported by the presented *in vitro* and *in vivo* data.

F.M. Lupinetti: The authors have shown that the chemical composition of early in vitro calcific deposits are very similar to those of early in vivo calcific deposits. However, there is a marked difference in the location of the deposits surface versus subsurface. How does the presence of blood vessels surrounding the subcutaneous implant affect the location of the calcium deposition?
Authors: We have no evidence that the presence of blood vessels surrounding the subcutaneous implant affects the location of the calcium deposition. The demonstrated subsurface mineralization *in vivo* is more likely attributable to necrotic tissue containing dead cells whose surfaces and membrane components can serve as suitable nucleating sites for the deposition of diffusing inorganic components from extracellular fluid. This situation is comparable to crystallization in gels, which allow slow diffusion of ions and the formation of calcium phosphates, whose composition depends on the physicochemical parameters of the gel medium [17]. Once formed, the crystals can transform, proliferate, and even penetrate through the tissue. This is the most common case in advanced calcification of tissue-derived bioprosthetic devices.