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Hydrogen bonds between protein side chains and phosphates and their role in biological calcification

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Poly(L-histidine)-phosphate ($H_2PO_4^-$, HPO_4^{2-}) and poly(L-glutamate)-phosphate systems (residue/phosphate, 1:1) in the presence of Ca^{2+} are studied by infrared spectroscopy. In the poly(L-histidine)-phosphate systems $N \ldots HOP \rightleftharpoons NH^+ \ldots O^- P$ hydrogen bonds are formed where most phosphate protons are found at the histidine ring. With an increase in the degree of hydration the proportion of the proton limiting structure $NH^+ \ldots O^- P$ increases. In the poly(L-glutamate)-dihydrogen phosphate system most phosphate protons are found at the carboxylate groups. Different behavior is observed for poly(L-glutamate)-hydrogen phosphate mixtures, where the residence time of the phosphate proton at the hydrogen acceptor carboxylate group is very short. This residence time increases, however, with increasing humidity. All these results support the triphasic theory of biological calcification involving a tripartite protein-calcium-phosphate complex where these hydrogen bonds can be present. The behavior of these hydrogen bonds can also explain the formation of a nidus of calcium phosphate salts in calcium oxalate-containing urinary calculi.

1. Introduction

Hydrogen bonds between polar groups in protein side chains and phosphates are of great interest for studying the function of many proteins [1,2]. The proton transfer equilibria of these hydrogen bonds have been bound to be dependent on the cation fields of ions present in the environment. Thus, Zundel's group [3–5] has studied poly(L-glutamic acid)- and poly(L-histidine)-phosphate hydrogen bonds in the presence of Li^+, Na^+ and K^+ and found that the stronger the cation field, the greater is the shifting of protons from phosphate to the organic acceptors.

In this work we have studied by means of infrared spectroscopy the hydrogen bonds in the following systems: poly(L-glutamate)-phosphate ($H_2PO_4^-$, HPO_4^{2-}) and poly(L-histidine)-phosphate, in the presence of Ca^{2+} (residue/phosphate, 1:1). This is of significant interest because one theory of biological calcification [6–8] proposes a tripartite protein-phosphate-calcium complex as the nucleation center, where these hydrogen bonds may be present. On the other hand, these hydrogen bonds may be involved in the nucleation of calcium phosphate salts present in urinary calculi. One of the problems in studying the nucleation of these stone-forming salts in the urinary tract has been the determination of whether nucleation is triggered off by some nucleating organic substances present in urine. In this connection, elucidation of the nucleation mechanisms of calcium phosphate salts in vivo is hindered by the complexity of the molecular structures and diversity of functional groups usually found in such organic compounds. It seems more appropriate to use, as an initial step, molecules with a restricted number of functional groups. Glutamic and

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aspartic acids and histidine are abundant amino acids in the mucoprotein-like materials which constitute the organic fraction of these calculi [9,10]. Carboxylate and histidine ring residues may influence the nucleation of calcium phosphate salts whenever they are hydrogen-bonded to phosphate ions whereby protons may be shifted from phosphate to these organic acceptor groups.

2. Experimental

2.1. Materials

Poly(L-histidine), $(\mathrm{His})_n$, and sodium poly(L-glutamate), NaPG, were purchased from Sigma and had molecular weights of 5000–15 000 and 2000–15 000, respectively. Aqueous solutions of calcium poly(L-glutamate), CaPG, were prepared by dialyzing NaPG solutions (1% polymer) against 2 M $CaCl_2$. The resultant solution was then dialyzed against water.

$CaCl_2$, Na_2HPO_4 and $Ca(H_2PO_4)_2$ were of analytical grade and supplied by Merck and Sigma.

2.2. Methods

The samples for infrared spectroscopy were prepared as follows. The $(\mathrm{His})_n$-$Ca(H_2PO_4)_2$ films were obtained by mixing 0.03 N aqueous solutions of $(\mathrm{His})_n$ containing acetic acid and the respective phosphate (mole ratio, 1 residue : 1 phosphate). The precipitation of the films were then performed upon vacuum drying over P_2O_5. Acetic acid was added to the $(\mathrm{His})_n$ solution because this polymer is soluble only when protons are present. After repeating drying and rehydration of the film the acid is quantitatively desorbed. A similar method was used to prepare the CaPG-$Ca(H_2PO_4)_2$ films. The samples of the $(\mathrm{His})_n$-$CaHPO_4$ and CaPG-$CaHPO_4$ systems were prepared by mixing equal volumes of two solutions: one containing the polymer (0.03 N) and 0.03 M $CaCl_2$, and the other a solution of 0.03 M Na_2HPO_4. Polycrystalline films were obtained after vacuum drying. To remove the NaCl the films were carefully washed with distilled water.

Infrared spectra were recorded on a Perkin-Elmer 599B infrared spectrophotometer and were acquired using an on-line computer at 1.0 cm^{-1} intervals. Measurements for the film samples were performed using special infrared cells with silicon windows [11]. Particular degrees of hydration of the samples were achieved via definite vapor pressures over saturated salt solutions. The relative intensities of the $\nu(C{=}O)$ and $\nu_s(COO^-)$ bands in the CaPG-phosphate systems were used to determine the percentage proton transfer. As these bands and those from the amide I and $\delta(CH_2)$ modes overlap, a fitting program for resolution of the spectral profiles was used [12]. The same procedure was followed to determine the degree of proton transfer in the $(\mathrm{His})_n$-phosphate systems, since the relative intensities of the $(\mathrm{His})_n$ bands at 1432 and 1400 cm^{-1} depend on the degree of protonation of the polymer [13].

Turbidimetric measurements were carried out by means of a Cary 17D ultraviolet-visible spectrophotometer. Equal volumes of solutions of 70 mM $CaCl_2$ and 70 mM Na_2HPO_4 at pH 5.7 were mixed in a vessel. The mixed solution was immediately transferred to a spectrophotometric cuvette of 1 cm path length. The first measurement was taken 1 min after mixing and thereafter readings of the transmittance were taken at 1 min intervals at 550 nm. The desired amounts of $(\mathrm{His})_n$ were premixed with the phosphate solutions to give a final concentration of 0.02 N. The precipitate obtained in these experiments was a mixture of dicalcium phosphate dihydrate (brushite) and calcium-deficient hydroxyapatite as shown by the infrared spectra.

3. Results and discussion

3.1. CaPG-phosphate systems

In the dried sample of the CaPG-$Ca(H_2PO_4)_2$ system (fig. 1) the $2\delta OH$ vibration near 2400 cm^{-1} of the OH groups in the hydrogen phosphate is observed as a marked band, which demonstrates that the hydrogen phosphate ions are cross-linked via hydrogen bonds [3]. An intense $\nu(C{=}O)$ band in the 1750–1700 cm^{-1} region is observed, and the relative intensity of the

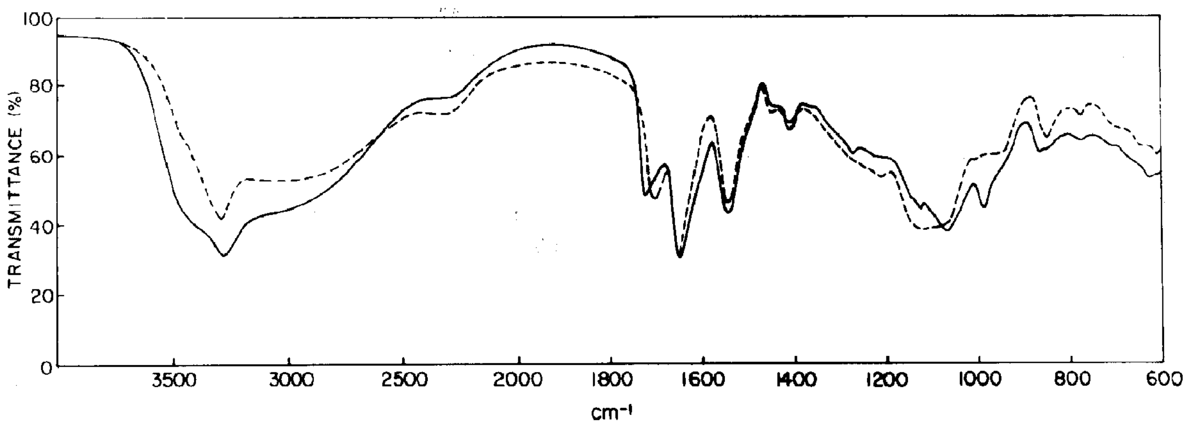

Fig. 1. Infrared spectra of the CaPG + $Ca(H_2PO_4)_2$ system thoroughly dried (— — —) and at 84% relative humidity (———).

$\nu_s(COO^-)$ band at 1400 cm^{-1} has decreased with respect to that of the pure homopolymer salt. This result shows that some of the protons have transferred from dihydrogen phosphate to carboxylate in the (I) $COO^- \ldots HOP \rightleftharpoons COOH \ldots O^-P$ (II) hydrogen bonds. This proton transfer is strongly confirmed by the presence of vibrations of $-PO_3^{2-}$ groups. $\nu_{as}(PO_3^{2-})$ is split due to removal of degeneracy, caused by the asymmetric environment of this group. One component of this doublet is found at 1125 cm^{-1} and the other at 1075 cm^{-1}. The first component may also be due to the $\nu_{as}(PO_2^-)$ vibration of the $H_2PO_4^-$ involved in the above-mentioned proton transfer equilibrium. The symmetric vibration $\nu_s(PO_3^{2-})$ appears at a relatively low frequency, 950 cm^{-1}, demonstrating that HPO_4^{2-} strongly interacts with its environment. The intense $\nu(C{=}O)$ band at 1708 cm^{-1} suggests that the proton transfer equilibrium $COO^- \ldots HOP \rightleftharpoons COOH \ldots O^-P$ is shifted to the right. The position of the equilibrium can be determined as described above and it can be estimated that about 80% of the protons in $H_2PO_4^-$ have transferred to carboxylate groups. Hence, the relative fraction of the proton limiting structure (II) in the proton transfer equilibrium is nearly 0.80. Burget and Zundel (submitted) have recently studied the poly(L-glutamic acid)-Na_2HPO_4 system and found a value of 0.55 for the relative fraction of the proton limiting structure (II). Instead of this system we have prepared a film of NaPG + NaH_2PO_4 (fig. 2) and obtained a value (0.50) similar to that of those authors. The different behavior of the Ca^{2+} system compared with the Na^+ system (fig. 2) can be explained as follows. The OH groups of $H_2PO_4^-$ involved in the hydrogen bonds become increasingly polarized with stronger electrostatic fields of the cations, and thus the loosening of protons in $H_2PO_4^-$ is increased.

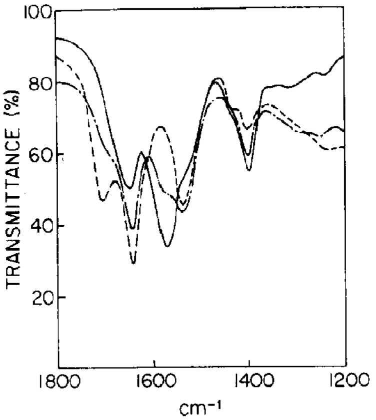

Fig. 2. Infrared spectra of the systems: NaPG (———), NaPG + NaH_2PO_4 (·-·-·), and CaPG + $Ca(H_2PO_4)_2$ (— — —), thoroughly dried.

In the hydrated system (fig. 1) a $\nu(C{=}O)$ band at 1730 cm^{-1} arises. This result demonstrates that the $C{=}O \ldots HOC$ or $C{=}O \ldots HOP$ bonds in which the protons are localized are broken due to hydration water molecules. Instead of these bonds C=O...HOH bonds are now present which are weaker and therefore ν(C=O) is shifted toward higher frequencies. The $\nu_s(PO_3^{2-})$ band is shifted from 950 to 986 cm^{-1}, probably due to an increasing degree of hydration of HPO_4^{2-}. Furthermore, the band at 1125 cm^{-1}, the one component of the doublet caused by removal of degeneracy of $\nu_{as}(PO_3^{2-})$ due to a strongly asymmetrical environment, decreases.

Concerning the secondary structure of the homopolymer, this is present mainly as α-helix. Actually, the amide A band appears at 3290 cm^{-1} as a relatively sharp band, indicating an ordered structure. This result and the position of the amide I band at 1652 cm^{-1} prove that an α-helical polypeptide structure is present. This remains with increasing humidity. This conformational structure in the polymer is consistent with the high-percentage proton transfer from phosphate to carboxylate groups described above, since it is well known that the polymer helix is formed in the presence of undissociated carboxyl groups.

In the CaPG-CaHPO$_4$ system (fig. 3) no noticeable ν(C=O) band is found about 1710 cm^{-1}. This result indicates that no protons of the phosphate OH groups are removed. The COO$^-$... HOP $\rightleftharpoons$ COOH...O$^-$P hydrogen bonds are asymmetrical with the proton present at the phosphate ion. This is also confirmed by the fact that the only visible phosphate bands correspond to HPO_4^{2-}. Thus, the $\nu_{as}(PO_3^{2-})$ vibration is found as an intense band at 1075 cm^{-1} and $\nu_s(PO_3^{2-})$ appears at 998 cm^{-1}. At 84% relative humidity a ν(C=O) shoulder is observed, indicating that proton transfer from phosphate to the carboxylate group has occurred to some extent. This is supported by the appearance of $\nu(PO_4^{3-})$ bands at 1025, 975 and 960 cm^{-1} [14]. We assign a value of 0.22 to COOH...O$^-$P for the fraction of proton limiting structure as deduced from the relative intensity of the ν_s(COO$^-$) band.

3.2. *(His)$_n$-phosphate systems*

Fig. 4 shows the infrared spectra of the (His)$_n$-Ca(H$_2$PO$_4$)$_2$ system, thoroughly dried and at 84% relative humidity. Broad bands are observed near 2800, 2400 and 1235 cm^{-1} which are caused by the ν(OH), 2δ(OH) and δ(OH) vibrations, respectively. These bands prove that the phosphate ions are cross-linked through hydrogen bonds. Moreover, the intense continuum that appears at about 1900 cm^{-1} is consistent with the formation of N...HOP $\rightleftharpoons$ NH$^+$...O$^-$P hydrogen bonds [3].

The position of the proton transfer equilibrium can be determined by means of bands of the histidine ring as described above. The quantitative estimation of the 1400 cm^{-1} band indicates that about 60% of the phosphate protons are present at the histidine ring. In the presence of Na$^+$ the fraction of the proton limiting structure NH$^+$...

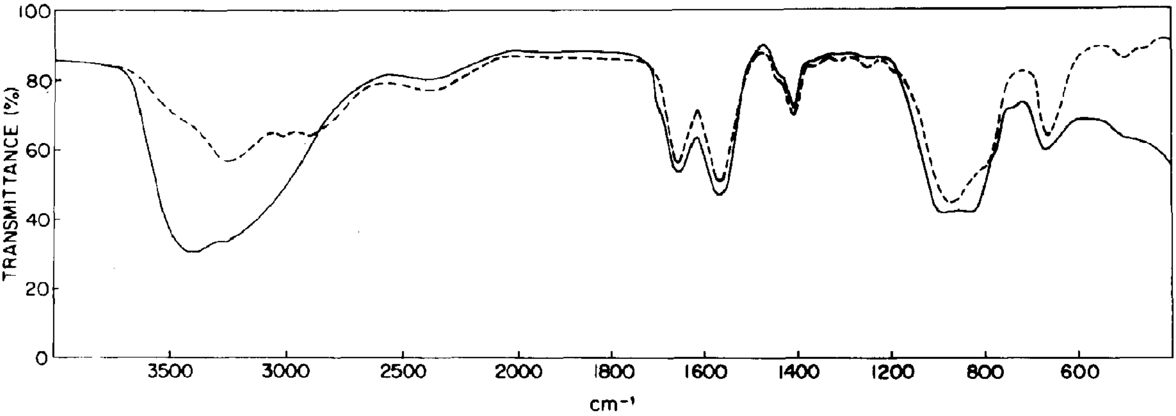

Fig. 3. Infrared spectra of the CaPG+CaHPO$_4$ system thoroughly dried (— — —) and at 84% relative humidity (———).

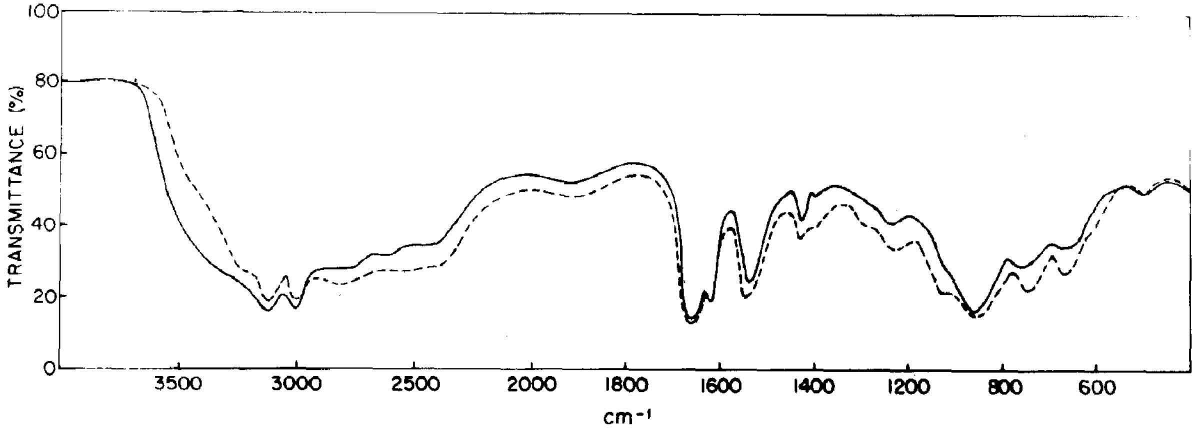

Fig. 4. Infrared spectra of the $(His)_n + Ca(H_2PO_4)_2$ system thoroughly dried (— — —) and at 84% relative humidity (———).

$O^{-}P$ was found to be 0.41 [3]. This difference in behavior due to the presence of Ca^{2+} is caused by the fact that this ion strongly polarizes the OH bonds of $H_2PO_4^-$ due to its strong cation field, as described above for the poly(L-glutamate)-phosphate systems. That the proton limiting structure $NH^+ \ldots O^-P$ is predominant is also supported by some phosphate bands. Thus, the $\nu_{as}(PO_3^{2-})$ shows a doublet structure with maxima at about 1055 and 1130 cm^{-1}, which demonstrates that the degeneracy of $\nu_{as}(PO_3^{2-})$ is removed by an asymmetrical environment of the $-PO_3^{2-}$ groups. At 84% relative humidity these groups are in a largely symmetrical environment, the $\nu_{as}(PO_3^{2-})$ degeneracy is then recovered and the resulting $\nu_{as}(PO_3^{2-})$ band at 1060 cm^{-1} is predominant with respect to a shoulder near 1122 cm^{-1}. This shoulder is due to the remaining $H_2PO_4^-$ in the proton limiting structure N ... HOP. It should also be noted that the proton transfer equilibrium is shifted to the right upon hydration, as deduced from the relative intensity of the 1400 cm^{-1} band. A quantitative estimation shows that about 85% of the protons have transferred from dihydrogen phosphate to histidine residue.

Finally, the spectra of the $(His)_n$-$CaHPO_4$ system (fig. 5) show the band of protonated $(His)_n$ at 1625 cm^{-1} and phosphate bands at 1075 and 1030

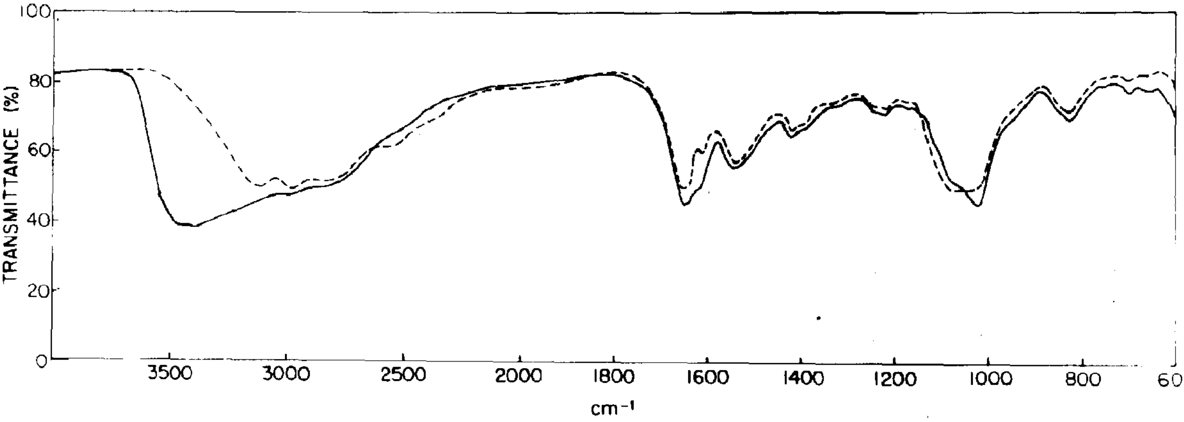

Fig. 5. Infrared spectra of the $(His)_n + CaHPO_4$ system thoroughly dried (— — —) and at 84% relative humidity (———).

cm^{-1} corresponding to $\nu_{as}(PO_3^{2-})$ and $\nu_{as}(PO_4^{3-})$, respectively. The latter increases in intensity with increasing hydration, and the fraction of the proton-boundary structure $NH^+ \ldots O^-P$ was found to be nearly 0.3.

In summary, we can state that in spite of the pK_a values of the protein side chain carboxylate group (pK_a 4.4) [15] and histidine ring (pK_a 6.0) [13] these groups remove protons from $H_2PO_4^-$ (pK_a 7.21) and HPO_4^{2-} (pK_a 12.4) [16] upon hydrogen bond formation. This may be due to the interactions of these hydrogen bonds with the environment [3] and obviously may facilitate the crystallization of calcium phosphate salts, as shown in fig. 6. It is well known that water breaks hydrogen bonds and consequently the percentage of phosphate ions hydrogen-bonded to the polymer chains may be low under these experimental conditions. However, the nucleation sites formed in these scarce hydrogen bonds cause observable accelerating effects on the precipitation of calcium phosphates. Fig. 6 includes the curves of transmittance vs. time of crystallization. Curve a corresponds to the crystallization in the absence of $(His)_n$. In another solution of the same ionic concentrations and pH as for curve a but containing $(His)_n$, the precipitation is accelerated. We also observed an accelerating effect of sodium poly(L-glutamate) on the precipitation of calcium phosphate (brushite) [17].

A question arises as to the connections of the aforementioned results with biological calcification and the structure of the nucleus in some urinary calculi.

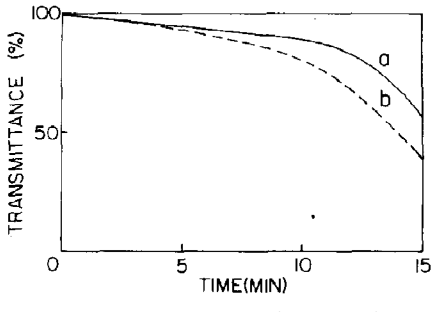

Fig. 6. Transmittance curves of calcium phosphate precipitation in the absence (———) and presence (— — —) of $(His)_n$.

3.3. *Influence of these hydrogen bonds on biological systems*

Calcium-deficient hydroxyapatite is the primary constituent of bone and teeth and of most pathological mineralized deposits, and its formula is $Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x} \cdot xH_2O$; $0 \leqslant x \leqslant 2$. Recent studies [18] have reported the presence of HPO_4^{2-} groups in bone, these ions being in a brushite configuration. In any case, the concentrations of $H_2PO_4^-$ and HPO_4^{2-} must be of the same order of magnitude at physiological pH because the pK_a of $H_2PO_4^-$ is 7.21. Consequently, the formation of hydroxyapatite or brushite-like calcium phosphate requires the removal of phosphate protons. For example, the binding of Ca^{2+} to these phosphate ions so that hydroxyapatite is formed must involve a release of H^+ described by the following equation:

$$\begin{aligned}(10-x)Ca^{2+} + 3H_2PO_4^- + 3HPO_4^{2-} + 2H_2O \\ = Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x} \cdot xH_2O \\ + (11-2x)H^+\end{aligned}$$

Thus, to make the reaction go from left to right the hydrogen ions must be removed. If the rate of crystallization is very slow, the diffusion of hydroxyl ions towards the crystal surface and of hydrogen ions away from it, especially in the presence of buffers, such as $NaHCO_3$, may suffice. When bone or teeth mineral is rapidly being laid down, however, diffusion must be supplemented by other processes, as suggested by some authors [19]. In this connection our spectroscopic results support the contention that the scavengers of protons can be some proteins rich in glutamic, aspartic and histidine residues. Our results also support the theory of calcification that proposes as initiator a tripartite protein-phosphate-calcium complex produced by ion association [6–8]. This theory also assumes the removal of a phosphate proton which might then become attached to the protein in the complex. Therefore, this tripartite complex is the crystal nucleation site which lowers the activation energy barrier or catalyzes the initial reaction between Ca^{2+} and phosphate ions of the extracellular fluid. The results presented here demonstrate that these effects on calcium phosphate crystal nucleation can be caused by the $COO^- \ldots HOP \rightleftharpoons COOH \ldots O^-P$ and $N \ldots HOP \rightleftharpoons NH^+ \ldots O^-P$ hydrogen bonds.

The behavior of these hydrogen bonds can also explain some structural aspects of the initial mineralization in some urinary calculi. The study in our laboratory of a large number of calcium oxalate stones [20] revealed that these contained a nidus or growth point where the main component was found to be apatitic calcium phosphate. On the other hand, brushite ($CaHPO_4 \cdot 2H_2O$) has been found to precipitate at pH values as high as 7 with subsequent rapid hydrolysis to apatitic calcium phosphate [21]. Moreover, brushite has been considered to be the first precipitate that appears in supersaturated urine [22] and to be the precursor of apatitic calcium phosphate in urinary calculi. In any case, there must be some organic components which make calcium phosphate precipitate earlier than calcium oxalate does, this being less soluble, however [23,24]. It is likely that the organic components which could accelerate the nucleation of calcium phosphate in urinary calculi are some proteins present in these stones. In fact, the amino acid analyses [9,10] of the organic fractions from urinary calculi revealed that glutamic and aspartic acid residues were predominant. As the carboxyl groups in the side chains of these residues are dissociated at physiological pH, these predominant groups can remove protons from phosphate ions, as described above, and consequently cause calcium phosphate to appear firstly as nidus in oxalate calculi. Once the nidus or growth point is formed, calcium oxalate precipitates on it because of heterogeneous nucleation.

Acknowledgements

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