

Synthetic Amorphous Calcium Phosphate and Its Relation to Bone Mineral Structure

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Bone, a mineralized connective tissue, not only acts as a supportive framework for the body but enters into metabolic interrelationship with it. About 35% of the dry weight of mature bone is organic in nature, mostly collagen, while the remainder is a complex inorganic calcium phosphate system. As early as 1926¹ it was known that bone mineral gave a poorly resolved X-ray diffraction pattern resembling that of the mineral hydroxylapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. A chemical analysis² of bone mineral shows it to be a calcium phosphate with about the Ca:P ratio of hydroxylapatite and containing substantial amounts of CO_3^{2-} (4–6%), citrate (0.9%), Mg^{2+} (0.5%), and Na^+ (0.7%) and trace amount of Cl^- , F^- , K^+ , Sr^{2+} , and other metal ions. Thus, the study of bone mineral has been closely tied to the investigation of hydroxylapatite and related calcium phosphates.

Bone mineral consists of finely divided particles which have a large specific surface (100–200 m^2/g) and a high surface reactivity. A recent review³ of the surface chemistry of bone mineral and related compounds deals with the measurement of specific surface and the bonding of certain molecules to the surface of these materials. The high specific surface of bone mineral provides an efficient interface for chemical interchange with body fluids and for bonding to the organic phases of bone.³

The extremely small size of bone crystals (200–300 Å) precludes the usual single-crystal atomic structure analysis by diffraction techniques. Thus, many workers have studied well-crystallized, prototype apatites to shed light on the nature of bone apatite. These structural investigations on well-crystallized apatites have been summarized in recent review articles.^{4,5} Investigations of the substitution of F^- for OH^- in mineral hydroxylapatite and the resulting increase in chemical stability have provided, in part, an explanation for the resistance resulting from fluoride in-

gestion to certain degenerative bone and tooth diseases.^{6–9} In addition, convincing evidence has indicated that CO_3^{2-} can substitute in well-crystallized apatites^{10,11} although the substitution results in changes in the lattice parameters and structural distortion.¹⁰ Atomic position parameters have yet to be determined by X-ray or neutron diffraction studies on well-crystallized carbonate-containing apatite. It has been assumed by analogy that the CO_3^{2-} found in bone apatite is at least partially substituted in the crystal interior; however, a substantial fraction of the CO_3^{2-} apparently remains on the mineral surface, easily available for exchange reactions.¹²

Finely divided, synthetic hydroxylapatite can be prepared which has a Ca:P molar ratio lower than the stoichiometric 10:6. It has been suggested that these precipitated hydroxylapatites are Ca deficient and that the electronic charge balance is achieved by some combination of hydrogen bonds between oxygens of adjacent PO_4^{3-} groups and missing OH^- ions.¹³ Calcium-deficient apatites form pyrophosphate, proportional to the extent of deficiency, on heating to 300–400°,¹³ while stoichiometric hydroxylapatite does not. In addition, these deficient hydroxylapatites are more reactive chemically than stoichiometric hydroxylapatites.¹⁴ Parallel experiments have suggested that bone apatite is about 10% Ca deficient with the accompanying increase in reactivity related to this condition.¹³ The presence of other ions, such as Na^+ or CO_3^{2-} , complicates the charge balancing but, presumably, one still has a Ca-deficient system.

Observations on the precipitation of hydroxylapatite from highly supersaturated solutions have shown that an amorphous calcium phosphate precursor phase always appears.¹⁵ Unless stabilized in some

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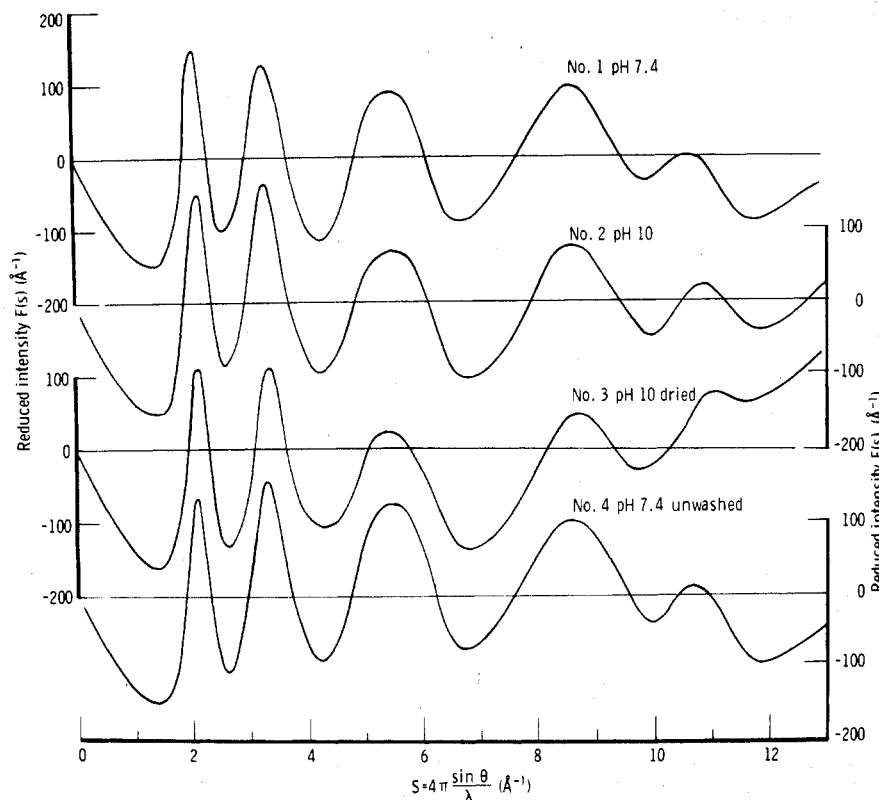


Figure 3. Reduced intensity, $F(s)$, for ACP samples.²⁶

Mg:Ca ratio the induction period is increased, but, once HA nucleation begins, the rate of this first-order transformation reaction proceeds with the same rate constant as in the absence of Mg. Thus, the presence of Mg lengthens the induction period but does not affect the kinetics of the conversion. While other ions such as CO_3^{2-} and certain macromolecules have also been shown to delay or completely prevent the ACP conversion,²³ only the Mg mechanism has been studied in any detail.

X-Ray Diffraction Study of ACP

The possibility existed that synthetic ACP was, in fact, HA of such small crystal size that its X-ray diffraction pattern was broadened so as to appear amorphous in character. Calculated diffraction patterns assuming that ACP consisted of small groups of HA unit cells, or even a single HA unit cell, did not match the observed ACP diffraction data.²⁴ The likelihood, then, that ACP was structurally distinct from HA led to a study of this material by the X-ray radial distribution method²⁵ which is used to obtain information about short-range atomic order in poorly crystallized and amorphous materials.

There were a number of questions about the nature of ACP which needed clarification. First, was there a reproducible structural unit, independent of a wide range of preparation conditions, as suggested by chemical data? What was the nature of the atomic arrangement in ACP and was there any relationship to the structure of HA? How does this ACP structure

compare with other so-called amorphous solids? Finally, what is the structural role of the 10 to 20 wt % of water found in ACP?

X-Ray diffraction data on several different ACP preparations are shown in Figure 3.²⁶ The reduced intensity, $F(s)$, is obtained from the familiar diffraction pattern by subtracting Compton scattering, structure-independent scattering, and correcting for the decrease in the scattering power of atoms with increasing angle.²⁵ Use of the scattering variable $s = 4\pi \sin \theta / \lambda$ makes the shape of the pattern independent of the X-ray wavelength. Thus, $F(s)$ contains diffraction information related only to the three-dimensional atomic arrangement, with other scattering removed. The reduced intensity functions of the four ACP samples, differing in composition and water content, are seen in Figure 3 to be essentially identical. Thus, all four samples contain the same basic arrangement of Ca^{2+} and PO_4^{3-} ions regardless of the Ca:P ratio. The adsorbed PO_4^{3-} and occluded Ca^{2+} which lower or raise the Ca:P ratio, respectively,²⁰ are present randomly and do not affect the $F(s)$ function. Furthermore, water is not an essential part of the ACP structure since its removal by vacuum drying did not change $F(s)$. A further analysis of the $F(s)$ data by calculation of the reduced radial distribution function $G(r)$ was carried out for a more detailed understanding of the structure of ACP.²⁶

In general, a radial distribution function (RDF) is a plot of atomic density vs. atomic separation, which affords information about atomic structure in a material. Positive values of the reduced radial distribution function, $G(r)$, correspond to densities of atoms greater than the average and negative values to densities lower than the average. Thus, peaks in the re-

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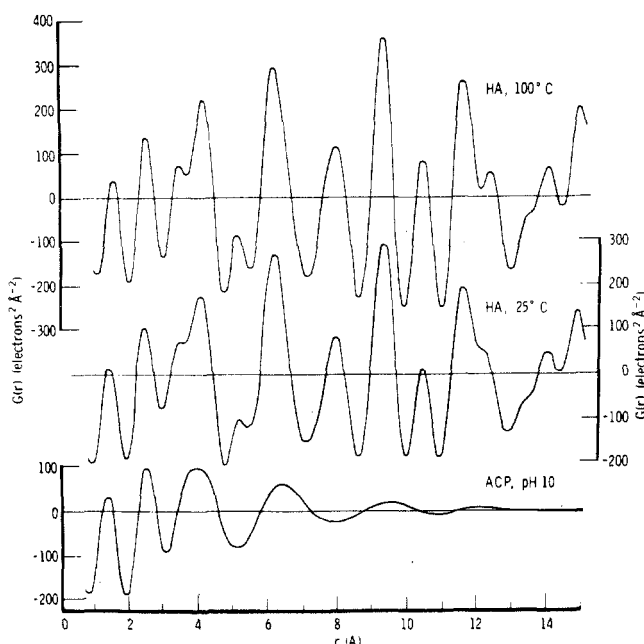


Figure 4. Reduced radial distribution functions, $G(r)$, for HA and ACP samples.²⁶

duced radial distribution function correspond to pairs of atoms with a separation equal to the r values at which the peak occurs.

Figure 4²⁶ shows experimental $G(r)$ curves for two HA samples (prepared by ACP hydrolysis at 100° and 25°, respectively) and for ACP sample 2. The two HA radial distributions are very similar, although the peaks in the RDF of the 100° HA are greater in amplitude and resolution than those of the 25° HA function. The smaller crystal size of the 25° compared to the 100° HA would contribute to the reduction in amplitude of the RDF, but would not affect the sharpness or resolution.²⁷ The better resolution of $G(r)$ for the 100° HA, as compared to the 25° HA, reflects the greater regularity of atomic arrangement in the 100° compound. From the structure of HA we can identify peaks in the radial distribution function as corresponding to the following ion pairs: P-O (1.5 Å), O-O and Ca-O (2.3 Å), Ca-Ca, Ca-P, and P-P (3 to 6 Å). The peaks at larger r values represent a composite of different types of ion pairs and are difficult to identify uniquely.

The reduced intensity functions of all the ACP samples are essentially identical (Figure 3) and, hence, their radial distribution functions must be the same. Thus, Figure 4 only gives the $G(r)$ plot for ACP sample 2. The first two peaks of the RDF for ACP in this figure are very similar to the HA functions. This is an expected result, since ACP and HA are both calcium orthophosphates of somewhat similar composition. The most important feature of the ACP radial distribution function is the fact that, unlike the crystalline HA functions, successive peaks decrease rapidly in amplitude with increasing r values. This suggests the existence of ordered domains or clusters of ions which are randomly packed with respect to one another within the larger ACP particles which we see in the electron microscope. From the decay in

amplitude of the $G(r)$ function in Figure 4 we can estimate that the ordered domains are large enough to contain pairs of atoms separated by 9.5 Å. The very small peaks at higher r are probably due to interparticle correlations which are present in all real substances.

ACP Structure Proposal

From the above chemical and radial distribution information, each cluster of ions in ACP was assumed to have a Ca:P molar ratio of 1.5, to be roughly spherical with a diameter of 9.5 Å, and to be free of water. Reasoning from the approximate sizes of the ions, a formula of $\text{Ca}_9(\text{PO}_4)_6$ was deduced.²⁶ The similar location of the peaks in $G(r)$ (Figure 4) for ACP and HA, as well as the ease of conversion of ACP to HA, suggested that a $\text{Ca}_9(\text{PO}_4)_6$ portion of the HA unit cell might be a possible model for the cluster (Figure 5). The reduced intensity function calculated for this model cluster, using the HA atomic position parameters, matched the observed ACP data (Figure 6).²⁸ The reduced intensity, calculated for a distortion of the atomic parameters of the cluster, such as might occur upon removal from the surrounding HA crystal, resulted in the improved agreement seen in the lower curves of Figure 6. Attempts to improve the already good agreement of these $F(s)$ functions by further small changes in the cluster model could be made, but there is little physical basis for such procedure. It is sufficient to say that the X-ray data were consistent with the view that synthetic ACP consists of roughly spherical $\text{Ca}_9(\text{PO}_4)_6$ clusters close-packed to form the larger spherical particles with water being held in the interstices.

In the process of ACP formation in solution, one can assume that $\text{Ca}_9(\text{PO}_4)_6$ clusters form first and then are aggregated randomly to produce the larger spherical particles with the inter-cluster space filled with water. The density of ACP obtained from pycnometry agrees well with the value calculated from this proposed model.²⁸ When ACP transforms, it has been shown that it must dissolve and renucleate as HA.²¹ It would be interesting to determine if the cluster remains intact in this dissolution process or whether it must dissociate completely before HA is formed.

Radial Distribution Studies of Bone

X-Ray diffraction measurements on mature mammalian bone were interpreted as showing that about 35% of the bone mineral did not contribute to the X-ray maxima of the single recognizable phase, a poorly crystallized apatite.¹⁷ In light of these results and by analogy to the synthetic systems, it was proposed that bone mineral contained a stabilized amorphous phase.¹³ As previously discussed, synthetic ACP is different from HA in stoichiometry, does not consist of extremely small crystals of HA, and has a distinctive radial distribution function from which a structural model has been proposed. Questions have been raised as to whether bone mineral was analogous to the synthetic case or, in fact, consisted of an apatite phase alone with little, if any, amorphous

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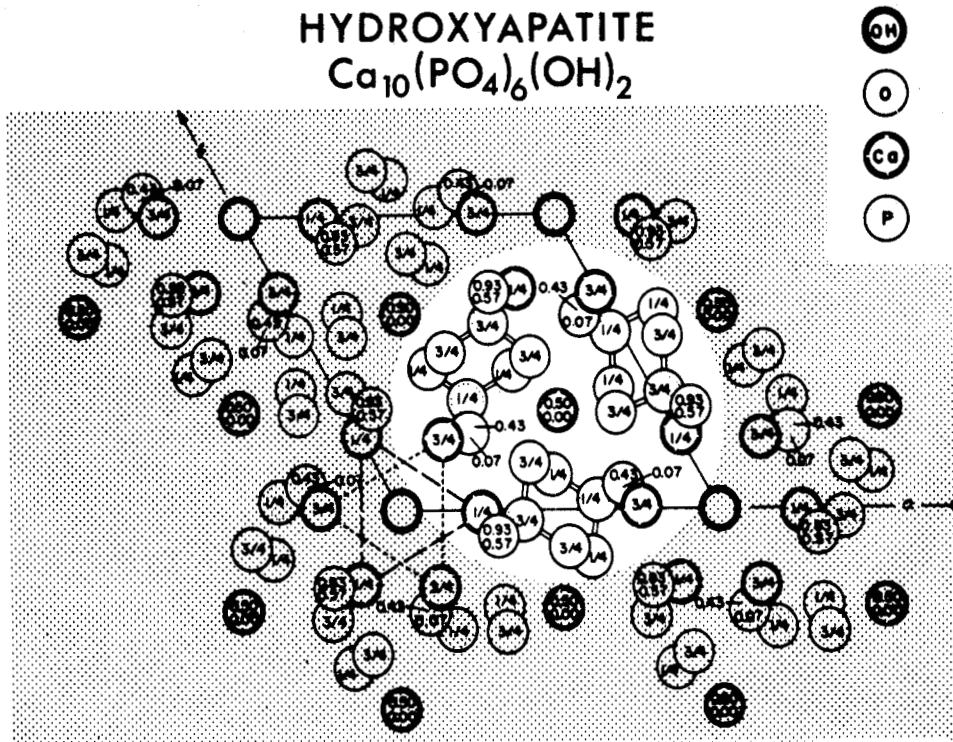


Figure 5. The HA atomic structure projected on the X-Y plane showing in the unshaded area the postulated $\text{Ca}_9(\text{PO}_4)_6$ cluster. Reproduced from ref 28 with permission of the publisher.

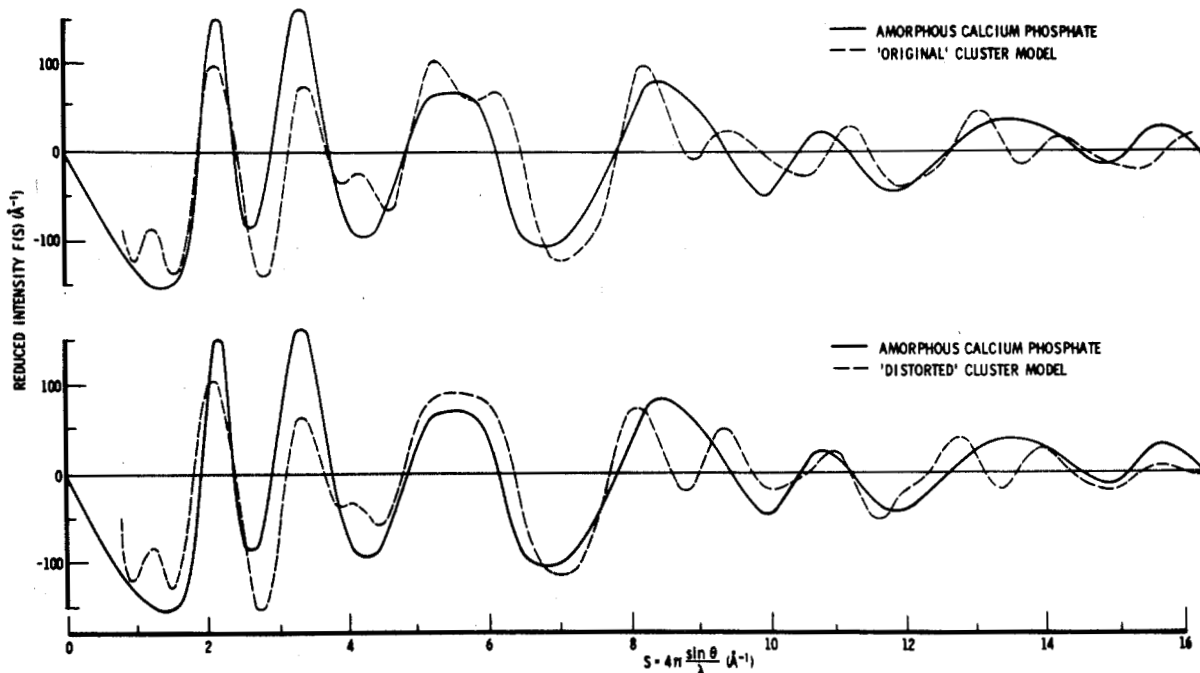


Figure 6. Reduced intensity functions for ACP model clusters.

phase present. It was suggested that the presence of a large percentage of bone apatite crystals appreciably smaller than the average size²⁹ and/or crystal disorder⁵ could account for the so-called amorphous contribution to the X-ray diffraction patterns of bone. Radial distribution studies gave us a method to test the hypothesis that bone contains an ACP analog admixed with some form of apatite. In addition, this technique provided information on the role of carbonate in bone apatite and apatite minerals in general.

It was shown³⁰ that incorporation of carbonate in the structure of synthetic HA caused the radial distribution function to be less well resolved and produced a decrease in the amplitude of this function with increasing interatomic distance. The radial distribution functions for a series of apatites with increasing CO_3^{2-} content are shown in Figure 7. An earlier suggestion that structural distortion resulted from carbonate inclusion in HA¹⁰ was confirmed by the radial distribution studies.³⁰ Increasing quantities of CO_3^{2-} can be seen to reduce the resolution of

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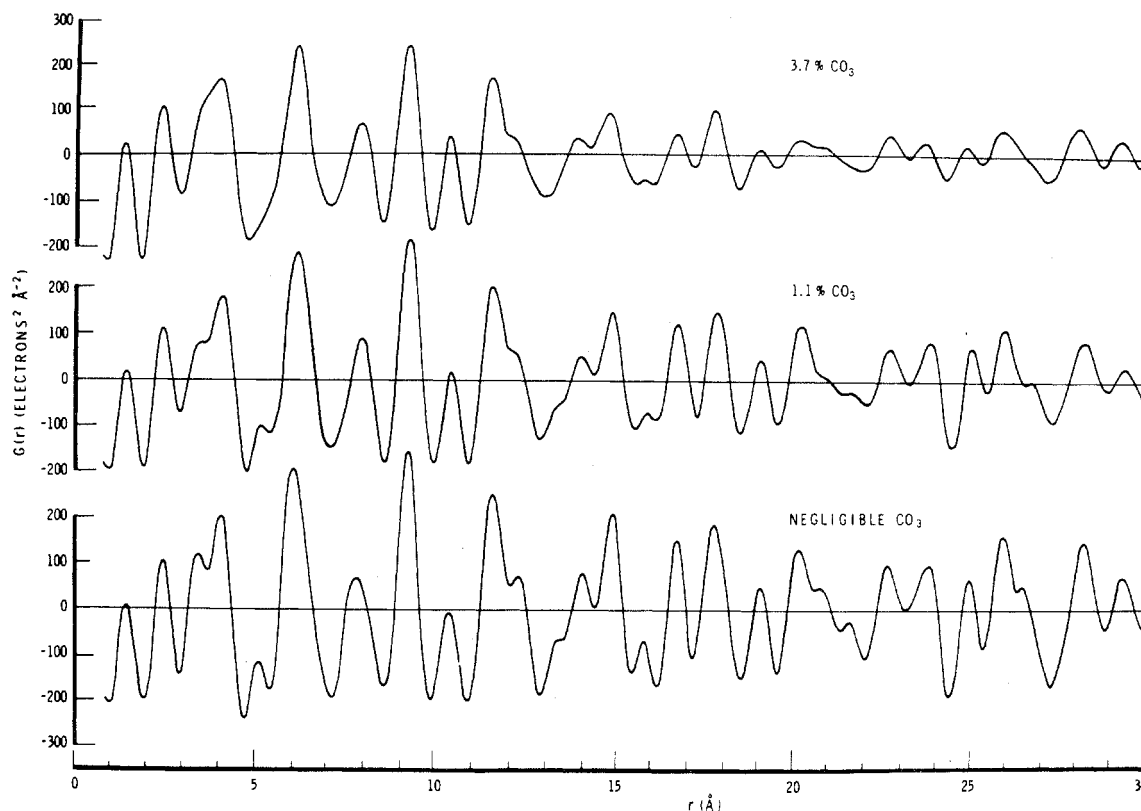


Figure 7. Reduced radial distribution functions for HA samples containing different amounts of CO_3 . Data collected from ref 26 and 30.

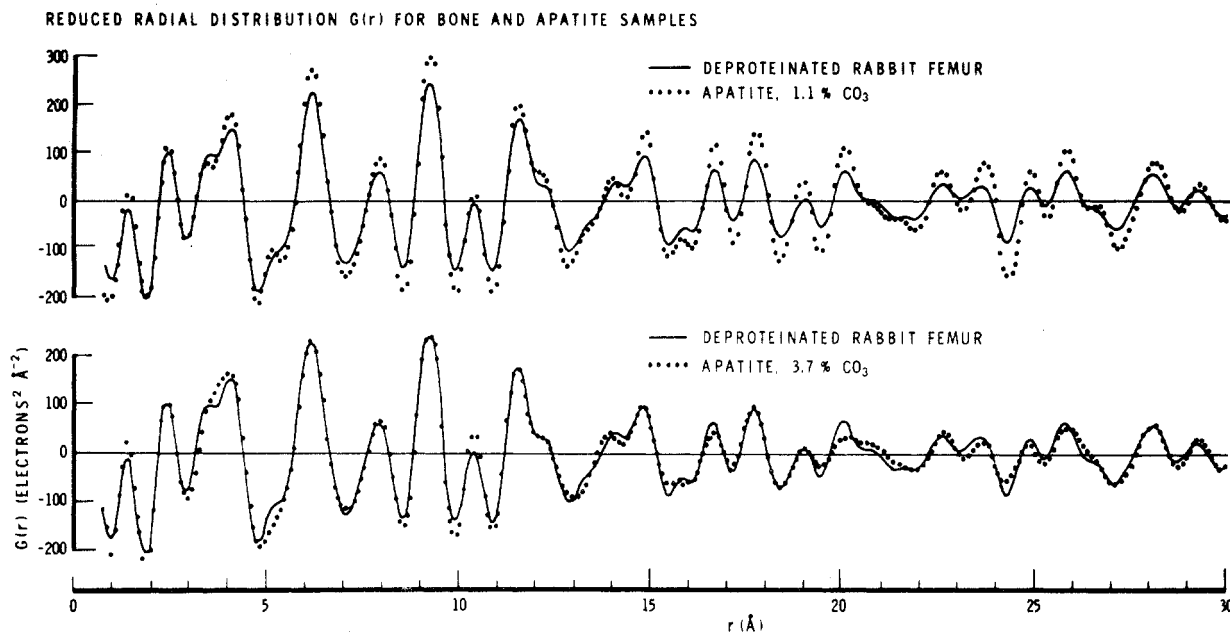


Figure 8. Comparison of the reduced radial distribution function $G(r)$ of 1.1% CO_3 -HA and 3.7% CO_3 -HA with deproteinized mature rabbit bone.

the $G(r)$ peaks in the 3 to 6 Å region which are related to interatomic distances involved with the phosphate groups. Also, the decrease in amplitude with increasing r is more pronounced as CO_3^{2-} content increases. The decrease in crystal size resulting from the presence of carbonate during precipitation³⁰ accounts for only a small part of this reduction in amplitude and does not contribute to the alteration of the shape (i.e., resolution) of this function.²⁷ Since bone mineral contains of the order of 4–6% CO_3^{2-} , any interpretation of a radial distribution of bone must take the effect of this ion into account.

The two sets of curves in Figure 8 show a comparison of the radial distribution functions obtained from mature rabbit bone mineral with those of HA containing 1.1 and 3.7% CO_3^{2-} , respectively. The general shape of the bone mineral RDF is very similar to that for the 3.7% CO_3^{2-} HA, falling off in amplitude with increasing r value at about the same rate. However, the shape of the peaks in the bone mineral RDF in the range of 3 to 6 Å (where CO_3^{2-} produced the greatest effect on resolution) is more like that of the 1.1% CO_3^{2-} HA. Since bone mineral contains at least 3.7% CO_3^{2-} these results suggested that part of the

CO_3^{2-} is incorporated differently in bone mineral than in synthetic HA. For smaller r values, $G(r)$ of bone resembles more closely that of the 1.1% CO_3^{2-} HA and for the higher r values it is closer to the 3.7% CO_3^{2-} HA. Thus, carbonate incorporation alone does not seem to account for all of the differences between the radial distribution functions of bone mineral and the synthetic carbonate-containing hydroxyapatites.

Figure 9 represents the data we used to test the hypothesis that bone contained an analog of synthetic ACP. The dashed line represents the RDF of deproteinized mature rabbit bone which was determined to be 63% crystalline by the X-ray diffraction method.¹⁷ The solid line in this figure is the $G(r)$ function obtained from a mixture of 37% by weight of synthetic ACP with 63% of 3.7% CO_3^{2-} HA. The bone mineral does not show as great a decrease in the amplitude of $G(r)$ with increasing r as does the synthetic mixture. Had we chosen the 1.1% CO_3^{2-} HA as the standard for the synthetic mixture, one can infer from Figure 8 that the match between the $G(r)$ values of bone mineral and mixture would have been improved slightly, because the drop-off in $G(r)$ with increasing r for the 1.1% CO_3^{2-} HA is less than that of the 3.7% CO_3^{2-} HA. These results throw doubt on the premise that 37% of this fully developed bone (as per the X-ray assay) is similar to if not identical with synthetic ACP. In fact, taking into account the errors inherent in the RDF method, no more than 10% of mature bone mineral could be present as an exact ACP analog.

Percent Crystallinity Measurement

In view of the doubt cast by the radial distribution function studies on the existence of a separate amorphous calcium phosphate phase in mature bone mineral, it is appropriate at this point to reevaluate the X-ray percent crystallinity method¹⁷ which suggested the bone model containing an amorphous phase. The basis for the method is seen in Figure 10, which shows a comparison between the X-ray patterns of equal weights of synthetic hydroxylapatite (100% crystalline) and whole bone; the latter shows no crystalline phase present other than a poorly crystallized apatite. The integrated intensity, above background, of the bone apatite pattern in Figure 10, even after an upward correction for the absorption by protein and water, is appreciably less than the integrated intensity of the 100% crystalline pattern. The ratio of the corrected intensity to that of the synthetic HA yields the percent crystallinity value for bone mineral. The validity of the method depends upon choosing a 100% crystalline apatite standard which is crystallographically identical with the apatite portion of bone mineral.

In the earliest use of the percent crystallinity method¹⁷ it was recognized that the hydroxyapatite standard must be close to bone apatite in crystal size as estimated by the broadening of the 002 X-ray maxima, since the integrated intensity of a given diffraction peak in this range of crystal size was seen to fall with increasing broadening. Because it was difficult to produce standards to match all bone samples in line width, an empirical correction factor for the integrated intensity as a function of line width of the standard was employed.³¹ More recent work²⁹

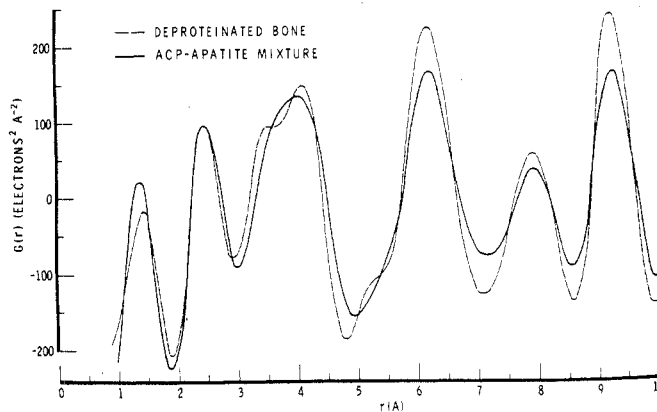


Figure 9. Comparison of the reduced radial distribution functions, $G(r)$, of deproteinized rabbit bone and a 0.33:0.67 ACP-HA mixture. The HA contained 3.7% CO_3 .

showed that by choosing a standard which gave the same X-ray line width and had the same CO_3^{2-} content as bone the measured percent crystallinity was substantially higher than when a CO_3^{2-} -free standard was used. Apparently lattice defects such as those due to CO_3^{2-} can affect the X-ray integrated intensity of the finely divided apatites and thus change the calculated percent crystallinity. Since we do not know the nature of the defects in bone apatite, or whether all bone apatites are similar, we can only guess which standard to choose for this percent crystallinity measurement. However, as we adopt a standard that seems closer to the bone apatite structure we find that the percent crystallinity rises. Electron microscopy showed that rat bone has a crystal size distribution with a substantial proportion of its crystals present in a size range far smaller than the average.²⁹ Thus, an X-ray percent crystallinity estimate of such bone would require the use of a standard which matched the bone in size distribution as well as in other aspects. As noted by these workers,²⁹ it is apparent that as we improve the crystallographic match of the standard to the bone we find that the percent crystallinity goes up, that is, our estimate of the amount of amorphous phase present goes down.

In light of these problems does the X-ray percent crystallinity measurement have validity or use in bone studies? In fact, the method has proved useful as a tool to follow changes in bone mineral with varying biological conditions. It is clear that this method provides a useful relative measurement. Thus, as noted earlier, using this method we find that bone mineral from young animals is lower in percent crystallinity than bone from adult animals.¹⁸ Also, as cited above, during the process of bone resorption the amorphous to X-ray phase is used up, leaving behind the more crystalline phase.¹⁹ In addition, preliminary work in our laboratory has shown that bone from patients with Paget's disease measured higher in amorphous content than normal bone. Studies are in progress where percent crystallinity measurements on bone biopsies are being used to ascertain the effectiveness of treatment of this disease.

Discussion

The existence of a synthetic noncrystalline orthophosphate is not limited to the calcium system.

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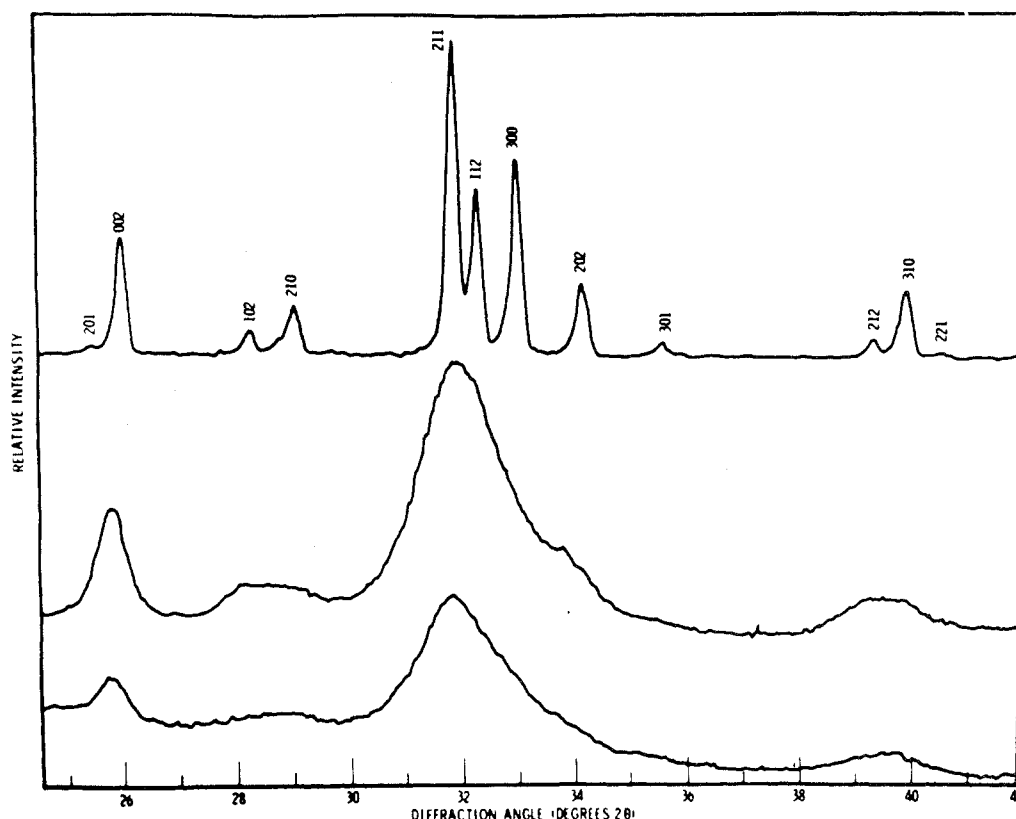


Figure 10. X-Ray diffraction patterns (copper $K\alpha$ radiation) of powdered human bone femur diaphysis (lower pattern); 100% crystalline, synthetic hydroxylapatite with small crystal size comparable to bone apatite (middle pattern); and for comparison well-crystallized, synthetic hydroxylapatite with diffraction peaks indexed (upper pattern). The two lower patterns are reproduced to the same scale, while the upper pattern is shown at a convenient arbitrary scale. The poor resolution of the 211, 112, 300, and 202 reflections resulting from small crystal size is evident in the two lower patterns. The lower intensity above background of the bone apatite pattern as compared to the poorly crystallized hydroxylapatite standard indicates that the bone mineral is not fully crystalline but contains a large amorphous fraction. Reproduced from ref 17 with permission of the publisher.

Amorphous copper(II) orthophosphate, magnesium phosphate, and magnesium ammonium phosphate have been prepared under comparable conditions.^{32,33} In addition, amorphous zirconium and ferric phosphates have been prepared.^{34,35} Preliminary X-ray radial distribution analysis of amorphous magnesium phosphate and amorphous ferric phosphate show them to have ion cluster structures smaller in cluster diameter than ACP.

An earlier experiment³³ on the preparation of alkaline earth phosphates from basic solution has relevance to the results of this paper. An attempt was made to precipitate separately the apatites of Mg, Ca, Sr, and Ba from basic supersaturated, phosphate solutions containing a 10:6 divalent cation:phosphate molar ratio. The first phase which precipitated in the case of Mg, Ca, and Sr had a 3:2 ratio while only the Ba went directly to the 10:6 apatite phase. As noted, the magnesium phosphate was amorphous and did not convert to an apatite. The amorphous tricalcium phosphate (ACP), of course, converted to $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. The $\text{Sr}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}$ which formed was poorly crystallized and converted in solution to $\text{Sr}_{10}(\text{PO}_4)_6(\text{OH})_2$, the apatite form. Thus, the smaller cation systems tended to form the more stable amorphous 3:2 compounds.

Limiting our discussion to calcium, it would seem there is something fundamental about the formation

of a $\text{Ca}_9(\text{PO}_4)_6$ cluster even though the Ca: PO_4 ratio of the precipitating solution varies from the 3:2 stoichiometry. One might speculate that in the ACP to HA transformation the ACP need only dissociate into these clusters rather than undergo complete ionic solvation. The clusters would then rearrange more slowly into the HA configuration, taking up OH^- and Ca^{2+} in the process. It is possible that the $\text{Ca}_9(\text{PO}_4)_6$ cluster is the transient solution precursor to ACP formation, and in fact a large proportion of the Ca and PO_4 ions found in neutral and basic calcium phosphate solution may be present in this form. One is tempted to speculate that in blood and in body fluids some proportion of the Ca and PO_4 is present as ion clusters, possible in this $(\text{Ca}_9(\text{PO}_4)_6)$ configuration, which are small enough to pass through biological membranes.

The biological relevance of the ACP work described in this paper takes on a new meaning with the results of a recent study on cytoplasmic amorphous calcium phosphate deposits.³⁶ A series of experiments suggest a calcium transport role by cell mitochondria in biological calcification.³⁷ It was shown that there are large deposits of stable amorphous calcium phosphate sequestered in the mitochondria of cells involved in normal tissue mineralization, such as osteoclasts³⁸ and chondrocytes.³⁹ It has been sug-

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gested that these amorphous deposits which are seen in the mitochondrion and the cytoplasm are transported in some way through the cell membrane to be deposited at the appropriate extracellular calcifications site.³⁷

The X-ray radial distribution function study³⁶ on cytoplasmic ACP from the hepatopancreas of the blue crab suggested that, like the synthetic ACP, these granules consist of ion clusters of the order of 10 Å in largest dimension. The same amount of adenosine triphosphate (ATP) found in the cellular amorphous granules was effective in stabilizing synthetic ACP in solution. The high Mg content of these cytoplasmic deposits suggests that the stabilization of this amorphous mineral may be due to some cooperative effect between the Mg and ATP.

Early electron microscope studies on newly forming bone showed that the first mineral to be deposited was morphologically amorphous.⁴⁰ A recent electron microscope study showed that a mineral phase in newly formed embryonic chick tibia was mor-

phologically amorphous and gave an electron diffraction pattern identical with that of synthetic ACP; older bone in this embryonic system appeared microcrystalline and gave an apatite electron diffraction pattern.⁴¹ In addition, an electron microscope study⁴² on metabolically active medullary bone in pigeon femur, which was shown to be highly amorphous by the X-ray diffraction percent crystallinity method,¹⁹ showed the morphology of a large portion of this bone mineral to be spherical in nature, similar in size and shape to the appearance of synthetic ACP.

From our radial distribution studies it is probable that fully developed bone mineral cannot contain over 10% of a phase which is a close analog of the synthetic amorphous calcium phosphate. It appears that the amorphous phase which is present in the early stages of tissue mineralization does not persist during the biological maturation process. It is probable that the bulk of mature bone mineral is a carbonate-containing analog of hydroxylapatite which is characterized by internal structural disorder.

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Mechanisms of Action of Naturally Occurring Irreversible Enzyme Inhibitors

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Naturally occurring competitive inhibitors which specifically block biological receptors are principally macromolecular in nature. For example, the naturally occurring protease inhibitors are proteins, as are the exceedingly potent snake neurotoxins. The soybean trypsin inhibitors, leupeptin and pepstatin, belong in the former class, and α -bungarotoxin is of the latter class.¹⁻³

Since these inhibitors are macromolecular in nature, it is not too difficult to understand, in a general way, the factors to which they owe their remarkable specificity. Simply, these macromolecules can make a large number of noncovalent contacts with the appropriate receptor. This assures tight binding, and thus selectivity.

On the other hand, there is another group of specific toxins⁴ functioning principally as noncompetitive inhibitors of enzymes, whose specificity of action

cannot be understood in terms of isosterism with normal substrate (effector) with a concomitant large number of noncovalent interactions with the target enzyme. The relatively more complex mechanism of action of this latter class of toxins is the subject of this Account.

I would like to confine myself to an examination of those naturally occurring toxins which act on enzymes as receptors, and show that the specificity of these toxins is a consequence of the fact that they must be converted into the active form of the inhibitor by the target enzyme itself. That is, these molecules are substrates for the target enzymes, and in the process of or as a consequence of this conversion the enzyme becomes irreversibly inhibited. I am interested here not in the molecules of nature that are chemically highly reactive and hence general poisons,

(1) M. Laskowski and R. W. Sealock, *Enzymes*, 3rd ed., **3**, 375 (1971).

(2) H. Umezawa, "Enzyme Inhibitors of Microbial Origin". University Park Press, London, 1972, Chapter 4.

(3) C. C. Chang and C. Y. Lee, *Arch. Int. Pharmacodyn.*, **144**, 241 (1963).

(4) Toxin is used here in the broad sense without reference to organisms; that is, antibiotics are considered as toxins.

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