*Proc. Nat. Acad. Sci. USA*
Vol. 72, No. 6, pp. 2088–2090, June 1975

# Atomic Structure of Intracellular Amorphous Calcium Phosphate Deposits

**(x-ray diffraction/radial distribution analysis/ion clusters/soft-tissue calcification/micropackets)**

F. BETTS*, N. C. BLUMENTHAL*, A. S. POSNER*, G. L. BECKER†, AND A. L. LEHNINGER†

*Hospital for Special Surgery, Cornell University Medical College, New York, N.Y. 10021; and †Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

*Contributed by A. L. Lehninger, March 27, 1975*

**ABSTRACT** **The radial distribution function calculated from x-ray diffraction of mineralized cytoplasmic structures isolated from the hepatopancreas of the blue crab (*Callinectes sapidus*) is very similar to that previously found for synthetic amorphous calcium phosphate. Both types of mineral apparently have only short-range atomic order, represented as a neutral ion cluster of about 10 Å in longest dimension, whose probable composition is expressed by the formula $Ca_9(PO_4)_6$. The minor differences observed are attributed to the presence in the biological mineral of significant amounts of $Mg^{2+}$ and ATP. Synthetic amorphous calcium phosphate in contact with a solution containing an amount of ATP equivalent to that of the biological mineral failed to undergo conversion to the thermodynamically more stable hydroxyapatite. The amorphous calcium phosphate of the cytoplasmic mineral granules is similarly stable, and does not undergo conversion to hydroxyapatite, presumably owing to the presence of ATP and $Mg^{2+}$, known inhibitors of the conversion process. The physiological implications of mineral deposits consisting of stabilized calcium phosphate ion clusters are discussed.**

Earlier work has shown that substantial deposits of amorphous calcium phosphate are formed in the inner compartment of mitochondria after the inward transport of $Ca^{2+}$ and phosphate coupled to electron transport as the energy source (1). From this and other lines of investigation, one of us has proposed the hypothesis that in calcifying tissues such intramitochondrial deposits of amorphous calcium phosphate are released from the mitochondria and transported as small, stable aggregates to other sites, where they serve as precursors for the larger and more stable mineral deposits characteristic of calcified tissues (2).

Studies on the preparation of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], the synthetic prototype of bone mineral, showed that an amorphous calcium phosphate precursor appears under conditions of high supersaturation (3). This precursor amorphous calcium phosphate, unless stabilized in some way, transforms autocatalytically in solution to hydroxyapatite. X-ray radial distribution analysis suggested that synthetic amorphous calcium phosphate particles, which appear as 300–1000 Å spheres in the electron microscope, the exact size depending on preparation conditions (4), consist of a random assembly of ion clusters 9.5 Å in diameter, dimensions consistent with the chemical composition $Ca_9(PO_4)_6$ (5, 6). The 15–20% of water found in synthetic amorphous calcium phosphate was shown to be mostly in the interstices between, and not within, the individual $Ca_9(PO_4)_6$ clusters (5). Although the exact mechanism of stabilization of amorphous calcium phosphate is not understood, the presence of $Mg^{2+}$ (7), carbonate (8), pyrophosphate (9), diphosphonates (10), or polyphosphorylated metabolites or nucleotides (11), in sufficient quantity will prevent the transformation of synthetic amorphous calcium phosphate to hydroxyapatite.

Recent work has shown that during intermolt the hepatopancreas of the adult male blue crab *Callinectes sapidus* contains numerous extramitochondrial mineral deposits, which are predominantly calcium phosphate and are stable enough to be isolated as a relatively pure subcellular fraction (12). This paper presents the results of an x-ray radial distribution analysis of the atomic structure of these cytoplasmic deposits and compares their structure with well-characterized synthetic calcium phosphates.

## METHODS

*Specimen Preparation.* The cytoplasmic mineral granule fraction of the hepatopancreas of the blue crab was collected as described earlier (12). Differential centrifugation by this method yielded a pellet of high mineral content in the first, low-speed step. This dense material was enriched in mineral by skimming off and discarding the less dense portions of the pellet. Repeated suspension in cold buffered isotonic medium and low-speed sedimentation of the chalky residue, followed by lyophilization, yielded a white, gritty, grossly homogeneous powder. Chemical analyses confirmed the relatively high mineral content of this fraction, as reported earlier (12).

*X-Ray Diffraction.* The dry mineral granule fraction was ground to pass a 325 mesh (128 openings per cm) sieve and a smooth-surfaced disc was pressed in a 22 mm diameter die for x-ray study. X-ray data were taken using molybdenum radiation, a graphite monochromator, and a scintillation detector with a pulse height analyzer set to eliminate $\lambda/2$ radiation. The x-ray pattern obtained consisted of a few broadened maxima typical of non-crystalline substances, in agreement with earlier diffraction studies of this material (ref. 13; E. D. Eanes, J. D. Termine, and G. L. Becker, unpublished data). A reduced radial distribution function (RDF) was calculated from the pattern by means of a method outlined in earlier publications (5, 6). A similar analysis was performed on synthetic hydroxyapatite and synthetic amorphous calcium phosphate.

## RESULTS AND DISCUSSION

The radial distribution method has long been used to obtain information about the short range atomic arrangement in poorly crystallized and amorphous materials. Essentially, a

Abbreviation: RDF, radial distribution function.

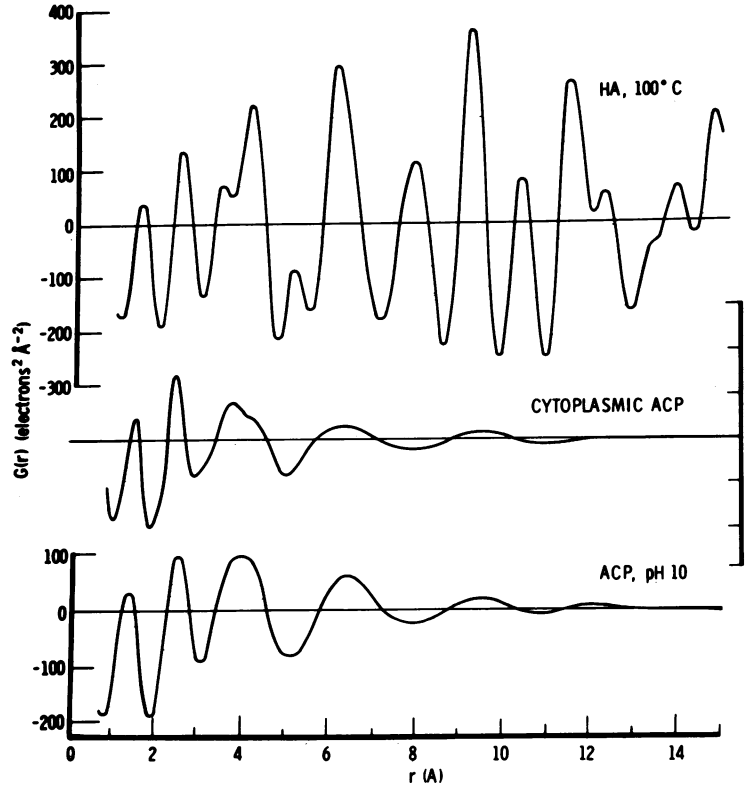


FIG. 1. Comparison of the reduced radial distribution functions, G(r), calculated from the x-ray diffraction patterns of well-crystallized hydroxyapatite (HA), cytoplasmic mineral granules (cytoplasmic ACP), and synthetic amorphous calcium phosphate (ACP). The ordinate values of the cytoplasmic ACP function are given in arbitrary units scaled to match the other two functions.

reduced RDF is a plot of atomic or electronic density versus atomic separation. Positive RDF values correspond to atom densities greater than the average, while negative RDF values correspond to atom densities lower than average. Thus, a peak in the RDF corresponds to one (or more) atom pairs separated by the distance on the abscissa of the function.

Fig. 1 shows a comparison of the RDF's of hydroxyapatite, the cytoplasmic mineral granule fraction, and synthetic amorphous calcium phosphate. It is clear that the cytoplasmic granules from the blue crab and synthetic amorphous calcium phosphate have similar atomic structures, and both differ from hydroxyapatite. The cytoplasmic calcium phosphate granules have a structure built up of close-packed ion clusters similar in size (about 9.5 Å) to those in synthetic amorphous calcium phosphate. The rapid drop-off of atomic periodicity in both the cytoplasmic and synthetic amorphous calcium phosphate samples is typical of amorphous cluster structures. Short-range order exists in these amorphous structures but no long-range order such as that in crystalline hydroxyapatite. There are some differences in resolution and amplitude between the RDF's of the cytoplasmic mineral and synthetic amorphous calcium phosphate structures. While a detailed interpretation of the RDF of the cytoplasmic granules has not been completed, the smaller amplitudes of peaks in the vicinity of 4, 6.3, and 9.5 Å relative to those at 1.5 and 2.5 Å suggest a non-spherical ion cluster. The cluster is 9.5 Å in largest dimension and it is quite likely of smaller mass than the $Ca_9(PO_4)_6$ cluster in synthetic amorphous calcium phosphate. It is possible that this structure difference results from the presence of ATP, $Mg^{2+}$, and other foreign ions in the cytoplasmic mineral.

The synthetic amorphous calcium phosphate is essentially pure $Ca_9(PO_4)_6$ with 15–20% interstitial water. The cytoplasmic amorphous calcium phosphate, on the other hand, contains in addition to interstitial water, substantial amounts of $Mg^{2+}$, ATP, and ADP (1), which probably cooperatively stabilize the biological material in the amorphous form, since purification procedures were used which would have rapidly converted unstabilized amorphous calcium phosphate to hydroxyapatite. We have, in fact, confirmed that synthetic amorphous calcium phosphate in contact with a solution containing 7–8% by weight of ATP relative to amorphous calcium phosphate (the same ratio as found in the cytoplasmic granules) is stabilized for 3–4 days at physiological pH, whereas the conversion is complete within several hours in the absence of ATP.

The data presented in this paper indicate that the cytoplasmic mineral deposits from the blue crab hepatopancreas are very similar in short-range atomic structure to synthetic amorphous calcium phosphate. This finding establishes the validity of synthetic amorphous calcium phosphate as a model for at least one type of biological mineral phase. In addition, the cytoplasmic mineral deposits are shown to have definite structural organization at a level of approximately 10 Å. Thus, individual units of cytoplasmic mineral, if stable, are of dimensions such that packaging by, and perhaps even passage through, intracellular membranes is not improbable. Furthermore, their apparent stabilization by ATP offers the possibility that intracellular metabolic processes, particularly phosphorylating respiration by mitochondria, may influence the stability and ultimately the biological fate of calcium phosphate deposits through change in the level of mineral-

associated nucleotide. Although the site of origin of the cytoplasmic granules is not known, earlier work has established that the formation of electron-dense deposits of calcium phosphate in liver mitochondria is accompanied by accumulation of ATP, in about the same Ca:ATP ratio (14) as observed in the cytoplasmic calcium phosphate granules from crab hepatopancreas.

This work was supported by grants from the National Institutes of Health (DE-01945, A.S.P.), (GM-05919, A.L.L.) and the National Science Foundation (GB-36015, A.L.L.).

1. Greenawalt, J. W., Rossi, C. S. & Lehninger, A. L. (1964) *J. Cell Biol.* **23**, 21–38.
2. Lehninger, A. L. (1970) *Biochem. J.* **119**, 129–138.
3. Eanes, E. D., Gillessen, I. H. & Posner, A. S. (1965) *Nature* **208**, 365–367.
4. Blumenthal, N. C. & Posner, A. S. (1972) *Mater. Res. Bull.* **7**, 1181–1190.
5. Betts, F. & Posner, A. S. (1974) *Mater. Res. Bull.* **9**, 353–360.
6. Betts, F. & Posner, A. S. (1974) *Trans. Am. Crystallogr. Assoc.* **10**, 73–84.
7. Eanes, E. D. & Posner, A. S. (1968) *Calcif. Tissue Res.* **2**, 38–48.
8. Bachra, B. N. (1959) *Ann. N.Y. Acad. Sci.* **109**, 251–255.
9. Fleisch, H. (1964) *Clin. Orthop. Relat. Res.* **32**, 170–180.
10. Francis, M. D. (1969) *Calcif. Tissue Res.* **3**, 159–162.
11. Termine, J. D. & Conn, K. M. (1975) *J. Dent. Res.* **54** (A), L26.
12. Becker, G. L., Chen, C.-H., Greenawalt, J. W. & Lehninger, A. L. (1974) *J. Cell Biol.* **61**, 316–326.
13. Becker, G. L. (1974) *Fed. Proc.* **33**, 1258.
14. Carafoli, E. C., Rossi, C. S. & Lehninger, A. L. (1965) *J. Biol. Chem.* **240**, 2254–2261.