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EXTENDED X-RAY ABSORPTION FINE STRUCTURE (EXAFS) STUDIES OF THE CALCIUM ION ENVIRONMENT IN BONE MINERAL AND RELATED CALCIUM PHOSPHATES.

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Summary EXAFS spectra have been recorded from bone mineral and related calcium phosphates. Fourier transformation of a spectrum, using theoretically calculated phase shifts, yields a good approximation to the radial distribution of atoms around a calcium ion. Comparison of the results shows that bone mineral is appreciably different from crystalline synthetic hydroxyapatite and geological apatites, which are similar to each other, but closely resembles hydroxyapatite obtained by maturation of amorphous calcium phosphate.

Introduction We have used extended X-ray absorption fine structure spectroscopy (EXAFS) to provide new information on the structure of bone mineral. Although it is often referred to as "hydroxyapatite" the chemical structure of bone mineral remains unknown (1,2). Amorphous calcium phosphate has been prepared which, when moist and not chemically stabilised, gradually matures to poorly crystalline hydroxyapatite (3). It has been suggested that this maturation is implicated in the development of bone mineral (4) but the suggestion has recently been questioned (2). In any case amorphous calcium phosphate could account for less than 10% of the mineral in adult bone (5).

The absorbance of X-rays plotted against photon energy is a smooth curve of negative slope, except in the vicinity of an absorption edge; on the high energy side of the edge the absorbance is oscillatory - these damped oscillations are the EXAFS spectrum. Although the phenomenon has been known for many years it has only been exploited for solving structural problems in biology since the availability of synchrotron radiation (6). We have recorded EXAFS spectra, above the calcium K absorption edge, from bone mineral, synthetic hydroxyapatite, geological apatites and amorphous calcium phosphate in varying stages of maturation

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to hydroxyapatite. Analysis of each spectrum yields a good approximation to the distribution of atoms around a calcium ion (7).

Materials and methods Femurs were dissected from three different adult rats and two samples of geological apatite were investigated. Amorphous calcium phosphate was prepared and stabilised by drying (3). Crystalline hydroxyapatite was prepared by igniting a stoichiometric mixture of calcium carbonate and calcium orthophosphate (1400°C for 2 hours) which was then ground, mixed with water (tenfold excess) and heated, in a sealed platinum crucible at elevated pressure (950° C, 1 kbar for 48 hours); the mixture was rapidly cooled by compressed-air quenching. Some amorphous calcium phosphate was allowed to mature into poorly crystalline hydroxyapatite - one sample for about 2 hours, the other for about 2 weeks. All samples were ground to a fine powder in an agate mortar. Their X-ray diffraction patterns were recorded before and after EXAFS spectra were obtained and the identity of the crystalline samples was checked by comparison of observed with expected spacings (8). No attempt was made to measure the fraction of crystalline material in maturing amorphous calcium phosphate because the methods which have been used (9) are now considered unreliable (5). Chemical analyses (for Ca and P) were performed as a final check by the Microanalytical Laboratory of the Department of Chemistry, University of Manchester.

Incident (I_o) and transmitted (I) X-ray intensities were measured as a function of photon energy (E) in the usual manner (6) with apparatus at LURE, Orsay and used to calculate absorbance ($\log\{I_o/I\}$). The damped oscillations of the EXAFS spectrum, $\chi(E)$, were extracted by subtracting out the smoothly varying background (10). Each spectrum was transformed into k-space where

$$k = \{2(E-E_o)\}^{\frac{1}{2}}$$

and E_o is the energy of the absorption edge. The Fourier transform

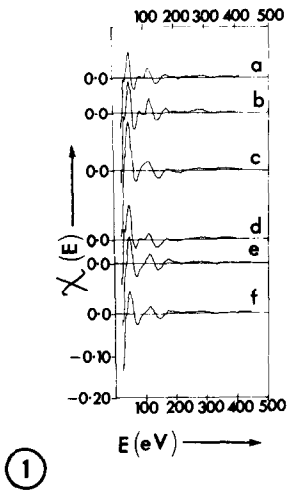
$$f(r) = \int_o^\infty \chi(k)\exp\left[-i\{2kr + \phi(k)\}\right]dk$$

was then calculated. Here $\phi(k)$ is a phase shift arising from backscatter of the emitted photoelectron; it was calculated theoretically (11) from the first coordination sphere of the hydroxyapatite structure (12). The modulus of f(r), denoted by $|f(r)|$, provides a good approximation to the radial distribution of atoms around a calcium ion (7). In order to remove rapid oscillations due to Fourier transformation of a finite data range $\chi(k)$ was first multiplied by a Gaussian window function

$$\exp\ \{-A(k-k_o)^2\}$$

with k_o placed at the centre of the EXAFS range and A chosen so as to eliminate the oscillations (7). The same values of A (0.10) and k_o (corresponding to an energy of 220 eV) were used for all spectra to allow strict comparison. No attempt has been made to extract coordination numbers or to identify the types of atoms surrounding the calcium ion.

Results Fig.1 shows that the spectra obtained from a geological apatite and crystalline hydroxyapatite were similar but different to those from rat bone; all three bone samples yielded almost identical spectra which most



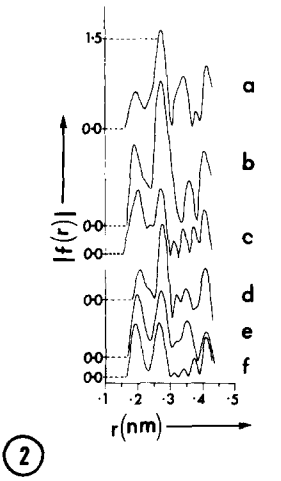


Fig.1. EXAFS spectra, $\chi(E)$, obtained from (a) synthetic crystalline hydroxyapatite, (b) a geological apatite, (c) amorphous calcium phosphate, (d) amorphous calcium phosphate in an early stage of maturation, (e) further matured calcium phosphate and (f) bone mineral. Energies are measured from the absorption edge, i.e. $(E-E_o)$. Note that E_o was defined by the mid-point of the edge and may not correspond exactly to the true value (10).

Fig.2 Radial distribution of atoms, $|f(r)|$, surrounding the calcium ion in (a) synthetic crystalline hydroxyapatite, (b) a geological apatite, (c) amorphous calcium phosphate, (d) amorphous calcium phosphate in an early stage of maturation, (e) further matured calcium phosphate and (f) bone mineral calculated from their EXAFS spectra.

closely resembled the spectrum obtained from hydroxyapatite formed by maturation of amorphous calcium phosphate. Comparisons are more easily made by first calculating $|f(r)|$, which represents the calcium environment, from each spectrum - the results are shown in Fig.2.

EXAFS results on crystalline hydroxyapatite (Fig.2a) are then comparable with those obtained by X-ray crystallography (12). Two peaks are readily interpretable; the first, at 0.2 nm, corresponds to the first coordination sphere of six oxygen atoms (crystallographic result 0.24 nm) and the second, around 0.3 nm, probably correspondends to the other surrounding oxygen (at 0.28 nm), phosphorous (at 0.32 nm) and calcium (at 0.34 nm). Further peaks have too many contributors to be readily interpretable.

Similarly the calcium ion environment in amorphous calcium phosphate (Fig.1c) is comparable with results obtained from a radial distribution function calculated from X-ray diffraction data (13). We obtained an X-ray diffraction pattern from our sample which was largely diffuse with only a feint trace of the strongest rings expected from crystalline hydroxyapatite

- all these rings were much more diffuse than those obtained from our crystalline samples. Since X-ray diffraction emphasises the presence of ordered phases at the expense of any disordered phase it appears that our sample was almost entirely amorphous. Once again the first peak, at 0.2 nm, corresponds to the oxygen atoms (diffraction result 0.24 nm (13)). The difference in relative peak heights and peak positions between Fig. 2a and 2c shows that the calcium ion environment in amorphous calcium phosphate is not identical to that in crystalline hydroxyapatite. Furthermore increased peak widths in Fig. 2c are consistent with a higher Debye-Waller factor (7), i.e. greater disorder, in amorphous calcium phosphate.

As amorphous calcium phosphate was allowed to mature so the hydroxyapatite rings in its X-ray diffraction pattern became more intense. Calcium ion environments were calculated from EXAFS spectra corresponding to an early (a few hours) and later (a few weeks) stage of maturation. It is difficult to draw any quantitative conclusions about the structure because of the proximity of the different coordination shells. However the overall appearance of $|f(r)|$ changes during maturation showing that changes occur in the environment of calcium.

Finally the results obtained from bone mineral (Fig.2f) most clearly resemble those from matured amorphous calcium phosphate (Fig.2e); in particular the positions and shapes of the first two peaks are almost identical. Although the first peak is at almost the same position as for crystalline hydroxyapatite (Fig.2a) and geological apatite (Fig.2b), the second peak seems to correspond to a shorter distance.

Conclusions Our results show that (i) fully matured amorphous calcium phosphate does not have exactly the same structure as crystalline hydroxyapatite, although this has often been assumed to be the case, (ii) the calcium environment in bone mineral is not the same as in apatite structures i.e. it is not identical to synthetic crystalline hydroxyapatite or to geological apatites and (iii) the structure of bone mineral closely resembles that of matured amorphous calcium phosphate. The third conclusion does not imply that maturation of amorphous calcium phosphate is the mechanism by which bone mineral develops in vivo. But it does provide new structural information which is consistent with the view that bone mineral is intermediate in structure between an amorphous solid (a glass) and a perfect crystal rather than simply consisting of hydroxyapatite crystals with distortions such as point defects and dislocations (14).

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