The local environment of metal sites in intracellular granules investigated by using X-ray-absorption spectroscopy

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We report the use of X-ray-absorption spectroscopy (x.a.s.) to study the local atomic environment of cations in intracellular granules from the hepatopancreas of *Helix aspersa*. Both the calcium *K*-edge in these concretions and the manganese *K*-edge in doped specimens were measured. Electron-microprobe measurements confirm that the introduced Mn^{2+} is concentrated in irregular growths on the surfaces of the granules. The near-edge structure (x.a.n.e.s.) of calcium is similar to that of manganese, indicating that the oxygen-co-ordination spheres of both cations share a similar symmetry. From the extended structure (e.x.a.f.s.) the metal–oxygen bond lengths of 0.230 nm (2.30 Å) for Ca–O and 0.218 nm (2.18 Å) for Mn–O [± 0.004 nm (0.04 Å)] were determined, reference being made to a variety of model compounds. The low density of the granules (2.07 g/cm³), together with the local atomic distribution, suggest an open hydrated structure for these phosphate deposits. Detailed analysis of the distribution of nearest-neighbour oxygen atoms demonstrates that this is asymmetric and considerably broader for Ca^{2+} than for Mn^{2+} . Compared with the model compounds, the Ca^{2+} environment in the granules is similar to that observed in $Ca_2P_2O_7$. I.r. spectra indicate the presence of condensed phosphate groups in the granules, with the strong possibility these are pyrophosphate ($P_2O_7^{4-}$) groups.

A variety of different types of cells in a wide range of organisms secrete intracellular deposits of inorganic material. These deposits often occur in considerable numbers (approx. 100) within a single cell, and they are typically spherical, 1–10 μ m in diameter, concentrically layered and formed within membrane-bound vesicles (Simkiss, 1976). The frequency with which these granules are discovered in different animals suggests they must be the product of a common cellular activity, but it is difficult to pursue this concept since the composition and function of these deposits is incompletely known. Two of the most interesting characteristics of the granules are that they have a variable metal-ion content and that much of the phosphate present is condensed. The granules are also amorphous to X-rays, and are therefore

of considerable interest since in the past little attention has been paid to the physical and chemical properties of amorphous phases in biochemical studies of mineral deposition. Biogenic amorphous minerals may have several functional roles, and Mann (1983) suggests they may variously act as precursors of crystalline phases, ion stores or structural materials. In order to investigate some of these possibilities we have studied in some detail the granules formed in the basophil cells of the hepatopancreas gland of the snail *Helix aspersa*. The results obtained from these particular deposits will obviously not be applicable to all other forms of granules, but they do provide a basis for considering some of the detoxification functions that have been ascribed to these concretions.

Physiological experiments have shown that the hepatopancreas is capable of removing a variety of metals (e.g. manganese, cobalt, zinc) from the blood of the snail within a few hours of their injection (Simkiss, 1981). These metals can sub-

Abbreviations used: x.a.s., X-ray-absorption spectroscopy; x.a.n.e.s., X-ray-absorption near-edge structure; e.x.a.f.s., extended-X-ray-absorption fine structure.

sequently be recovered from the intracellular granules of the basophil cells. Ultrastructural studies show that, during this time, the metals have traversed the cytoplasm and become incorporated in the inorganic deposits formed within the vesicle membrane (Mason & Simkiss, 1982). Chemical analysis shows that the deposits are partly hydrated, containing about 18% (w/w) water, that 5% (w/w) is organic matter and that the remainder is inorganic. The inorganic material is phosphate-based, and under normal circumstances the main cations present are Ca^{2+} and Mg^{2+} (Howard *et al.*, 1981). After take-up of metal ions in the blood, however, these cations are added to by the introduced metal ions (Simkiss, 1981). Interestingly, i.r.-absorption spectra of the granules show a medium broad band at 925 cm^{-1} with a weaker but sharper band at 745 cm^{-1} . Equivalent modes have also been observed by Raman spectroscopy (J. C. Merlin & E. Payen, unpublished work). These features are typical of stretching vibrations of the bridging P–O–P unit and are a 'fingerprint' of condensed phosphate groups (Corbridge & Lowe, 1954; Corbridge, 1969; Palmer, 1961). Enzymic assays have established that these include an appreciable fraction of the pyrophosphate anion $\text{P}_2\text{O}_7^{4-}$. On the basis of these findings it has been suggested that the granules may be a way of immobilizing a variety of metals from the cytoplasm of the cell by incorporating them into these highly insoluble phosphate salts (Howard *et al.*, 1981).

The details of such a detoxification process are difficult to pursue from a structural standpoint, since the deposits are amorphous and standard crystallography techniques cannot be used. The absence of electron spin for calcium and magnesium means that e.s.r. cannot be used to probe the local atomic environment of the natural deposits. E.s.r. can be used for studying the local structure of transition metal-dopants, but initial experiments have proved to be relatively insensitive. It has therefore been necessary to turn to X-ray-absorption spectroscopy (x.a.s.) to examine the short-range order in these phosphate concretions. This technique has the advantage of being atom-specific, so that several cations, for instance, can be studied independently in the same system. Accordingly, the present paper describes the X-ray fine structure measured at the calcium *K*-edge in the granules, comparing it with the fine structure measured at the *K*-edge of an introduced cation, namely Mn^{2+} , as a way of investigating how such metals become incorporated into these deposits. X.a.s. has been used previously to study the local atomic structure of calcium in various biological systems [notably bone, milk and calcium ATP (Hasnain, 1983, and references cited therein)], but

this is the first application to intracellular calcium and the first attempt to use this technique to probe the local atomic structure of a foreign metal.

Experimental and results

Specimen preparation and X-ray-spectroscopy measurements

Specimens of intracellular granules were obtained from the hepatopancreas of snails (*Helix aspersa*). The techniques used have been described previously (Howard *et al.*, 1981). The doped specimens were taken from animals that had been given a diet sprayed with 50 mM- MnCl_2 over a period of 2 weeks. Electron micrographs of typical natural and doped specimens are shown in Fig. 1. They are equivalent to granules obtained from injected animals (Simkiss *et al.*, 1982). The undoped granules are spherical, with diameters ranging from 1 to $5\text{ }\mu\text{m}$. Although the same type of granule can be detected in the doped specimens, most are encrusted with an irregular surface growth. Electron-microprobe analyses are also included in Fig. 1. The inorganic ingredients of Mg, Ca and P can be clearly seen in both the undoped (a) and the doped granules (b), with the addition of Mn showing up strongly in the doped granules. Notably, the manganese is more highly concentrated in the rough edges of the doped granules rather than in their centres [compare Fig. 1(d) and Fig. 1(e)]. The indication, then, is that manganese is incorporated in preference to calcium in the fresh growth of the doped granules, the magnesium content staying roughly constant (Table 1). Although the ionic radius of Mn^{2+} [0.080 nm (0.80 Å)] is intermediate between that of Mg^{2+} [0.066 nm (0.66 Å)] and that of Ca^{2+} [0.099 nm (0.99 Å)], manganese phosphates are often isostructural with magnesium phosphates rather than calcium phosphates (see, e.g., Lukaszewicz & Smajkiewicz, 1961; Durif, 1971). This may be the reason why the encrusted shells of the granules consist mainly of a mixture of phosphates of magnesium and manganese. It may also be the reason for the irregular nature of the growth at the surface, if epitaxy is seeded by those regions on the granules' surface that are rich in magnesium.

Table 1. Ratios of elements present in intracellular granules from normal and manganese-doped snails (see Fig. 1)

Values are peak-background counts from X-ray-microprobe samples but are uncorrected for counting efficiencies at the different energies.

	Mg/P	Ca/P	Mn/P
Normal snails: granule	0.20	0.55	0.00
Mn-fed snails: granule centre	0.15	0.54	0.15
Mn-fed snails: granule edge	0.17	0.17	0.45

The phosphate model compounds were obtained in the following way. $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (brushite) was prepared by the method described by Jensen & Rathler (1953). $\text{Ca}_2\text{P}_2\text{O}_7$ (calcium pyrophosphate) was prepared by ignition of brushite at 900°C . $\text{MnHPO}_4 \cdot 3\text{H}_2\text{O}$ (manganese hydrogen phosphate) was synthesized by following the method described by Palmer (1954), and the analytical method described by Klement & Haselheck (1964) was used for $\text{Mn}_2\text{P}_2\text{O}_7$ (manganese pyrophosphate). Samples of CaO and MnO were prepared carefully to avoid contamination with H_2O or CO_2 . Starting materials were obtained from BDH Chemicals (Poole, Dorset, U.K.). CaO was prepared by heating CaCO_3 at 1000°C . MnO was 99.5% pure and was then heated at 300°C in dry H_2 . Both CaO and MnO were contained in sealed tubes before measurement.

X.a.s. measurements of the granules and model compounds were made by using synchrotron radiation from the Synchrotron Radiation Source (SRS) at Daresbury Laboratory. X-ray-absorption spectra were obtained from the transmission of powdered samples. If I_0 and I are the incident and transmitted intensities respectively, the X-ray absorbance, α , is given by:

$$\alpha = -\ln(I/I_0)$$

Measurements were made at room temperature at the K -edges of calcium [0.307 nm (3.07 Å)] and manganese [0.190 nm (1.90 Å)]. During the experiment, the Synchrotron Radiation Source ran with a circulating beam current of approx. 100 mA and an energy of 1.8 GeV. A silicon (111) monochromator was used, and harmonic contamination (particularly at the calcium K -edge) was minimized by running the ionization chambers semi-transparent ($T_{I_0} \sim 90\%$ and $T_I \sim 50\%$), and this was also helped by the relatively low machine energy.

E.x.a.f.s. and x.a.n.e.s. results

An X-ray absorption spectrum is customarily subdivided into two regions. The first 40–50 eV, the X-ray-absorption near-edge structure, is usually referred to as x.a.n.e.s.; the acronym e.x.a.f.s. (extended X-ray-absorption fine structure) is reserved for the remaining structure, which generally runs out to many hundreds of electronvolts above the absorption threshold. For an introduction to x.a.s. for structure determination several reviews can be recommended, notably Stern (1978), Teo (1980) and Gorman (1982).

Normalized e.x.a.f.s. spectra, $\chi(k)$, for the calcium and manganese K -edges of normal and doped granules are presented in Fig. 2. The existence of fine structure points to well-defined cation sites. Site variability cannot be ruled out, but is clearly limited for the fine structure to be resolved. The

obvious differences between the calcium and manganese spectra demonstrate immediately that the local atomic structures for the two cations are not the same. The different periods of the fine-structure oscillations, for instance, stem from different cation–oxygen bond lengths for calcium and manganese. These differences in e.x.a.f.s. period are indicated by the sequence of arrows in Fig. 2, but are particularly clear in the partial e.x.a.f.s. spectra shown below in Fig. 6(a).

Measurements of the calcium K -edge were also made for the doped granules, and the fine structure was found to be essentially identical with that of the undoped granules shown in Fig. 2(a). This is in line with the X-ray microanalysis, which, as we have seen (Fig. 1), demonstrates the bulk of the calcium in doped granules resides in the core, where the concentration of introduced manganese is least.

Information about the nature of the cation site, i.e. its symmetry and oxidation state, can be deduced from the x.a.n.e.s. and the absolute position of the absorption threshold (Greaves *et al.*, 1981b, and references cited therein). The manganese x.a.n.e.s. for the granules is plotted in Fig. 3(a), where it is compared with the x.a.n.e.s. of metallic manganese (α -Mn), MnO and KMnO_4 . In the metallic structure manganese atoms have 12 or 13 near-neighbour atoms with a peak at 0.275 nm (2.75 Å) (Wyckoff, 1964). MnO has the rock-salt structure, with each manganese atom octahedrally co-ordinated to six oxygen atoms at 0.222 nm (2.22 Å). In the MnO_4^- anion manganese is tetrahedrally co-ordinated with an Mn–O bond length of 0.155 nm (1.55 Å). From Fig. 3(a) there is an obvious difference in the x.a.n.e.s. for these three cation sites. The dipole selection rule dictates that K -shell absorption edges are dominated by transitions of $1s$ electrons to empty states with p -like symmetry. The strong pre-edge spike for KMnO_4 is due to transitions to empty d -states, which are drawn into hybridization with p -states by the tetrahedral symmetry. This feature is diminutive for MnO and becomes absorbed into the absorption edge as a shoulder for the metal. It is clear from Fig. 3(a) that the x.a.n.e.s. for the doped granules closely resembles that of MnO , indicating the local symmetry is close to octahedral. The x.a.n.e.s. of $\text{Mn}_2\text{P}_2\text{O}_7$ and $\text{MnHPO}_4 \cdot 3\text{H}_2\text{O}$ are compared with those of the granules in Fig. 3(b). These crystalline phosphates are considerably disordered, although the local symmetry of manganese is always close to octahedral. The overall similarity between the manganese x.a.n.e.s. for the crystalline phosphates and the granules is clear and underlines that the local symmetry is broadly similar. The differences in detail derive from the degree of disorder in the first shell of atoms and

Fig. 1. Electron micrographs showing the spherical granules isolated from the hepatopancreas of *Helix aspersa* (a) Granules of normally fed animals; (b) granules of animals whose diet was sprayed with aqueous MnCl_2 . Magnification $\times 10000$. Electron-microprobe analyses are also shown: (c) normal granules; (d) the rough edges of doped granules; (e) the centre of doped granules. Cation/P ratios are given in Table 1.

also the atomic arrangement outside the 'octahedral' ligand.

Evidence for the oxidation state of manganese in the granules can be drawn from the absolute position of the X-ray-absorption edge (Belli *et al.*, 1980; Brown *et al.*, 1984). This is customarily defined as the location of the first major peak in the derivative spectrum. The positions of the *K*-edges for the granules and the two crystalline phosphates $\text{Mn}_2\text{P}_2\text{O}_7$ and $\text{MnHPO}_4 \cdot 3\text{H}_2\text{O}$ are presented in Table 2. At 6547.2 eV the *K*-edge of the granules falls midway between those for $\text{Mn}_2\text{P}_2\text{O}_7$ and $\text{MnHPO}_4 \cdot 3\text{H}_2\text{O}$, indicating that the oxidation state is most likely to be Mn^{2+} .

The position of the absorption edge is strongly influenced by the screening of the core potential by the valence electrons. This is greatest for metallic manganese (6538.0 eV) and least for manganese covalently bonded in permanganate (6557.2 eV), an edge shift of over 19 eV (Brown *et al.*, 1984). Edge shifts for other oxides are in between. Absolute *K*-edge positions for Mn, MnO, Mn_2O_3 , MnO_2 and MnO_4^- are also assembled in Table 2.

Table 2. Absolute positions of the *K*-edges of manganese for the granules compared with various oxides and phosphates. Results for the oxides are taken from Brown *et al.* (1984). Edge shifts are relative to metallic manganese (α -Mn). Formal oxidation states for the simple oxides are given. (The instrumental energy resolution is 0.4 eV.)

Sample	Valence state	<i>K</i> -edge position (eV)	Edge shift (eV)
α -Mn	0	6538.0	0
MnO	2	6543.6	5.6
$\text{Mn}_2\text{P}_2\text{O}_7$		6545.9	7.9
Granules		6547.2	9.2
$\text{MnHPO}_4 \cdot 3\text{H}_2\text{O}$		6547.8	9.8
Mn_2O_3	3	6548.4	10.4
MnO_2	4	6551.2	13.2
KMnO_4	7	6557.2	19.2

Although phosphates of manganese will follow a similar trend, absolute values will differ because, in addition to the screening of the core hole, the X-

Fig. 2. Normalized e.x.a.f.s. for normal and doped granules

Electron micrographs of the extracts are shown in Figs. 1(a) and 1(b) respectively. E.x.a.f.s. is plotted as a function of photoelectron energy, E , and wavevector, k (see the text for details). The calcium K -edge of the undoped granules is shown in (a) and the manganese K -edge of the doped granules in (b). The calcium K -edge of the doped granules (not shown) was essentially identical with (a). The arrows highlight the underlying oscillation due to the back-scattering of the photoelectron by nearest-neighbour oxygen atoms.

ray-absorption edge is also affected by the type and configuration of the surrounding anions. The edges for the phosphates in Table 2 are between those of MnO and Mn_2O_3 , which suggests that phosphate groups affect the electronic structure differently to oxygen atoms, shifting the K -edge to higher energies by several electronvolts.

By comparison the position of the K -edge of calcium for the undoped and doped granules is the same as that of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ as well as of $\text{Ca}_2\text{P}_2\text{O}_7$ (i.e. within the 0.4 eV instrumental resolution). This is to be expected for a group-II

bivalent cation sharing a similar oxygen ligand. The x.a.n.e.s. profiles for these calcium phosphates are very similar to the calcium near-edge structure of the granules. Interestingly, all show a marked similarity to the x.a.n.e.s. of manganese, thereby demonstrating a common local symmetry for the two cations. The overall equivalence of features in the x.a.n.e.s. spectra of isostructural materials has been well demonstrated for a range of oxides (Knapp *et al.*, 1982). Fig. 3(c) shows the x.a.n.e.s. of manganese and calcium for the granules plotted on the same energy scale. Despite these similarities in overall local symmetry obtained from inspection of the x.a.n.e.s., it is shown below that the calcium and manganese sites are in fact structurally distinct. Analysis of the e.x.a.f.s. reveals that the average shell radius as well as the local disorder for calcium and manganese are quite different.

Analysis

General atomic distributions

A picture of the local atomic structure surrounding calcium and manganese can be obtained by Fourier-transforming the normalized fine structure $\chi(k)$ with respect to k , the photoelectron wave vector. k is given by

$$k = \frac{\sqrt{2m(E - E_0)}}{\hbar}$$

where m is the free electron mass and $\hbar = h/2\pi$, h being Planck's constant. E_0 is the energy zero of the ejected electron. For all calcium spectra E_0 was taken 16 eV below the position of the absorption edge. E_0 for the manganese spectra was 29 eV below the absorption edge. The Fourier transform used, $F(r)$, is given by the expression:

$$F(r) = \frac{1}{\sqrt{2\pi}} \left| \int_{k_{\min.}}^{k_{\max.}} \frac{W(k) \cdot k \cdot \chi(k) \cdot \exp[-i(kr + \phi)] dk}{f(k)} \right| \quad (1)$$

where $f(k)$ is the back-scattering amplitude (for oxygen in this case) and ϕ is the combined phase shift for the scattered photoelectron from calcium or manganese with an oxygen environment. ϕ was calculated by using the SRS MUFOT program. $W(k)$ in the above expression is a Gaussian window function introduced to help minimize termination ripples resulting from the finite wave-vector range [for details see Gurman & Pendry (1976)]. $W(k)$ was centred around 70 nm^{-1} ($7 \text{ \AA}^{-1}$) or 170 eV above the absorption edge, i.e. roughly in the centre of the data. The precise position of $W(k)$ and the accompanying value of E_0 were chosen for

Fig. 3. X.a.n.e.s. of manganese for the doped granules compared with various model compounds

In (a) the doped granules are compared with metallic α -manganese, MnO and KMnO_4 ; in (b) the doped granules are compared with $\text{Mn}_2\text{P}_2\text{O}_7$ and $\text{MnHPO}_4 \cdot 3\text{H}_2\text{O}$; in (c) x.a.n.e.s. of manganese is compared with x.a.n.e.s. of calcium for the doped and the undoped granules respectively. The similarity stems from the similar local symmetry of the two cations.

the Ca and Mn *K*-edges to provide the best agreement with the known structures of CaO and MnO (see below). The width of the window was 160 eV for both edges.

The local atomic environments, $F(r)$, of calcium and manganese for the granules are presented in Fig. 4. The overall structure in either case is qualitatively similar to that found around modify-

Fig. 4. Atomic distributions, $F(r)$, surrounding (a) calcium and (b) manganese for the natural and the doped granules. These were obtained by Fourier-transforming the normalized e.x.a.f.s., $\chi(k)$, given in Fig. 2 (see the text for details).

ing cations in other disordered oxides, such as silicate glasses (Greaves *et al.*, 1981a; Greaves & Raoux, 1983). The first peak in each distribution is due to the oxygen ligand. Ca–O bonds can be clearly seen to be longer than Mn–O bonds, the distances being 0.230 nm (2.30 Å) and 0.218 nm (2.18 Å) respectively. It should be noted also that the oxygen peak is sharper for manganese than for calcium, suggesting a less-disordered site. Beyond the first shell the structure for the granules is comparatively diminished. It relates to the first shell of phosphorus atoms, shells of calcium or manganese atoms and further oxygen shells. Compared with the calcium environment, structure in this region for the manganese distribution is drawn to shorter atomic distances by the shorter metal–oxygen bond length. The relative strength of these outer shells is a combination of the type of atom in each shell, the degree of disorder present and the atomic density.

Although manganese has previously not been studied by using e.x.a.f.s. in a biological system, several studies of calcium have been made (Hasnain, 1983, and references cited therein). The local

atomic structure surrounding calcium measured in the present work in the granules is distinct from that found in bone. There is a strong second peak in $F(r)$ for bone in place of the two other smaller peaks at 0.34 and 0.40 nm (3.4 and 4.0 Å) found in the granules. On the other hand, calcium distribution has similarities with that measured in bovine casein micelles of milk (Holt *et al.*, 1983).

In Fig. 5 the Fourier transforms, $F(r)$, for CaO, $\text{Ca}_2\text{P}_2\text{O}_7$, $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, MnO, $\text{Mn}_2\text{P}_2\text{O}_7$ and $\text{MnHPO}_4 \cdot 3\text{H}_2\text{O}$ are compared with the granule data. These were obtained by using identical spectral ranges, the same values of E_0 and the same Fourier windows, $W(k)$, as for the granule profiles presented in Fig. 4. Each group is therefore internally numerically consistent. Where the crystal structure is known, the radial distribution of atoms around each cation is shown underneath. The accuracy of the e.x.a.f.s. radial distances can be judged by comparing the positions of the peaks in $F(r)$ with the crystallographic shell radii. This is done for the first oxygen-atom shells in Table 3. E.x.a.f.s. values agree with crystallographic values for CaO and MnO to 0.002 nm (0.02 Å). Agreement with the phosphates is less good. In most cases, however, accurate crystallographic studies have not been made. The exception is $\text{Ca}_2\text{P}_2\text{O}_7$ (Webb, 1966), where the agreement with e.x.a.f.s. is closest. It is also worth noting from Table 3 that the Mn–O bond lengths for the granules and the two phosphates are practically equal [0.218 ± 0.003 nm (2.18 ± 0.03 Å)] and lie close to the Mn–O bond length in MnO [0.220 nm (2.20 Å)]. By comparison the bond length in Mn_2O_3 is significantly shorter [0.199 nm (1.99 Å)], which is further evidence that the oxidation state of manganese in the granules is Mn^{2+} .

The accuracy of shells more distant than nearest neighbours is less precise [± 0.01 nm (0.1 Å)]. This is partly due to the phase shifts ϕ , which are inappropriate for cation shells, and also the precise position of $W(k)$. Nevertheless individual shells can be readily identified out to 0.4–0.5 nm (4–5 Å), particularly for the simple cubic structures of CaO and MnO.

It can be seen from the histograms in Fig. 5 that crystalline phosphate structures like silicates are considerably disordered. The first shell of oxygen atoms is often spread over 0.05 nm (0.5 Å) or more, and the atomic distribution beyond is one of increasing complexity. The different types of atom add some simplification: calcium and manganese are stronger back-scatterers than phosphorus, and this in turn scatters more strongly than oxygen. So for $\text{Ca}_2\text{P}_2\text{O}_7$ the strong second peak in $F(r)$ shown in Fig. 5 is largely due to phosphorus and calcium, phosphorus giving rise to the shoulder at around 0.32 nm (3.2 Å) and calcium to the peak at 0.37 nm

Fig. 5. Atomic distributions, $F(r)$, surrounding calcium (a) and manganese (b) for CaO (i), $\text{Ca}_2\text{P}_2\text{O}_7$ (ii), $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (iii), MnO (v), $\text{Mn}_2\text{P}_2\text{O}_7$ (vi) and $\text{MnHPO}_4 \cdot 3\text{H}_2\text{O}$ (vii) compared with the calcium (iv) and manganese (viii) distributions of the granules

Note the different vertical scales. The diminishing contribution of structure beyond the first shell of oxygen atoms evident from frames (i) to (iv) and from frames (v) to (viii) follows the decrease in density (Table 3) due to the increase in water content. The histograms are drawn from the crystallographic determinations (see Table 3 for details). □, Oxygen shells; ▨, phosphorus shells; ■, calcium or manganese shells.

(3.7 Å). $\text{Mn}_2\text{P}_2\text{O}_7$ is qualitatively similar. The peak at 0.32 nm (3.2 Å) is a combination of a phosphorus-atom shell at 0.31 nm (3.1 Å) and a man-

ganese-atom shell centred at 0.34 nm (3.4 Å). Detailed crystal structures for $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{MnHPO}_4 \cdot 3\text{H}_2\text{O}$ are not available, but it is

Table 3. *Densities and metal–oxygen bond lengths for the granules compared with oxides and phosphates of calcium and manganese*

Key to references: ^aWyckoff (1964); ^bWebb (1966); ^cBeevers (1958) and Jones & Smith (1982); ^dLukaszewicz & Smajkiewicz (1961); ^eDurif (1971), and see also the structure of $\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$ (Sutor, 1967).

Material	Density (g/cm ³)	Bond	Crystallography bond length [nm (Å)]	E.x.a.f.s. bond length [nm (Å)]
CaO	3.31	Ca–O	0.240 (2.40) ^a	0.238 (2.38)
Ca ₂ P ₂ O ₇	3.12	Ca–O	0.239 (2.39) ^b	0.243 (2.43)
CaHPO ₄ ·2H ₂ O	2.32	Ca–O	0.247 (2.47) ^c	0.238 (2.38)
Granules	2.07	Ca–O		0.230 (2.30)
MnO	5.45	Mn–O	0.222 (2.22) ^a	0.220 (2.20)
Mn ₂ P ₂ O ₇	3.72	Mn–O	0.208 (2.08) ^d	0.215 (2.15)
MnHPO ₄ ·3H ₂ O	2.34	Mn–O	0.212 (2.12) ^e	0.220 (2.20)
Granules	2.07	Mn–O		0.218 (2.18)

expected that a similar interpretation will apply. For hydroxyapatite, on the other hand, the calcium e.x.a.f.s. is significantly different (Holt *et al.*, 1983), such that the second peak in $F(r)$ due to calcium and phosphorus is substantially stronger than either of the phosphates measured in the present work, and this rules this mineral out as a model for the granules.

Turning back to the atomic distributions for the granules presented in Fig. 4, we see that the strengths of features beyond the first shell of oxygen atoms for both calcium and manganese are less than equivalent features in the anhydrous pyrophosphates and are comparable in magnitude with those in the hydrated hydrogen phosphates. To a first approximation this reflects the lower density of hydrated phosphates. The more water that is present in the structure, the fewer calcium/manganese and phosphorus atoms that will be co-ordinated to the central metal atom through nearest-neighbour oxygen atoms. This can be clearly seen in Fig. 5 by comparing the strength of the Ca/Mn (■) and P (▣) shells of the crystalline structures with the physical density. Table 3 lists the densities of the oxides and phosphates investigated, including the granules. The structures of CaO and MnO are cubic and ordered and constitute the most efficient packing of bivalent cations with oxygen. The second peak, which is due to metal–metal correlations for CaO and MnO [about 0.32 nm (3.2 Å)], is approximately 4 times the magnitude of the first peak due to nearest-neighbour oxygen atoms in the $F(r)$ values presented in Fig. 5. For phosphates, by comparison, the second peak in the atomic distribution is always substantially smaller. Phosphates, however, form layer and chain structures and have lower physical densities compared with the cubic rock-salt structure of CaO or MnO.

The presence of water decreases the density still more effectively by intercolating the phosphate

sheets. This is most obviously seen in the hydrated pyrophosphates. $\text{Ca}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ has a density of 2.55 g/cm³ compared with 3.12 g/cm³ for $\text{Ca}_2\text{P}_2\text{O}_7$ (Webb, 1966; Mandel, 1975); $\text{Mn}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ has a density of 2.95 g/cm³ compared with 3.72 g/cm³ for $\text{Mn}_2\text{P}_2\text{O}_7$ (Lukaszewicz & Smajkiewicz, 1961; Schneider & Collin, 1973). The considerable water content of the granules (18%, w/w) contributes to their low density (1.93–2.07 g/cm³). Furthermore the e.x.a.f.s. $F(r)$ values show only a very weak metal–metal/phosphorus peak. Taken together, this strongly suggests a hydrated open structure for the intracellular granules.

Profiles of nearest neighbour oxygen-atom distributions

E.x.a.f.s. is particularly sensitive to nearest-neighbour atoms. In most systems to which e.x.a.f.s. has been applied, the distribution of nearest-neighbour atoms is gaussian. However, asymmetry can be detected by using this technique provided that the static disorder is significantly greater than the disorder resulting from thermal vibrations. Previous studies in this area have been chiefly concerned with the non-gaussian distribution of nearest-neighbour atoms in liquid and amorphous metals and alloys (Eisenberger & Brown, 1979; Crozier & Seary, 1980; Greaves & Raoux, 1983). By comparison, disorder in the oxygen ligand of calcium in crystalline phosphates is far more extensive (see histograms in Fig. 5a). It results not so much from the packing together of metal atoms and oxygen atoms as from the steric hindrance generated by the structural organization of the phosphate component. Indeed, the particular disorder displayed in the nearest-neighbour oxygen atoms provides a signature of the overall structure. In amorphous phosphates like the granules the shape of the distribution of nearest-neighbour oxygen atoms can be used to distinguish one site from another.

Partial e.x.a.f.s. spectra, $\chi_1(k)$, for the oxygen shells of calcium and manganese are shown in Fig. 6(a). These were obtained by windowing the first peak in the Fourier transform and back-transforming from r -space into k -space. Unlike eqn. (1), phase shifts were not used [$f(k) = 1$, $\phi = 0$] and a rectangular window function was used for $W(k)$. A least-squares routine was then used to fit the partial oxygen-shell spectra to the spherical-wave approximation for e.x.a.f.s. (Lee & Pendry, 1975). Starting conditions were a single shell of oxygen atoms. For calcium this consisted of eight oxygen atoms at 0.23 nm (2.3 Å) and for the manganese six oxygen atoms at 0.22 nm (2.2 Å), the co-ordination numbers and shell radii suggested by the crystal-line phosphates and pyrophosphates (see Table 3 for references). (Although the curve-fitting least-squares routine used is sensitive in principle to relatively small changes in co-ordination number that would distinguish six from eight oxygen atoms

in the first shell, the disorder in these distances weakens this precision. Moreover the amplitudes of the e.x.a.f.s. oscillations that govern the co-ordination number are prone to several sources of error, some empirical and others associated with data reduction. For the present measurements the overall precision in total co-ordination number for calcium or manganese is likely to be between 20 and 30%, which means little distinction can be made between 6- and 8-fold co-ordination.) Individual radii for each spectrum were allowed to float along with the energy zero E_0 . The final oxygen-shell distributions for Ca and Mn are shown in Fig. 6(b), and the theoretical fit to experiment is given by the broken curves in Fig. 6(a). The thermal variance in shell radii used (the Debye-Waller factor) was 0.00009 nm² (0.009 Å²). This value is similar to that found for sodium e.x.a.f.s. in silicate glasses (Greaves *et al.*, 1981a) and is close to the values deduced in recent molecular-dynamics

Fig. 6. Nearest-neighbour oxygen-shell data for calcium and manganese for the granules

(a) Partial e.x.a.f.s. functions $\chi_1(k)k^3$ (shown by continuous lines). These were obtained by back-transforming the first peak in the Fourier transform (see Fig. 4). The broken lines are the results of a least-squares fitting of the spherical-wave approximation (Lee & Pendry, 1975). (b) Gaussian-broadened histograms for the oxygen-shell distributions (shown by continuous lines). These were obtained by curve-fitting the partial e.x.a.f.s. functions presented in (a). Individual atom positions are shown below by the broken lines.

calculations for CaF_2 (L. Moroney, unpublished work). Individual oxygen distances are shown in Fig. 6(b) by the broken curves, each of which has been gaussian-broadened by the same amount. The full-width-half-maximum of each line was $2.35 \sqrt{0.00009} = 0.022 \text{ nm}$ ($0.22 \text{ \AA}$). The total oxygen-shell profiles for calcium and manganese are shown by the continuous curves. Note that the peak positions for the total distributions are slightly different from those taken from Fig. 4 because different spectral ranges and E_0 values were used in the least-squares fitting.

It is clear from Fig. 6 that the distribution of nearest-neighbour oxygen atoms surrounding

introduced manganese in the granules is different from that surrounding calcium. The average bond length is shorter, as has already been noted (Table 3), but it is also clear that the static disorder in the oxygen distances is less for manganese than for calcium. The range of oxygen distances surrounding manganese (Fig. 6b), Δr , is 0.027 nm ($0.27 \text{ \AA}$) compared with 0.044 nm ($0.44 \text{ \AA}$) for calcium sites. The relatively narrow distribution of Mn–O distances in the granules mirrors the situation in phosphates of manganese. In $\text{Mn}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$, for instance, the spread in oxygen distances $\Delta r = 0.0201 \text{ nm}$ ($0.201 \text{ \AA}$) (Schneider & Collin, 1973). In the doped granules, manganese

Fig. 7. Gaussian-broadened histograms for the oxygen-shell distributions of calcium

These were obtained by curve-fitting the partial e.x.a.f.s. function $\chi_1(k)k^3$ for CaO (i), $\text{Ca}_2\text{P}_2\text{O}_7$ (ii), $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (iii) and the granules (iv). The broadened histograms from crystallographic studies are also shown: CaO (v), $\text{Ca}_2\text{P}_2\text{O}_7$ (vi) and $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (vii).

clearly occupies a site distinct from that of calcium. This demonstrates that manganese chemically bonds into the growing structure rather than ion-exchanges for Ca^{2+} in the existing granule material. All of this is consistent with the electron-micrograph results (Fig. 1), which indicate that introduced manganese is largely concentrated in the irregular surfaces of the doped granules rather than in the centres.

In Fig. 7 we show the broadened oxygen first-shell distributions for CaO , $\text{Ca}_2\text{P}_2\text{O}_7$ and $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, and alongside the distributions derived from single-crystal X-ray-diffraction results. Excellent agreement is obtained in the shape of the distributions for CaO and $\text{Ca}_2\text{P}_2\text{O}_7$. This is also true for the general atomic distributions shown in Fig. 5(a). The correspondence between e.x.a.f.s. and X-ray crystallography for $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ is less good. The crystal structure of brushite, however, is not accurately known. The refinement factor reported for $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ was 0.164 (Jones & Smith, 1982), compared with 0.068 for $\text{Ca}_2\text{P}_2\text{O}_7$ (Webb, 1966), which probably accounts for the poorer agreement with e.x.a.f.s. The oxygen first-shell distribution for the granules is also included in Fig. 7. Although agreement with either of the model compounds is not perfect, it is clear that the oxygen-shell distribution of the granules is modelled more closely by $\text{Ca}_2\text{P}_2\text{O}_7$ than by $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$, suggesting that many calcium atoms in the granules are bound to pyrophosphate groups. It is shown below that there is strong evidence from i.r. spectroscopy that pyrophosphate ($\text{P}_2\text{O}_7^{4-}$) groups are present in the granules (Fig. 8).

A similar exercise for the manganese first-shell e.x.a.f.s. spectra was carried out, but the results indicated little difference between the model compounds and the granules. This similarity in the shape of the oxygen nearest-neighbour distributions for manganese phosphates stems from the narrow range of Mn–O distances [≤ 0.02 nm (≤ 0.2 Å)] compared with the thermal disorder of 0.022 nm (0.22 Å).

I.r. spectroscopy

The identity of the phosphate groups bound to calcium or manganese is difficult to judge simply from the cation e.x.a.f.s. Phosphorus e.x.a.f.s. will give more direct information about this, but x.a.s. measurements require soft-X-ray radiation (Greaves & Raoux, 1983). The main evidence to date for the existence of pyrophosphate groups in the intracellular granules rests with the enzymic assay and the vibrational modes manifest in the i.r.-absorption spectra (Howard *et al.*, 1981). Fig. 8 compares the i.r.-absorption spectra in the range 1300 – 400 cm^{-1} for the granules with several

Fig. 8. I.r.-absorption spectra for $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (i), hydroxyapatite (ii), $\text{Ca}_2\text{P}_2\text{O}_7$ (iii), $\text{Na}_4\text{P}_2\text{O}_7$ (iv) and the intracellular granules (v).

phosphates and condensed phosphates, namely $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (brushite), hydroxyapatite and the pyrophosphates $\text{Ca}_2\text{P}_2\text{O}_7$ and $\text{Na}_4\text{P}_2\text{O}_7$. All spectra exhibit a strong broad structured band around 1000 – 1200 cm^{-1} , which is largely due to the P–O stretching modes within the phosphate anions. The particular modes associated with condensed phosphate chains fall between this band around 1100 cm^{-1} and the deformation modes centred around 550 cm^{-1} . In particular, both $\text{Ca}_2\text{P}_2\text{O}_7$ and $\text{Na}_4\text{P}_2\text{O}_7$ have prominent absorption bands close to 920 cm^{-1} and 735 cm^{-1} , which can be assigned to the asymmetric and symmetric vibrations of the bridging P–O–P linkage respectively. These features are notably absent from the absorption spectra of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ and hydroxyapatite. In the case of the granules, however, there are strong bands at 920 and 745 cm^{-1} . Identical features have also been recorded for both natural and manganese-doped granules. The strong similarity between the i.r.-absorption spectra of the granules and the two pyrophosphates shown in Fig. 8 indicates the presence of condensed phosphates in

the intracellular deposits and the likelihood that these are pyrophosphate ($\text{P}_2\text{O}_7^{4-}$) groups.

Conclusion

In the present study we have demonstrated the usefulness of e.x.a.f.s. for measuring the local atomic structure of calcium in the intracellular phosphate granules of *Helix aspersa* and of probing the binding of a foreign bivalent metal, manganese. As in other applications, e.x.a.f.s. benefits from being combined with additional analytical techniques. We have used electron microscopy to gauge the cation composition and its topography in the doped granules. I.r. absorption has been used to confirm the presence of condensed phosphates. The e.x.a.f.s. technique, on the other hand, has provided accurate cation-oxygen bond lengths [0.230 nm (2.30 Å) for Ca-O and 0.218 nm (2.18 Å) for Mn-O] for these amorphous materials. Cation-phosphorus and cation-cation shells have been distinguished in the granules by reference to crystalline model compounds. The strength of these features combined with the low density of the granules (1.93–2.07 g/cm³) points to a hydrated open structure. Finally, the shape of the nearest-neighbour oxygen-atom distribution has been used to distinguish the manganese site from the more disordered calcium site. Once manganese has entered the cell it is precipitated as a phosphate predominantly on to the surface of existing granules rather than substituting for calcium in the existing concretions. Moreover, the oxygen-atom environment of calcium in the granules displays a marked resemblance to that measured in $\text{Ca}_2\text{P}_2\text{O}_7$.

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