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CALCIUM MAGNESIUM PHOSPHATE GRANULES: ATOMISTIC SIMULATIONS EXPLAINING CELL DEATH

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Summary

1. A large number of invertebrates have cells that produce intracellular granules of amorphous calcium magnesium phosphates that are thought to act as ion stores or sites of metal detoxification.

2. The interatomic potentials and force constants have been calculated for these ions, and computer simulations of the crystal lattices have been used to determine the effects of ion substitutions on these lattice energies.

3. The results provide insights into the mechanisms of granule formation and the effects of ion substitutions on cell physiology.

Introduction

The cellular roles of the metals that form the main electrolytes (Na^+, K^+, Ca^{2+} and Mg^{2+}) are well understood and provide a basis for our understanding of such fundamental phenomena as osmoregulation, neuromuscular physiology, sensory transduction, acid–base balance, intracellular signalling and enzyme activation. The involvement of a variety of other essential trace metals, such as chromium, manganese, iron, cobalt, nickel, copper, zinc, tin and molybdenum, is also well established and their roles in specific biochemical events are clearly documented (da Silva and Williams, 1991). What is less clear, however, is how these trace elements enter cells (Simkiss and Taylor, 1994). There could be specific and well-regulated ion pumps that transport the ions across the plasma membrane on demand or, alternatively, they could enter by leakage through the main ion pumps and their intracellular activity could be regulated by detoxification systems. The ability to distinguish between these hypotheses is largely a technical one. Trace elements exist at ionic concentrations in the range 10^{-8}–10^{-10} mol l^{-1}, which is at the limit of many analytical methods. This makes it particularly difficult to study them *in vivo* and to understand the principles involved in their biological properties.

In an attempt to consider some of these questions, Simkiss (1981) exposed cells of the snail *Helix aspersa* to trace amounts of a number of metal ion pairs and then measured their relative concentrations in tissues, cytoplasmic fractions and particulate deposits. The discrimination processes that were revealed demonstrated two clear pathways for

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trace element metabolism. One of these accumulated the more covalent-binding group 'b' metals (Ahrland *et al.* 1958), such as zinc, cadmium and mercury, and retained them in the cytosol. Such a system would be in keeping with the known properties of the metallothionein molecules, which are sulphydryl-rich, inducible proteins. Metallothioneins possess two metal-binding clusters and occur in virtually all animals (Riordan and Vallee, 1991). The second pathway that was revealed involved the accumulation of group 'a' metals in membrane-bound inorganic granules of a type that occurs in cells of virtually every phylum of animals (Simkiss, 1976; Brown, 1982; Taylor and Simkiss, 1984; Hopkin, 1989). The granules are typically amorphous solids without any stoichiometric composition. They have the general formula $Ca_wMg_x(PO_4)_2$ or $Ca_yMg_z(P_2O_7)$.

Our understanding of both the metallothionein protein and the inorganic granule systems suffers from the same problem. It is not clear whether metallothioneins are storage systems for essential ions, such as Zn^{2+} and Cu^{2+} (Brady *et al.* 1982), or detoxification pathways for non-essential elements, such as Hg^{2+} or Cd^{2+} (Webb, 1987). The phosphatic granules may similarly either act as essential phosphate stores (Munk and Rosenberg, 1969) or provide detoxification routes for the ionic group 'a' metals (Simkiss, 1981). These interpretations are ways of describing the properties of these systems and great progress has been made in recent years in understanding the induction, binding properties and turnover rates of metallothioneins *in vitro*. In an attempt to extend this approach to the membrane-bound granules, we have studied their properties *in vivo* and *in vitro* in relation to both their chemical and structural forms (Howard *et al.* 1981; Mason and Simkiss, 1982; Taylor *et al.* 1988, 1990).

In the snail *Helix aspersa*, these granules occur in large numbers (approximately 10^3 per cell) in pyramid-shaped 'calcium cells' in the hepatopancreas. The granules are periodically shed from these cells and appear as white accumulations in the faecal strands. Chemically, they have an approximate composition of $CaMgP_2O_7$ in an amorphous glass-like form, i.e. they do not possess any long-range order of the type found in crystal lattices, but can be envisaged as random networks (Fig. 1). This model is based on Zachariasen's (1932) interpretation of silica glasses, where the network structure is favoured by tetrahedral anions joined at their corners but not by their edges or faces. With the advent of X-ray analysis, it was shown with silicate glasses that the incorporation of soda led to 'soda glasses' in which the sodium ions occupied the larger holes in the random network. The incorporation of such 'modifiers' is the basis for the disposal of waste metals by the industrial process of vitrification and it is clearly favoured by the absence of a rigid crystal lattice. It is interesting to note, therefore, that a similar process apparently occurs in a large number of living cells. The system is, however, not without its own chemical hazards. If snails are exposed to metal ions *in vivo* by feeding them on food contaminated with metals, the group 'a' elements become incorporated into the glass granule or react with it. In the case of manganese contamination, the metal corrodes the granule intracellularly, producing concretions on its surface by displacing calcium ions from its structure and killing the surrounding cell in a unique form of cell pathology (Taylor *et al.* 1988). If Zn^{2+} is fed to the snails it is assimilated and penetrates the granules, transforming the anion from pyrophosphate ($P_2O_7^{4-}$) to orthophosphate

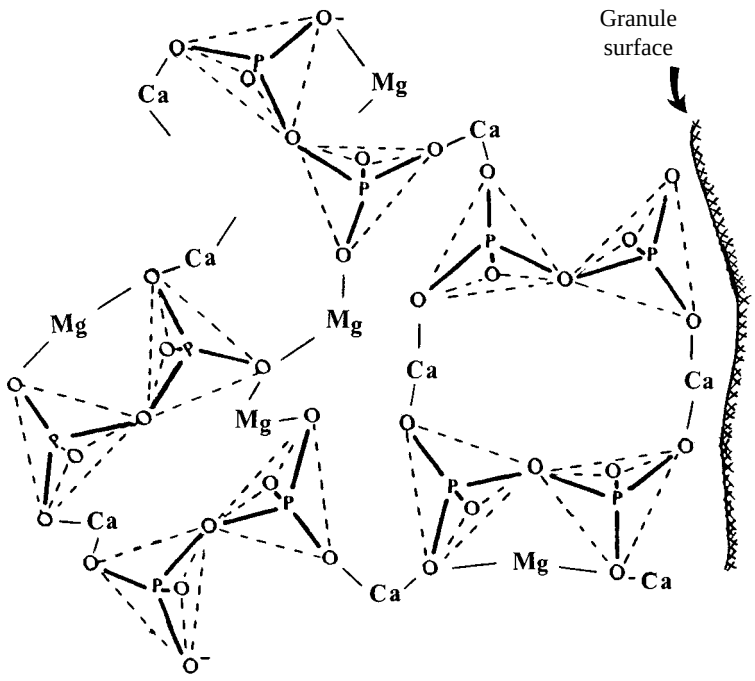

Fig. 1. Random network model of a $CaMgP_2O_7$ granule.

(PO_4^{3-}) (Taylor *et al.* 1990). In order to understand the properties of these granules and their interaction with the surrounding cells, atomistic simulations have been used to investigate the effects of these cation substitutions on the lattice energy of the solid.

Materials and methods

The chemical structures of pyrophosphates have been reviewed by Clark and Morley (1976). In some pyrophosphates the bridging oxygen is the first coordination sphere (e.g. β-$Ca_2P_2O_7$), while in others the conformation may be stabilized by the bonding of the cation to both halves of the pyrophosphate dimer (e.g. α-$Ca_2P_2O_7$). A feature of both structures is the range of bond orders and bond distances in the phosphorus–oxygen link. In the low-temperature phase, magnesium pyrophosphate normally exists in the α-$Mg_2P_2O_7$ form with a bridging P–O–P bond angle that is greater than 140°. We have, therefore, used β-$Ca_2P_2O_7$ and α-$Mg_2P_2O_7$ as model compounds for perfect static-lattice simulations. Energy minimization methods have been used where the lattice energy is minimized as a function of structural variables such as unit cell dimensions, atomic coordinates, bond lengths and bond angles. At the minimum, the derivatives of the energy with respect to the geometric factors will be zero.

The energy of interaction between each of a pair of ions *i* and *j* with charges q_i and q_j is given by the equation:

$$V_{ij}(r_{ij}) = q_i \times q_j/r_{ij} + A_{ij}\exp(-r_{ij}/p_{ij}) - \chi_D(r_{ij})C_{6(ij)}/r^6_{ij}\,.$$

The derivation of these terms and the procedures for determining their values in computer modelling are described in detail by Taylor *et al.* (1992, 1994). The first term involves the electrostatic interactions between each pair of ions, the second term represents the non-bonding repulsion term arising from the overlap of the electron clouds of the approaching ions and the third term represents van der Waals attractive forces. In addition, two- and three-body force constants have been used to model the P–O stretching and O–P–O bending modes. Parameters for the interatomic potentials have been calculated for manganese and zinc pyrophosphates and related to the calcium and magnesium salts. These methods involve the computer programs THBREL and PHONON (available through SERC CCP5 scheme), which enable the crystal lattices to be relaxed so as to test the calculated interatomic potentials and force constants in terms of lattice energy minimization.

By using these atomistic simulations, it is possible to investigate the effects of cation substitutions into a variety of biominerals. These calculations have been performed for the substitution of Ca^{2+} into α-$Mg_2P_2O_7$ and for Mg^{2+} into β-$Ca_2P_2O_7$ at particular lattice sites. This provides an insight into the formation of the $CaMgP_2O_7$ granules that are found intracellularly. It is then possible to determine the lattice energy changes on substituting Mn^{2+} or Zn^{2+} into these solids in a chemical simulation corresponding to the biological detoxification process in the cell.

In order to relate these simulations to *in vivo* events, snails were fed for 15 days on a diet of carrots and cabbage on which a 1:1 mixture of manganese and calcium or zinc and calcium carbonates had been sprinkled. Normal and metal-treated snails were decapitated and their granules extracted by homogenization and centrifugation of their hepatopancreas. Granules were analysed by atomic absorption spectroscopy of samples digested in nitric acid, as described by Taylor *et al.* (1992). Similar samples from a snail given a pulse of manganese were coated with carbon and examined in a JEOL 300M scanning electron microscope.

Results

Changes in the lattice energies ($kJ\,mol^{-1}$) of β-$Ca_2P_2O_7$ have been calculated for the original crystals and for substitution of 2, 4 and 8 cations per unit cell of Mg^{2+}, Mn^{2+} and Zn^{2+}. Similar calculations were made for α-$Mg_2P_2O_7$ with substitutions of Ca^{2+}, Mn^{2+} and Zn^{2+}. Full details of these procedures and of the values obtained are given in Taylor *et al.* (1994). The results for these substitutions are shown in Fig. 2. Note that substitutions into the $Mg_2P_2O_7$ crystal increase the lattice energy, resulting in a destabilizing effect. Substituting ions into $Ca_2P_2O_7$ decreases the lattice energy and therefore has a stabilizing effect.

These results suggest, therefore, that Mg^{2+} would substitute readily into the $Ca_2P_2O_7$ lattice since this is energetically favourable, and it is this process that probably leads to the formation of an amorphous intracellular granule. The opposite possibility, i.e. the substitution of Ca^{2+} into the $Mg_2P_2O_7$ lattice, is energetically unfavourable and, therefore, unlikely to occur spontaneously.

On the basis of these data it is apparent that exposure of the granules to Mn^{2+} would

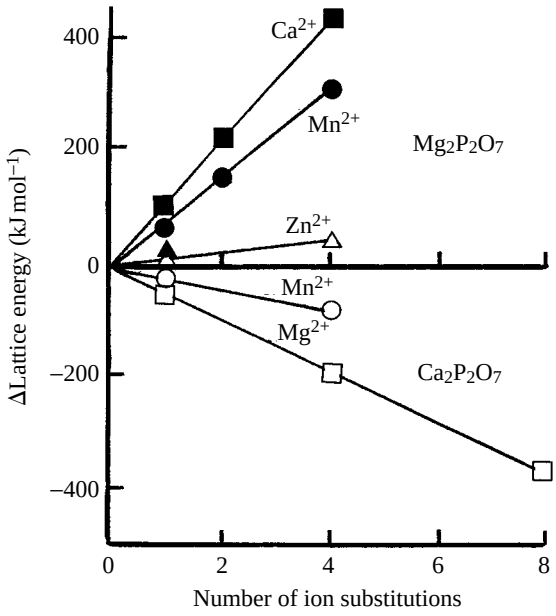

Fig. 2. Calculated changes in lattice energies ($kJ\,mol^{-1}$) involved in substituting 2, 4 or 8 foreign cations into $Mg_2P_2O_7$ (filled symbols) or $Ca_2P_2O_7$ (open symbols). Note that, for simplicity, ions with similar energy levels in a lattice are represented with a single symbol.

Table 1. *Cationic composition (mol %) of granules isolated from the hepatopancreas of snails*

	Granule composition (mol %)				Change in composition	
	Ca^{2+}	Mg^{2+}	Mn^{2+}	Zn^{2+}	ΔCa^{2+}	ΔMg^{2+}
Normal	49.9	49.1	0.2	0.8	–	–
Mn^{2+} diet	31.4	47.7	20.9	–	−18.5	−1.4
Zn^{2+} diet	28.2	37.6	0.2	33.9	−21.7	−11.5

Snails were fed either a normal diet or diets containing additions of either equimolar calcium and manganese (Mn^{2+} diet) or calcium and zinc (Zn^{2+} diet) carbonates for 2 weeks.
–, not measured.

lead to the substitution of Mn^{2+} for Ca^{2+}. In contrast Zn^{2+} has virtually no energetic effect on being substituted into the lattice. The analyses of granules from snails fed food contaminated with these ions are shown in Table 1. The structure of the granules was disrupted by feeding these ions to the snails. In the case of manganese, the granules became corroded by rosettes of manganese pyrophosphate (Simkiss *et al.* 1982) at specific sites on the granule surface (Fig. 3). Zinc had no effect on the structure of the granules when administered in the diet, but it facilitated the hydrolysis of pyrophosphate to orthophosphate (Taylor *et al.* 1990).

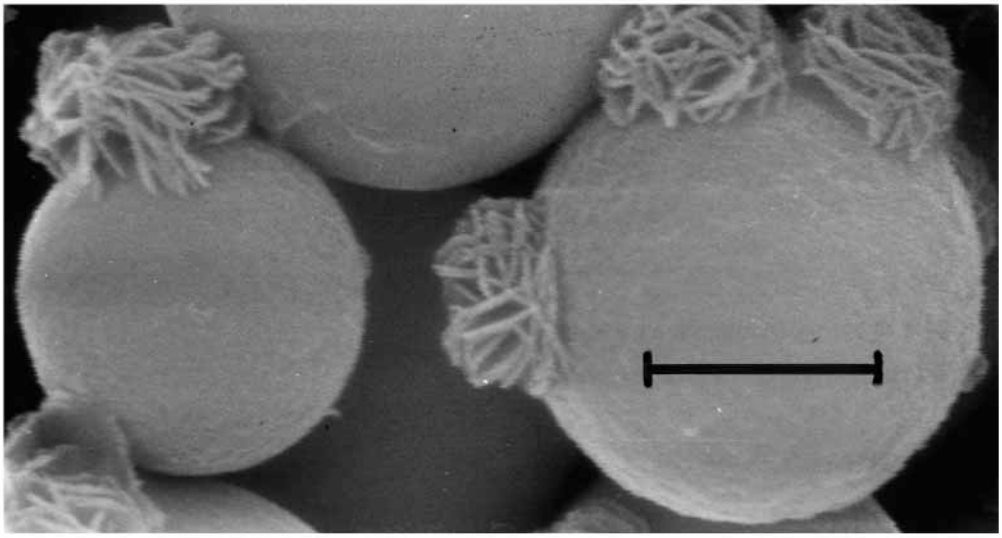

Fig. 3. Electron micrograph of granules removed from the hepatopancreas of a snail given a short pulse of manganese ions. The encrustation on the surface of the granule is composed of $Mn_2P_2O_7$. Scale bar, 1 μm.

Discussion

In the years since 1828, when Friedrich Wöhler synthesized urea, it has become accepted that biochemistry is a particular form of organic chemistry. The application of the kinetics of catalysed reactions, solute partitioning, stereochemistry and electron transfer reactions have, therefore, all been applied to reactions that explain particular problems of cell biology. The application of equivalent concepts in inorganic chemistry has been much slower, with emphasis being restricted mainly to electrolytes and metal ion–protein complexes. This situation will probably change quite rapidly with the application of computer simulation and molecular modelling techniques, but there is a dearth of experimental data upon which to build. Our previous experiment tracing the relative fluxes of metal ions in the snail *H. aspersa* (Simkiss, 1981) is a clear demonstration that the principles of the relative affinities of ligand atoms for acceptor molecules and ions expounded by Arhland *et al.* (1958) are directly applicable to biological systems, and these concepts are used by biologists in a more simplified form by reference to group ‘a’ and group ‘b’ metals in ecotoxicology studies (Nieboer and Richardson, 1980). In a similar study on the crayfish *Austropotamobius pallipes*, Lyon *et al.* (1984) were able to show that the rate of disappearance of metal ions from the blood included a fast component that could be explained on the basis of an Irving–Williams (1953) series for predicting the stability of transition-metal complexes. In both of these cases, therefore, it has been possible to show that the principles of inorganic chemistry can be applied directly to phenomena observed in biology.

In the current work, we have attempted to extend this approach to provide an understanding of the incorporation of intracellular metal ions into the solid phase of

amorphous minerals and its impact on cell biology. In a series of studies, we have used the method of static simulation of ionic lattices to model the phosphatic components of a variety of biominerals (Taylor *et al.* 1992, 1994). The parameters for the interatomic potentials have been determined by treating the mineral as an ionic solid with Mg^{2+} or Ca^{2+} as the cations and $P_2O_7^{4-}$ as the predominantly covalent anion that is bound to them by essentially electrostatic forces. Analyses of the intracellular granules formed by the snail *H. aspersa* reveal a surprising result in that these deposits have an approximate composition of $CaMgP_2O_7$ (Table 1), despite the fact that the intracellular ratios of Ca^{2+} (approximately 10^{-7} mol l^{-1}) and Mg^{2+} (approximately 10^{-3} mol l^{-1}) differ by about 10^{-4}. The studies shown in Fig. 2 suggest that magnesium stabilizes the calcium pyrophosphate structure, but with enough disruption to form an amorphous random network structure. The significance of these properties is that the amorphous structure clearly facilitates the movement of ions from the solid phase back into solution and also permits the incorporation of a variety of foreign ions. This enables the granules to act as phosphate stores or detoxification routes. It is apparent, however, from the chemical analyses shown in Table 1, that if these animals are exposed to manganese these ions are also incorporated into the network at the expense of calcium ions. This is clearly shown by the atomistic simulations given in Fig. 2. The result of this substitution is apparent *in vivo* since the intracellular granules actually corrode, and cells from animals that have been exposed to this treatment contain granules with large encrusted corrosion sites on their surfaces (Fig. 3). Zinc ions substitute for both calcium and manganese ions in the granules (Table 1), probably because there is very little difference in the energy of the substituted or unsubstituted compounds. The interesting difference between the incorporation of manganese and zinc is probably a kinetic one (Simkiss, 1981). The very rapid incorporation of manganese into the granules is probably the cause of cell toxicity and necrosis of the hepatopancreas (Taylor *et al.* 1988), since similar changes are not induced with zinc and there is no obvious change to the morphology of the granule (Taylor *et al.* 1990).

This study has shown that an understanding of the interatomic potentials in phosphatic biominerals provides three new insights into the biology of 'calcium cells' in the snail hepatopancreas. First, these cells appear to form intracellular deposits of $Ca_2P_2O_7$ in membrane-bound vesicles (Mason and Simkiss, 1982; Taylor *et al.* 1988), where magnesium ions partially substitute for some of the calcium ions to form amorphous $CaMgP_2O_7$ granules. The ability of magnesium to substitute into $Ca_2P_2O_7$ and the inability of calcium to substitute into $Mg_2P_2O_7$ provide an interesting insight into this form of biomineralization. Second, the amorphous state facilitates the movement of both anions and cations into and out of this random network structure, providing the possibility that such deposits could act both as 'transient stores' or as 'detoxification sites' for these ions. Again, the chemical structure of these granules provides a basis for their physiological functions. Third, it is predictable from the interatomic potentials that some cations will displace others from these granules. This is clearly seen with manganese, which can act as a Bronsted acid, $[Mn(H_2O)_5OH]^+ + H^+$, and which will therefore displace Ca^{2+} from the surface of these deposits, as is evident from the analyses of these granules (Table 1). As a result, the intracellular granules begin to corrode and to release calcium

ions at such a rate that they apparently overwhelm the calcium regulatory system and induce cell death in large regions of the hepatopancreas (Taylor *et al.* 1988). Thus, the atomistic simulations described in this study provide an explanation for some of the properties of intracellular granules and illustrate a hitherto unknown form of cell pathology and cell death.

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