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The composition of intracellular granules from the metal-accumulating cells of the common garden snail (*Helix aspersa*)

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Certain cells in the hepatopancreas of the common garden snail (*Helix aspersa*) contain intracellular granules that are sites of metal-ion accumulation. These granules have been extracted and investigated by u.v. and i.r. spectroscopy, atomic-absorption spectroscopy, X-ray microanalysis, thermogravimetric analysis, enzymic assay and microanalysis. The deposits contain about 18% (w/w) water, 5% (w/w) organic matter and 76% (w/w) inorganic material of which the main components are Ca^{2+}, Mg^{2+} and $P_2O_7^{4-}$. The possible origin of these granules is discussed, as is their role in detoxifying heavy-metal ions.

The past decade has produced extensive evidence for the occurrence of inorganic granules inside a variety of cells. Under the electron microscope they appear as remarkably similar, concentrically layered deposits up to 100μm diameter that occur in membrane-bound vesicles associated with the endoplasmic reticulum or Golgi system (Simkiss, 1976; Watabe *et al.*, 1976). Analyses of these deposits have shown that they are of variable composition, with Ca^{2+} and Mg^{2+} as the major cations and CO_3^{2-} and PO_4^{3-} as the principal anions (von Brand *et al.*, 1965; Burton, 1972; Becker *et al.*, 1974). The granules are most common in invertebrates, where it has been shown that they may also contain a variety of trace elements, with Ag, Al, B, Ba, Cd, Co, Cr, Fe, Mn, Sr and Zn having been reported to occur in various samples (Simkiss, 1977). The inorganic deposits occur in only one or two tissues, which frequently correspond to the organs that contain the main concentrations of trace elements in that particular species (Bryan, 1976). This has led to the suggestion that the granules may be part of a cellular storage or detoxification system. Some support for this concept comes from the fact that periodically the granules may be extruded into the alimentary tract, with a subsequent decrease in the body loads of the metal ions (Bryan, 1973).

The granule-secreting cells seem to pose a number of interesting biochemical problems. The metabolic pathways involved in granule formation are not known, and it has been difficult to envisage any simple system that would account for the very variable composition of these deposits. The minerals that are produced are also amorphous to both electron and X-ray diffraction (Simkiss, 1976), so that their formation cannot be considered as if they were simple crystals nucleated on an organic template in the way that is usually advocated in biomineralization. For these reasons we decided to analyse some of these deposits in considerable detail with a view to obtaining a more precise definition of their chemical composition and structure. It was hoped that the results could be used to relate the formation of granules to some defined biochemical pathway and hence facilitate further studies.

Cells capable of producing amorphous intracellular granules occur in considerable numbers in the hepatopancreas of pulmonate molluscs. The deposits occur in the so-called 'calcium cells' of this tissue, and since these have been well studied histologically (Abolins-Krogis, 1970; Walker, 1970), we decided to use this material as an easily available source of granules.

Experimental

Animals

Snails (*Helix aspersa*) were collected locally from a single site and maintained in the laboratory on a diet of lettuce, carrots and $CaCO_3$ for several months before use.

Ultrastructure and X-ray microanalysis

Pieces of hepatopancreas were dissected from animals and rapidly fixed in glutaraldehyde and OsO_4 before embedding in Araldite and sectioning

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on an ultramicrotome for ultrastructural studies. The presence of granules in the 'calcium cells' was confirmed with a JEOL model-100S electron microscope. Granules prepared as unfixed extracts were analysed in either a transmission or scanning mode by X-ray microanalysis with a Kevex detector and a Link analyser. Standards of $Ca_2P_2O_7$ and $Mg_2P_2O_7$ were synthesized from $Na_4P_2O_7$ and used for calibration in X-ray microanalysis.

Analyses

Granules for analysis were separated from the hepatopancreas by macerating the cells in water with a tissue homogenizer and repeatedly centrifuging at 3000 *g* and resuspending the pellet until a clear white deposit was obtained. This was checked in the scanning electron microscope to ensure that it was not contaminated with cell debris. The granules were insoluble in water and in NaCl (155 mmol·dm^{-3}) and were not completely soluble in HCl (1 mol·dm^{-3}). They were dissolved however by HNO_3 (16 mol·dm^{-3}) at 60°C, and this procedure was used to provide solutions that were then analysed for Ca, Mg, Zn and Mn by using a Varian model-175 atomic-absorption spectrophotometer. A nitrous oxide/acetylene flame and a 5 g·dm^{-3} solution of KCl were used as required to suppress ionization effects. K was analysed by flame emission spectroscopy. Total P_i was determined colorimetrically by using the vanadomolybdate method of Hansen (1950).

Granules were dissolved in cold (4°C) $HClO_4$ (70 mmol·dm^{-3}) to avoid excessive hydrolysis and were then immediately analysed for pyrophosphate ions ($P_2O_7^{4-}$) by using the enzymic method of Drake *et al.* (1979). This assay relies on the synthesis of ATP from $P_2O_7^{4-}$ by sulphurylase (sulphate adenylyltransferase, EC 2.7.7.4). The ATP released was measured by using hexokinase (EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) to produced $NADPH_2^+$, which was determined spectroscopically. Recoveries were checked by using standards of $Na_4P_2O_7$.

Samples of powdered granules were examined at a concentration of approx. 1% (w/w) in KBr discs on a Perkin–Elmer 457-grating i.r. spectrophotometer and by u.v.–visible reflectance spectroscopy with a Beckman Acta M IV spectophotometer with $BaSO_4$ as a standard.

The C, N and H contents of dry granules were determined microanalytically.

Granules that had been dried at 60°C were analysed thermogravimetrically in dry air on the Stanton Redcraft TG 750 thermobalance, with a heating rate of 50°C·min^{-1} up to a maximum of 1000°C. Differential thermal analysis was recorded against an alumina standard by using a Stanton Redcroft 673-4 furnace at a heating rate of 20°C·min^{-1}.

Results and discussion

Composition of the granules

The X-ray-microprobe analyses of the granules showed the presence of Ca, Mg and P, with smaller quantities of Si, K, Mn and Zn (Fig. 1*a*). Mean values for the chemical analyses of these elements in ten different samples of granules are shown in Table 1. The virtual absence of organic compounds was shown by the microanalysis (C, 4.23%, N, 0.89% and H, 2.21%, w/w) and u.v. spectra of these deposits. There was no evidence for CO_3^{2-} (i.r. spectra, thermal analysis and no CO_2 with acid) or sulphate groups (X-ray probe and i.r. spectra). It appeared, therefore, that the major components of the granules were in the form of inorganic phosphates.

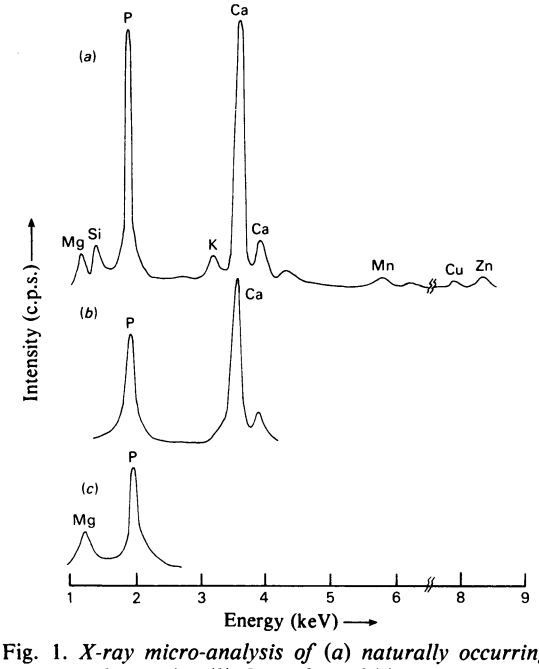

Fig. 1. *X-ray micro-analysis of* (*a*) *naturally occurring snail granules,* (*b*) $Ca_2P_2O_7$ *and* (*c*) $Mg_2P_2O_7$
Analyses were performed on graphite holders by using an accelerating voltage of 40 kV, a specimen tilt of 35° and a spot size of approximately 70 nm. The small Cu peak is an instrumentation artefact due to scattered electrons. The efficiency of X-ray micro-analysis decreases with the lighter elements, so that quantification of the peaks depends on the use of suitable standards of $Mg_2P_2O_7$ and $Ca_2P_2O_7$. The synthesized $Ca_2P_2O_7$ has a variable water content, but it was analysed and shown to contain 70% (w/w) $Ca_2P_2O_7$.

Table 1. *Mean values for the main inorganic ions in ten granule samples*

	Content ($\mu g \cdot atoms \cdot mg^{-1}$)					
Ion ...	Ca^{2+}	Mg^{2+}	K^+	Zn^{2+}	Mn^{2+}	P (as PO_4^{3-})
Mean	3.19	3.11	0.21	0.05	0.01	6.14
S.D.	±0.70	±0.77	±0.17	±0.03	±0.01	±1.43
Range	2.84–5.24	1.73–4.47	0.01–0.52	0.02–0.13	0.00–0.04	4.34–10.30

Table 2. *Pyrophosphate and orthophosphate content of two samples of granules in relation to the main cations and total mineral content*

	Content					
Sample Ion ...	$P_2O_7^{4-}$	PO_4^{3-}	Ca^{2+}	Mg^{2+}	K^+	Total (%)
1 ($\mu mol \cdot mg^{-1}$)	1.53	2.35	4.10	1.85	0.97	—
($\mu g \cdot mg^{-1}$)	269	226	164	44	38	74
2 ($\mu mol \cdot mg^{-1}$)	1.90	2.60	3.06	2.54	0.27	—
($\mu g \cdot mg^{-1}$)	334	250	122	61	10	78

Structure of the phosphate

The empirical formula derived from the analyses shown in Table 1 is $Ca^{2+}Mg^{2+}(PO_4^{3-})_2$, which clearly does not balance charges. The i.r. spectra also show a number of features that do not correspond to orthophosphates. Characteristic features that are absent from the spectra of the usual phosphate salts are the medium broad absorption bands at 920 cm^{-1} and the weaker bands at 745 cm^{-1} (Fig. 2). These are assigned to P–O–P vibrations (Corbridge & Lowe, 1954; Palmer, 1961) and spectra of $Ca_2P_2O_7$ samples showed a close correlation with those of granules. Thus the i.r. spectra indicate that some of the P is present as pyrophosphate $P_2O_7^{4-}$.

The granules were amorphous to X-ray diffraction.

Analysis of granule samples

Two separate preparations of granules were analysed in detail for total phosphate and pyrophosphate; the difference between them being attributed to orthophosphates (Table 2). Assuming that there is no hydrolysis of the pyrophosphate in dissolving the samples, then the granules consist of roughly 50% (w/w) $(M^{2+})_2P_2O_4^{4-}$. The analyses shown in Table 2 account for about 76% of the weight of the granules.

Thermogravimetric analysis (Fig. 3) showed that loss of water accounted for about 18% (w/w) of the granule. Thus, with a small content of 5% (w/w) organic matter, there was a recovery of approx. 99% (w/w) of the total granule material.

X-ray-probe analysis

The enzymic analysis of pyrophosphate is

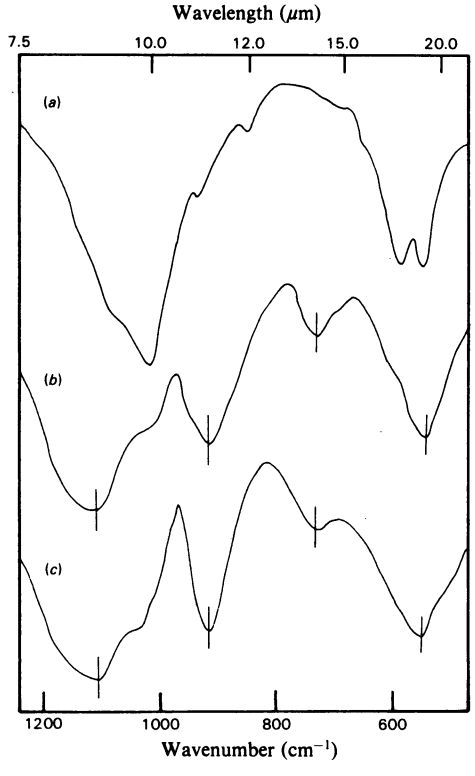

Fig. 2. *I.r. spectra of (a) hydroxyapatite, (b) snail granules and (c) $Ca_2P_2O_7$ in KBr discs*
The concentrations of materials used were (*a*) 1.7 mg of $Ca_{10}(PO_4)_6(OH)_2$ in 110.3 mg of KBr, (*b*) 1.8 mg of snail granules in 147.9 mg of KBr and (*c*) 1.8 mg of $Ca_2P_2O_7$ in 154.2 mg of KBr. Vertical lines indicate the main identifying peaks between the granules and $Ca_2P_2O_7$. The hydroxyapatite is provided for comparison.

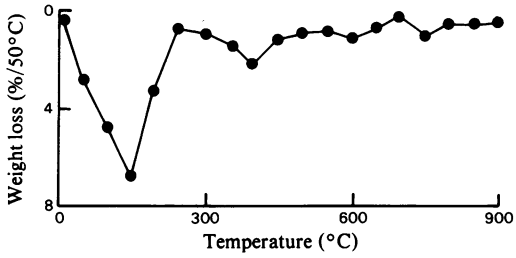

Fig. 3. *Thermogravimetric analysis of granules*
Weight loss per 50°C increases in temperature is plotted against temperature and shows a major loss up to 250°C, with minor losses at temperatures (T_m) of 375, 561 and 765°C. A differential thermal analysis of these data shows a strong broad endothermic peak, T_m, of 157 corresponding to dehydration, with smaller exothermic peaks at 373, 561 and 765°C assigned to the combustion of organic matter and the condensation of hydrogen phosphate. A sharp exothermic peak at 675°C, with no corresponding weight loss, is assigned to crystallization.

specific, but there may be some loss of the ion on dissolving the granules in acid before analysis. $Ca_2P_2O_7$ and $Mg_2P_2O_7$ were therefore synthesized and used to calibrate the X-ray microanalysis so that quantification of the solid granules could be attempted. If the P peak is normalized as 100 c.p.s. then Ca is 158 and Mg 33 c.p.s. in their respective pyrophosphates. X-ray microanalysis of the granules gives corresponding peaks of: P, 100; Ca, 104; and Mg, 10 c.p.s. If one assumed that the granules had the same composition as the standards this would correspond to a Ca/Mg ratio of 2.2:1 and the equivalent P-values would account for 96% of the P-peak as $P_2O_7^{4-}$. If the Ca and Mg were present as PO_4^{3-} the P-peak would be expected to be one-third smaller. This interpretation is probably beyond the purity of the standards used, but the X-ray-microanalysis data are in keeping with the other analyses in confirming $Ca_2P_2O_7$ and $Mg_2P_2O_7$ rather than $Ca_3(PO_4)_2$ and $Mg_3(PO_4)_2$ as the major components of the granules.

The occurrence of intracellular pyrophosphate

Inorganic $P_2O_7^{4-}$ is produced at one or more steps in the formation of proteins, lipids, phospholipids, nucleotides, nucleic acids, urea, steroids, structural polysaccharides and glycogen (Russell, 1976). According to Kornberg (1962), $P_2O_7^{4-}$ is rapidly hydrolysed by all cells and this enables these synthetic pathways to proceed. Some doubt has been expressed, however, as to whether the hydrolysis by inorganic pyrophosphatase (EC 3.6.1.1) ever reaches equilibrium *in vivo* (Flodgaard & Fleron, 1974). Despite this, $Ca_2P_2O_7$ only occurs in vertebrates in a few diseased states such as chondrocalcinosis and pseudo-gout (McGuire *et al.*, 1980).

A variety of micro-organisms are known to accumulate condensed phosphates as energy sources, and in the protozoan *Tetrahymena pyriformis* there is good evidence for the occurrence of intracellular granules of inorganic $P_2O_7^{4-}$ (Rosenberg, 1966). These deposits contained 69% mineral (as roughly equimolar $Ca_2P_2O_7$ and $Mg_2P_2O_7$), 5% organic material and 25% bound water, results very similar to those obtained with the *H. aspersa* granules.

Pyrophosphates as metal-detoxification systems

Analyses of snails from environments polluted by smelting works have shown that the hepatopancreas is the cellular site for the accumulation of many heavy metals (Coughtrey & Martin, 1977). Pyrophosphates would be excellent candidates for a metal-detoxification system, since they are easily formed *in vivo*, they react with metal ions at physiological pH values and they form insoluble salts with most metal ions except for those of group 1A. It is significant that the metal-binding constants of Mg^{2+} and Ca^{2+} are 6300- and 3700-fold greater for pyrophosphate than for orthophosphate (Chemical Society, 1964, 1971; see also da Silva & Williams, 1976). Thus $P_2O_7^{4-}$ would have clear advantages over many other anions in removing potentially toxic cations from the body fluids, providing first that pyrophosphatase inhibition was present and second that metal ions could penetrate to the sites of pyrophosphate accumulation. Ca^{2+} is known to inhibit a number of pyrophosphatases (Butler, 1971) and may act in this way in these cells. In addition, however, one might have to postulate some cytoplasmic protein as a carrier of metal ions to this detoxification site.

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