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Short Review

Cellular Discrimination Pathways

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Introduction

In 1955 the Belgian physiologist Christian de Duve applied the techniques of ultracentrification and electron microscopy to discover a group of new membrane bound organelles within animal cells. These structures were full of enzymes capable of breaking down a wide variety of biological polymers and the organelles became known as lysosomes. In subsequent years it slowly became apparent that these lysosomes were associated with a whole group of cellular storage diseases [1]. It was also discovered that calcium was involved with lysosomes and acted as a component in the cellular signalling and cell survival systems [2]. They were a totally unsuspected aspect of inorganic biochemistry.

Similar revelations occurred with the geologist's suggestion that the early Cambrian sea was extremely rich in mineral particles. The sea was saturated enough to produce deposits that would tend to coat any bottom living organisms with life threatening sediments [3]. It was, therefore, only after the Cambrian era, when the seas became diluted, that organisms could be found as shells in the fossil record, showing that they had developed a biomineralization system to protect themselves with a defensive shell. It was pointed out by the physiologist Jahnen-Dechent [4] that the descendants of such organisms would perhaps then be in danger of also mineralizing their own organs. This could result in the evolution of organisms that had to protect their own organs, such as the cardiovascular system, by circulating anti-calcification molecules such as fetuin-A [5,6]. It was a creative theme linking the benefits of bone formation to the need to evolve molecules that would protect the soft tissues from also mineralizing. It took its inspiration from the study of geology and the suggestion that the control of the human soft tissues probably required inhibitory molecules for use in the medical laboratory.

These two examples illustrated how diverse studies can contribute to new insights and it was suggested that since the study of metal ions in the environment was not based on any general theory it might need some approach other than simply monitoring an ecosystem. Perhaps taking snails into a chemical laboratory might be as interesting as taking inhibitors of calcification into a cardiovascular clinic.

Materials and Methods

We started when Williams [7] wrote an article on the problems of communicating an approach to biological research. He made an interesting distinction between two types of modelling. A 'first order' model simply asserted reality as it was thought to exist (e.g. some animals are being killed by metal contamination). A 'second order' model attempted to explain a phenomenon in terms of fewer but better understood components (e.g. tracking what happens to the distribution of metal ions if they are assimilated by a live mollusc). The following experiments were undertaken on the distribution of metal ions within an organism by using gamma radiations from metal ions that could be easily tracked and analysed.

The choice of metals was based on the availability of samples that could be commercially supplied by the Radiochemical Centre (Amersham, UK) and consisted of gamma-emiting $^{54}Mn^{2+}$, $^{59}Fe^{3+}$, $^{65}Zn^{2+}$, $^{85}Sr^{2+}$, $^{109}Cd^{2+}$ and $^{203}Hg^{2+}$ with an Ultragamma 1280 (LKB) analyser as the initial instrument for tracking them. We used pairs of metal ions so that comparative responses were possible. The animal involved was the common garden snail (Helix aspersa) 7-8 gm in size, fed on carrots and lettuce or cabbage and generally taken to be a well-studied and easily obtained organism. The snail has a large haemocoel for injections and a liver-like hepatopancreas for histological study.

Snails were injected with small doses (100 ul) of the gamma-emitting metals made up to a concentration of 1 umol by dilution with stable salts (chlorides) in a saline that could be introduced directly into the snail's blood (roughly 2-3 ml) through a catheter threaded through the snail tentacle. Direct injection into the haemocoel was intended to reduce the number of membrane systems that might be involved in a complicated pathway. The animals were killed and samples taken 6 hrs after they were injected. The hepatopancreas was examined histologically and included a JEOL 300M scanning electron microscope with analytical facilities for electron probe studies using a Link 290 system X ray microanalysis.

The animals were found to contain spherical granules about 1 um in diameter in the hepatopancreas of the snail cells. The composition of these intracellular granules was analysed by Howard et al [8] using ultraviolent and infrared spectroscopy, atomic-absorption spectroscopy, X-ray microanalysis, thermogravimetric analysis, enzyme assay and microanalysis. The granules contained about 18% (w/w) water, 5% organic matter (w/w) and 76% (w/w) inorganic material of which the main components were Ca^{2+}, Mg^{2+}, and $P_2O_7^{4-}$. Normal and manganese treated granules were studied by ultra-low angle scattering patterns using the Synchroton Radiation source (Daresbury Laboratory, UK.). The modelling of the chemical environment of the metal sites in the granules revealed an amorphous random network in a study that was initiated by Greaves et al [9] and developed by Taylor et al [10]. The hydration sites of these granules were studied using infra-red Raman and inelastic neutron scattering [11] using the Rutherford Appleton Laboratory (Didcot,UK).

Three treatments were used. (1) snails were simply injected in vivo with pairs of metal ions prior to killing the animals after 6 hrs and obtaining organ samples (2) the snails were killed 6 hrs after injection, blood samples were taken and granules separated from the hepatopancreas for separate analysis and (3) clean "donor" snails were bled and the blood spiked with radioisotopes. Samples of granules were then exposed to this oxygenated spiked snail blood for 6 hrs in vitro. All treatments involved all possible pairs of radioisotopes that were available. Radioactive samples were corrected for any spill-over of radiation between counting channels. All results were calculated as observed ratios (O/R) for tissue (t) to blood (b) samples of isotopes X and Y i.e O.R. = X_t/Y_t: X_b/Y_b and is a measure of the discrimination of the metals in each experiment.

The details of the distribution of radioactivity between different sets of paired samples is given in Simkiss [12] and the molecular modelling of the granules can be found in the references provided.

Results

The histological examination of the hepatopancreas showed that there were numerous so called 'calcium cells' containing roughly a thousand mineral deposits per cell in perfectly spherical membrane-bound organelles as has been reported in a large number of studies [12,13]. The snails that had been exposed to manganese ions had a large number of sites showing corrosion on the surfaces of these otherwise spherical deposits (Figure 1). Analysis of granules extracted from untreated snails showed that the granules were mainly the pyrophosphate $CaMgPO_7$ [8].

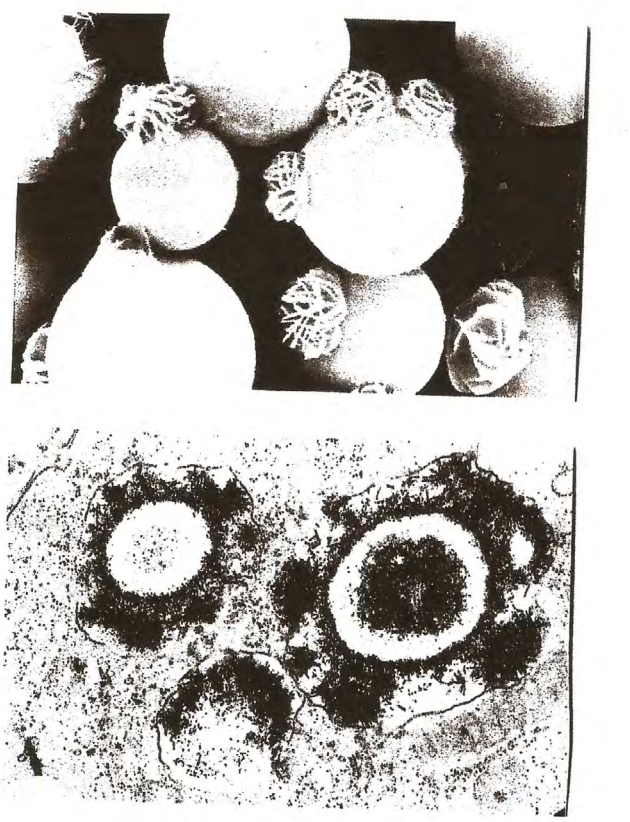

Figure 1. Scanning electron microscope photograpy showing sites of corrosion on the surface of $CaMgPO_7$ granules after exposure to manganese ions (top). A section across a granule showing the manganese deposits are on the surface of the granule within surrounding membranes (bottom). Granules are roughly 1 um in diameter.

An example of the observed molar ratios for two sets of ions taken from experiment 1 is given in table 1. This indicates that the concentration of Mn is 3.4 times higher than that of Fe; 35.7 times higher than that of Cd, 35.5 higher than Hg etc as recovered in the hepatopancreas. The data for the 7 metals in 3 different experiments are given in detail in Simkiss [12] but it is much easier to interpret these series as follows.

Experiment 1 (hepatopancreas in vivo)
$Mn^{2+}>Fe^{3+}>Cd^{2+}>Hg^{2+}>Zn^{2+}>>Sr^{2+}$

Experiment 2 (granules obtained in vivo)
$Mn^{2+}>Fe^{3+}>Zn^{2+}>Cd^{2+}>Hg^{2+}>Sr^{2+}$

Experiment 3 (granules treated in vitro)
$Mn^{2+}>Fe^{2+}>Sr^{2+}>Hg^{2+}>Cd^{2+}>Zn^{2+}$

The results of the uptake of ions by the hepatopancreas tissue and the granules exposed to combinations of metal ions are clearly different in each set of experiments although manganese treated granules always had the highest concentration and were therefore used to normalize the data between experiments. The content of Cd and Hg in experiment 1 shows a large move to the right in experiment 2. The Sr^{2+} in experiments 1 and 2 shows a placement far to the right but in experiment 3 it is shifted strongly to the left.

An explanation of these changes can be modelled in figure 2 that shows that Sr is not involved in both the in vivo experiments but otherwise follows Mn and Fe in the in vitro experiment. An interesting explanation is given by da Silva and Williams [14] who suggest antagonism between the strontium crystal form and the morphology of the cell. The movement of Hg, Cd, and Zn tended to form a separate group using the Ahrland et al [15] modified approach of Pearson's group's 'a' and 'b' hard and soft acids. Hard (a) Lewis group acids are characterised by small ionic radii, high positive charge, and strongly solvated. Soft (b) Lewis group acids are characterised as having a large ionic radii and low positive charge. Clearly the intracellular isotopes follow two distinct pathways in the snail hepatopancreas cells that are driven by the chemistry of hard 'a' and soft 'b' Lewis acids (Figure 2)

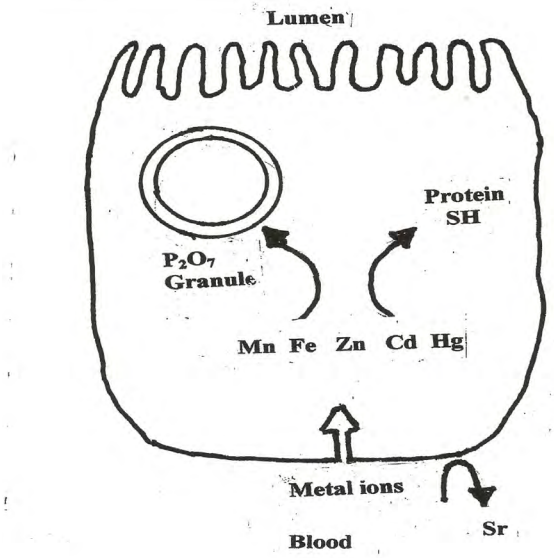

Figure 2. Illustration of metal ions entering a cell with Mn^{2+} and Fe^{3+} ions passing into the pyrophosphate granule while the Cd^{2+} and Hg^{2+} ions pass along a separate pathway and become attached to proteins with SH groups (such as the metallothioneins).

In order to further explore the properties of the granule Taylor et al [16] used static simulation of ionic lattices to model the phosphate components of a variety of biominerals. The parameters for the interatomic potentials have been obtained 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 electrostatic forces. This reveals an unexpected result as the intracellular Ca^{2+} and Mg^{2+} ions differ in concentrations that are about 10^{-4} times different, but Mg^{2+} appears to stabilize the Ca_2PO_7 structure with enough disruption to form a solid amorphous random network (Figure 3).

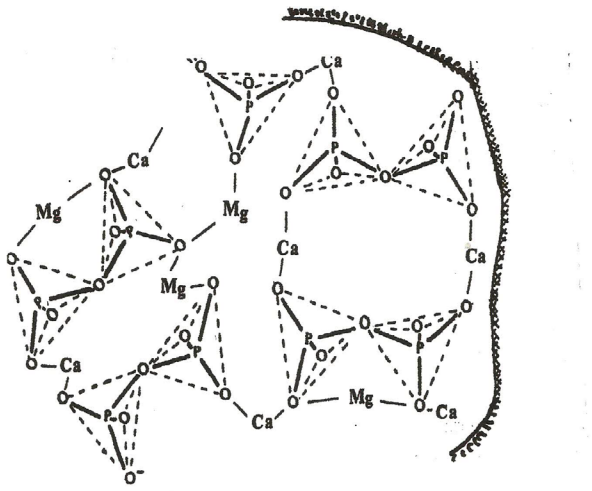

Figure 3. Model of a random network granule of pyrophosphate and metal ions. The granule is contained in a membrane system.

	Mn	Fe	Cd	Hg	Zn	Sr
Mn	-	3.4	35.7	35.5	63.5	35.2
Fe	0.29	-	1.1	2.8	3.3	36.2
Cd	0.03	0.87	-	10.9	3.2	39.6
Hg	0.03	0.35	0.09	-	0.44	4.9
Zn	0.02	0.31	0.31	2.3	-	2.7
Sr	0.003	0.03	0.03	0.1	0.37	-

Note the greater the O/R the weaker the interaction between the two metals i.e Mn : Sr shows Mn is 352 greater than Sr whereas the ratio Sr:Mn shows Sr is 0.003 times weaker than Mn. Complete sets of these interactions of all pairs of all metals in all three experimental treatments are available at Simkiss (1981 but an easier treatment of the same data is given in the text and in figure 2.

Table 1. Example of observed molar ratios (O/R) for the accumulation of metal ion pairs in hepatopancreas tissue treated *in vivo* (experiment 1)

This is an important effect as the random network would enable the movement of ions from the solid phase back into the cytosol and also facilitate the variety of foreign ions that could enter the pyrophosphate structure. The rapid movement of Mn^{2+} into the granule displaces and releases Ca^{2+} from the

random network of $CaMgP_2O_7$. By displacing Ca^{2+} the Mn^{2+} ion causes the corrosion of the intracellular granules and a rapid release of calcium that then exceeds the level of Ca^{2+} in the cytoplasm and triggers cell death in the surrounding cells.

Conclusion

Inorganic pyrophosphate is currently best understood for its involvement in the extracellular blocking of skeletal biomineralization and for controlling a number of soft tissue conditions such as the calcification of cardiovascular muscles in humans [17]. The biological involvement of an intracellular granular system is, therefore, somewhat surprisingly ignored as it only occurs with invertebrate organisms. According to Brown's [13] review of the literature, metal containing cells occur in 15 phyla from protozoa, to jellyfish, from tapeworms to nematodes and earthworms, from gastropod snails to bivalve mussels, with crabs and decapods to isopods like woodlice, from barnacles to butterflies and blood sucking lice. The granular system contain metals including calcium, copper, iron, lead, magnesium, manganese and zinc with anions of carbonate, chloride, sulphate, and phosphates. They are recorded in at least 68 different species although their observation is often casual. The intracellular granule system appears to involve a basic cellular organelle in the majority of organisms.

In the current study two chemical approaches (class a and class b, hard and soft Lewis acids) have been used to interpret a set of experiments that were performed in living animals i.e. it is possible to do inorganic chemistry inside live cells. Some cellular pathways are chemically very 'clean'. The $CaMgP_2O_7$ granules that we used were 94% inorganic with only 5% organic material and the chemical discriminations were very repeatable. It is possible to get substitutions of Mg into Ca_2PO_7 but not Ca into Mg_2PO_7. The Mg effect appears to stabilize the amorphous random network and open up spaces in the structure thereby facilitating anion and cation movements in and out of the cytoplasm [18]. This suggests that some of the biological functions of the intracellular granules may include either transient stores of ions or metal detoxification sites. Finally, the tracking of metal ion movements in vivo may be capable of following some other functions such as perhaps the expanding variety of ions involved in endosomal systems.

The results of these experiments indicate that there are inorganic biochemical pathways in particular organs that contribute to the physiological activities in the invertebrates that we studied These pathways may be part of a detoxification system based on amorphous minerals. They can, however also kill cells as Mn ions displace other components such as Ca ions that are released at ion storage sites with lethal effects in the cytoplasm.

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