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INVITED REVIEW

The future of stone research: rummagings in the attic, Randall's plaque, nanobacteria, and lessons from phylogeny

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Abstract The prevention or cure of stone disease will be achieved only by identifying biochemical, physiological and molecular mechanisms operating *before* the formation of a calculus. Yet, the gradual increase in the total number of papers devoted to the study of kidney stones that has occurred since the beginning of the 21st century can be attributed almost entirely to papers concerned with the investigation of factors associated with urolithiasis *after* stones have already formed. The need to prevent stones by discovering how the human body routinely stops their formation in those of us who do not suffer from them is therefore as exigent as ever and a new approach to investigating the causes of stones is urgently needed. In this paper, I develop the view that stone research will best progress by examining and understanding how healthy plants and animals control the formation of biominerals. In addition to structures like bones, teeth, shells and spines, many organisms spanning the entire phylogenetic tree form intra- and extracellular granules which are use as storage depots for calcium and other important ions, which they can reclaim to maintain homeostasis or to satisfy specific needs during periods of high demand, such as shell formation, moulting or skeletal development. These electron-dense granules, which also bear an uncanny resemblance to calcified nanobacteria, are remarkably similar in general structure, size and composition to particles observed in healthy human kidneys and in Randall's plaque. Therefore, it is likely that the granules in human kidneys fulfil analogous functions to those in other organisms—particularly in calcium homeostasis. Their study in a large range of creatures has already provided a deep well of information about their structure, movement, composition, macromolecular content, synthesis and resorption, from which we can draw to quench our thirst for knowledge of basic mechanisms and events involved in the formation of human kidney stones.

Keywords Urolithiasis · Randall's plaque · Nanobacteria · Biomineralization · Calcium homeostasis · Metal-containing granules · Nanoparticles

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Proem

The contents of this article bear scant resemblance to the subject that it was originally supposed to review. Asked to deliver a presentation at the 12th European Symposium on Urolithiasis, entitled "The Future of Stone Research", and knowing myself to be bereft of the breadth of scientific and clinical knowledge required to do justice to such a sweeping topic (to say nothing of the time it would take to rectify my deficiency), I decided to adopt a more philosophical approach. My longstanding belief in the economy of Nature, along with an amateur interest in biomimetics, convinced me that the phylogeny of calcium homeostasis—so central to human urolithiasis—might be more fascinating, more focused and less controversial than merely recommending specific areas of future research to colleagues whose knowledge of those areas far outstrips my own. And most important, it would take less time. I was wrong. The journey certainly proved to be fascinating, but principally because it led to the discovery of a connection between kidney stones, calcium, human beings and our phylogenetic siblings, of whose existence I had no previous inkling. And while the subject *eventually* reached a focus because of that connection, it was only after much meandering through a literary wilderness—a process that took an enormous amount of time. I did, however, start where I was supposed to.

Researching the stone

Any discussion on the future of stone research clearly rests on knowing what stone research actually *is*. A personal definition:

> Investigation undertaken with the specific intention of clarifying the understanding of renal stone disease, in order to effect its cure, prevention or alleviation.

It then becomes necessary to define exactly what stone research embraces. Of course, laboratory-based scientific enquiry into the factors that cause stone disease falls under the rubric of stone research; but a casual perusal of current and past literature immediately reveals a long list of equally mandatory headings. Papers devoted to epidemiology, aetiology, natural history, genetics, and diet are obvious contributions to stone research. So are those concerned with evaluation of stone patients. Incorporating studies on urine, stone and blood analyses for detection of metabolic derangements or calculation of risk indices, their aim is to discover ways to identify patients likely to suffer further stone episodes so steps can be taken to avoid them. Reports of the effects of new or existing medical treatments, including drug trials, are compulsory headings, as are surgical strategies such as open surgery, extracorporeal shock wave lithotripsy (ESWL), percutaneous nephrolithotripsy/lithotomy (PCNL) and ureteroscopy. Coupled with these are investigational tools like urine microscopy and imaging. Descriptions of stone-forming sequelae of calcium supplementation for osteoporosis, or drugs administered for the treatment of other diseases—protease inhibitors for HIV, for instance—can all be regarded as forms of stone research. It can even be argued that case studies, surveys and reviews like this help to further our knowledge of human urolithiasis.

Thus, stone research encompasses widely differing types of investigation involving a large range of scientific and clinical disciplines (which was one of my principal reasons for wanting to avoid writing a review on its future!). Furthermore, it's been going on since Hippocrates noticed the passage of solid material in urine. From then till now, so much effort, and money and time have been expended on stone research; so much information has been generated and so many papers have been written on the subject; so why do we *still* not have a guaranteed means of preventing recurrences? Have we been doing the wrong sort of research? If this is even remotely possible, it is vital that we take a look at what we have actually been doing.

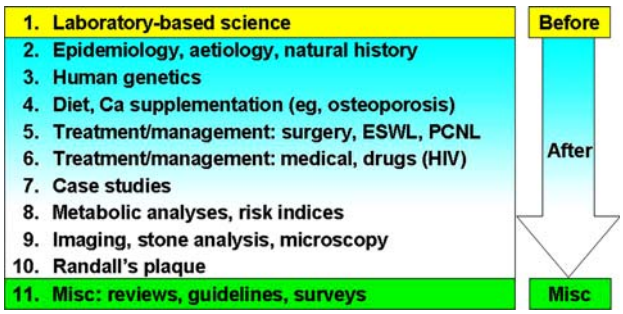
Stone research in the 21st century

It would be impossible to review and classify all the urolithiasis research that has been undertaken since the time of Hippocrates; but the dawn of a new millennium provided a convenient starting point for looking at contemporary stone investigation. Starting from January 2000, just three words, *human*, *kidney* and *stone* were fed, together, into PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez). This approach is far from ideal, since it cannot pick up all kidney stone papers—indeed, a number of my own papers did not show up! It will also not detect the many hundreds of papers published in stone conference proceedings. However, no single abstracting system will identify *all* stone research papers and PubMed, though imperfect, was more than adequate to the task in hand.

For each year from 2000 to 2006, each of the stone publications identified by PubMed was placed into one of the 11 categories shown in Table 1. Overlap was unavoidable, but classification was as consistent as possible. For instance, any paper concerned with ESWL, even if it entailed laboratory-based investigation, was included in the ESWL section. On scrutinizing the various categories it became clear that almost all of them—categories 2–10 were about research concerned with observation of factors *after* a stone had formed: studies on patient evaluation, treatment, imaging, and genetic and epidemiological analysis, for example, unavoidably involve individuals who have already declared themselves to be stone formers. The only category devoted to the study of events and mechanisms occurring *before* the formation of a calculus was laboratory-based research. The remainder—reviews, guidelines and surveys—were classified separately as miscellaneous.

Figure 1 shows the total number of papers published annually since 2000, together with the sum of papers falling into several individual key groups. Although overall the total number of stone papers increased between 2000 and 2006, it stabilised from 2004 to 2005, and even dropped slightly between 2005 and 2006. The cause for the fall in 2001 is unknown, but probably represents an international response to the events of September 11th of that year. It was more than compensated for by a rebound in 2002. The sum of the papers placed into the *after* category parallels

Table 1 Stone research in the 21st century



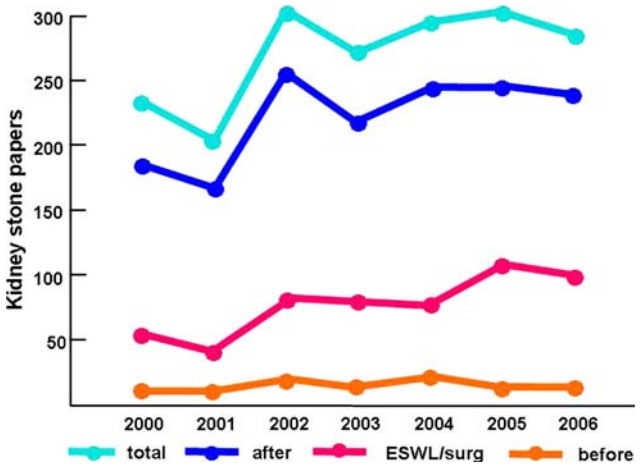


Fig. 1 The number of kidney stone research papers published between 2000 and 2006. See text for details of research categories. The total number of papers, as well as those classified as *ESWL/surg* and *after*, has risen consistently since the beginning of the 21st century. Papers devoted to basic stone research (*before*) have not risen

that of the total stone research papers—the same blip between 2001 and 2002, but a gradual increase that declines after 2004. A similar trend is seen with papers concerned with ESWL and surgery. The only group that did not show a similar pattern was that containing the *before* papers. In Fig. 2 are shown the data expressed as a percentage of the total papers published per year. As expected from Fig. 1, most papers (78–86%) fell into the *after* category, while miscellaneous publications accounted for 9–15%. The *before* studies—the laboratory-based investigations—made up the remaining 6–8%. It is obvious therefore, that stone research has focused on examining factors

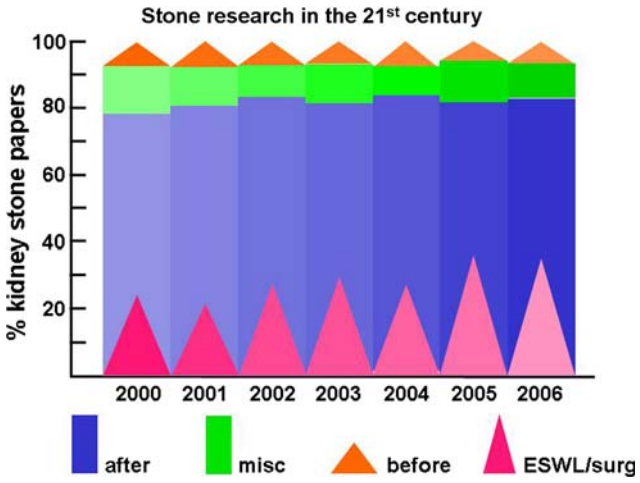


Fig. 2 The number of stone research papers published between 2000 and 2006, separated into individual categories (see text for details), expressed as a percentage of the total papers per year. The proportion of papers classified as *ESWL/surg* has risen consistently since 2000. That increase probably accounts for the slight upward trend in the percentage of papers categorised as *after*. The percentage of papers devoted to basic scientific research has remained constant

connected with stone pathogenesis after the stone has already formed. This is best exemplified in Fig. 2 by papers concerned with ESWL or surgery, which comprised 21–36% of the total papers. Perhaps even more importantly, it is clear that the number of publications in that category, with minor fluctuations, has risen consistently since 2000. This is hardly surprising and, for the time being at least, also entirely necessary. Because we have not yet found a means of preventing stone recurrences, or even better, of obliterating them entirely from the human race, we have no choice but to find ways of treating them.

Introduced more than 25 years ago, ESWL revolutionized stone treatment, as did the other modern surgical treatment modalities it heralded. Profitless hours languishing in hospital recovering from painful surgery, along with its accompanying complications, became a thing of the past. Yet ESWL has not been all beer and skittles. Many of the publications represented in Figs. 1 and 2 were concerned with short and long term problems caused by ESWL itself. It is hard to imagine returning to the dark ages of open stone surgery, but the easier it has become to blast or remove stones, the more blasé we have become about distinguishing between treatment and cure. Despite its indispensable value, ESWL's unwitting legacy has been to create the impression that stones are a medical nuisance that can be easily cured by a short trip to a day surgery unit and a few thousand uncomfortable shock waves. They cannot. ESWL merely removes, with misleadingly apparent ease, a painful by-product of an underlying metabolic abnormality, and in so doing it effectively discourages the performance of basic research aimed at finding means of preventing the disease. There is, therefore, as there has always been, an urgent need to *prevent* stones using strategies based on sound scientific knowledge of the biochemical, physiological and molecular causes of urolithiasis. Unfortunately, however, it is dismally apparent from Figs. 1 and 2 that the very kind of research that needs to be performed is simply not being done. Nor is it likely to be, given the current trend. But of one thing we can be sure: more of the same is not going to achieve our goal, which is to prevent or cure stones by discovering how the human body stops their formation *every single day* in those of us who do *not* suffer from them.

Pretending the rest of the world isn't there

At least as far as urolithiasis is concerned, our species has always been shamelessly anthropocentric, absorbing ourselves in the study of *human* stones and largely ignoring the fact that their formation is but one example of a process that occurs in manifold guises throughout phylogeny—biomineralization. The manufacture by living organisms of a

galaxy of mineral structures, particularly those based on calcium, is ubiquitous throughout our world and absolutely indispensable to life on earth. Using mechanisms only barely understood, a huge range of creatures, from bacteria to mammals, artfully sculpt simple chemicals into weapons, armoury, scaffolding, dwellings, flotation devices, navigational instruments, photoreceptors, hearing aids, gyroscopes—even sex aids [1]! Plants, from the simplest algae to the largest gymnosperms, deposit crystals of calcium salts for protection against herbivory, calcium and heavy metal detoxification, osmoregulation, tissue support and rigidity, and light gathering and reflection [2]. Our own teeth and bones exemplify the utility and necessity of well-controlled biomineralization. Unfortunately, however, the exquisite fine-tuning required to regulate these processes sometimes goes haywire, leading to a spectrum of distressing pathological conditions [3]—even in dinosaurs [4]! One thing is clear, though. To correct or treat what is abnormal, it is first necessary to define and understand what is *normal*. For too long, we urolithiasists have been preoccupied with stones, which are manifestations of a process that has clearly gone wrong, instead of concentrating our efforts on one that has obviously got it right.

Biomineralization and calcium homeostasis

> The physiological roles of calcium are so numerous and of such fundamental importance that it may properly be considered the most important mineral in the maintenance of life.
>
> Stini [5]

Because calcium occurs in approximately half of the 60 or so known biominerals, calcification is often used as a synonym for biomineralization [6]. The unique properties of the calcium ion—its size, charge, ubiquity, ionization potential and ability to bind avidly yet still move freely [7]—established it as a key player in biological processes from an early point in evolution [5]. Nonetheless, calcium's essentiality to the maintenance of an organism's survival and health becomes more important the higher its position on the evolutionary scale [7]: in humans, the ion is critical to the healthy functioning of the musculoskeletal, nervous and immune systems [5] and is fundamental to the clotting of blood. But it is also toxic: excesses are as detrimental to all organisms as are deficiencies, so its levels must be very carefully controlled. Calcium homeostasis, which is vital for survival and maintenance of health, requires tightly regulating intra- and extracellular calcium ion flux and finely balancing the deposition of calcium into mineral structures with its uptake from the surrounding environment. In other words, it is necessary to have free access to a steady supply of calcium while being able to dispose of any excess [7] …*safely*. In humans, this is achieved by ensuring that the amount absorbed in the intestine is sufficiently high to accommodate obligatory losses and low enough to avoid kidney damage from intolerably high urinary calcium concentrations [7]. However, clearly we are not always successful—or I would have had no reason to write this review. It would appear therefore, that one price some of us have had to pay—at least in modern times—for our roost atop the phylogenetic tree has been an inability to achieve the same fine balance between calcium husbandry and calcium disposal that comes so easily to the organisms occupying the branches below us.

Travelling the silk route: *Bombyx mori* and calcium oxalate crystals

The majority of kidney stones are composed principally of calcium oxalate (CaOx), but the deposition of this mineral is by no means confined to the human species. As mentioned above, it is also precipitated and stored in a highly controlled manner by most higher green plants. Because the salt is barely soluble under physiological conditions, formation of CaOx crystals represents a nifty way of packaging up potentially harmful excess supplies of calcium in an inert, innocuous form. The silkworm might seem an unlikely subject to feature in a review on the future of kidney stone research, but it has several features that single it out as a calcium manager *extraordinaire*. The development of holometabolous insects, from egg to imago, must surely be one of Nature's wonders and the silk moth *Bombyx mori* is a fine exemplar. *Bombyx* starts life as a tiny egg that hatches into a larva, which is nothing more than a mobile eating machine whose sole *raison d'être* is to gorge itself silly until it spins itself a cocoon and pupates. Hidden in its silken oubliette, a remarkable transformation takes place: the fat grub's tissues are broken down into a primordial metabolic soup for reassembly into the tissues and structures of the adult moth. The pupa, however, is a closed system. It cannot eat, so its nutritional needs must be satisfied by the food ingested by the greedy caterpillar, which can excrete any materials that the pupa will not require, but must store—*safely*—those it will later need for construction of the adult's body parts. And calcium is perhaps its greatest problem.

Anyone who has kept silkworms as a child will know that they are fussy eaters. They really thrive only on mulberry leaves, which are full of CaOx and calcium carbonate crystals [8]. While some of that calcium is dispatched in the grub's faeces, a great deal of it is stored in its Malpighian tubules, whose functions are developmentally and physiologically equivalent to those of the mammalian kidney.

Within a day of hatching, small CaOx crystals can be seen attached by microvillous filaments to the larval Malpighian tubule walls (Webb, personal communication), in a pattern remarkably similar to the adhesion of CaOx crystals to the proximal tubular lumina of hyperoxaluric rats [9]. As the larvae grow, the number and size of the crystals increase. Figure 3 shows a transverse section of crystals accumulated within the Malpighian tubule of a second instar *Bombyx* larva, which again, are similar to those seen in the proximal tubules of hyperoxaluric rats [10], except that they completely fill the lumina of the Malpighian tubules. It is difficult to imagine how the insect manages to dispose of its nitrogenous and electrolyte waste with its "kidney" effectively blocked, but it suffers no ill effects; indeed the process is entirely normal [11]. What is more extraordinary, however, is that as the larva develops and moults to its fouth instar, prepupal stage, the CaOx mineral is gradually resorbed, until all that remains is what appears to be its supporting organic matrix (Webb, personal communication). Curiously, the calcium is probably later re-formed into CaOx crystals once again.

The impregnation of cocoons with CaOx crystals is common in the Lepidoptera [12]. They have been observed scattered upon the surfaces of the silk fibres comprising the cocoon of the silk moth *Antheraea assama* [13] and between the filaments and fibrils of the cocoon of the tasar silk moth *Antheraea mylitta* where, because of the hardness they impart to the cocoon, they probably fulfil a protective function [14]. The silk moth thus appears to possess the amazing capability of safely sequestering the calcium required for its later development in the form of inert CaOx crystals. It then dissolves them in order to redeem the calcium temporarily… only to use it once more to manufacture yet more CaOx crystals! It seems that other insects avail themselves of the same mechanism for storing calcium. Some species of cockroach, for instance, store CaOx crystals in their oöthecae, while others incorporate them in their egg cases [15]. The white cabbage butterfly deposits and later resorbs them during larval development [16]. As a group, members of *Insecta* use a variety of sparingly soluble calcium salts, including calcium carbonate and citrate [15], tartrate and urate [17] as convenient ways of regulating, storing and disposing of calcium. The formation of insoluble crystals in various stages of insect development can probably be regarded as one mechanism for controlling calcium homeostasis, in an analogous manner to the urinary formation and expulsion of CaOx in humans, which has been previously proposed as a normal, healthy process designed to rid the body of excess quantities of calcium (and perhaps oxalate) in an innocuous form [18, 19]. *Bombyx* does, however, possess another ingenious mechanism for locking up superfluous calcium in a form that is harmless, and also from which the calcium can be easily released if later required for other purposes.

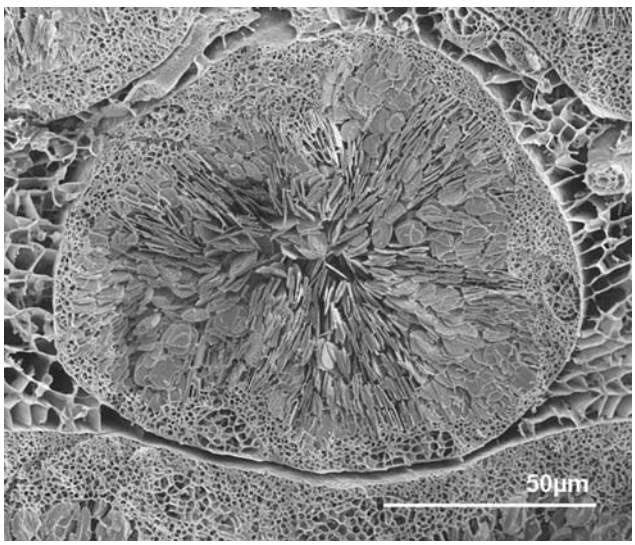


Fig. 3 Transverse section of the Malpighian tubule of a late second instar *Bombyx mori* larva. The tubule is crammed with calcium oxalate monohydrate (COM) crystals of similar morphology to human urinary COM crystals. Original image kindly provided by Dr. Mary Alice Webb

A curiosity of particles

> What are the strange particles?
>
> Feynman [20]

During my temporary literary sojourn into evolutionary aspects of calcium management I happened upon a paper reporting the study of cytoplasmic calcium salt deposits in fifth instar larvae of *Bombyx mori* [21], an example of which is shown in Fig. 4. I soon discovered a large body of literature, much of which had largely been relegated to the scientific attic, but some of it also recent, devoted to the study of calcium-containing granules in a wide range of organisms. Depending upon their source, the granules have been variously named—excretory concretions [22], concretion bodies [23], metal-containing granules [24], calcium granules [25, 26], storage vesicles [24], calcium spherules or calcospheres [27], calcospherites [21, 24], spherites [27, 28]; mineral concretions [24, 28, 29], mineral granules [30], spherocrystals [16, 31], intracellular granules [25, 32], intracellular mineral granules [33], calcium concretions [34], intracellular and lysosomal granules [22], calcium phosphate (CaP) granules [30, 35], CaP spherites [36], precipitation granules [37], calcareous concretions [38], calcareous corpuscles [39], crystalloid bodies [24], detoxification granules [40], electron-dense granules [41], calcium spherites and calcifying granules [42]. As we will see later,

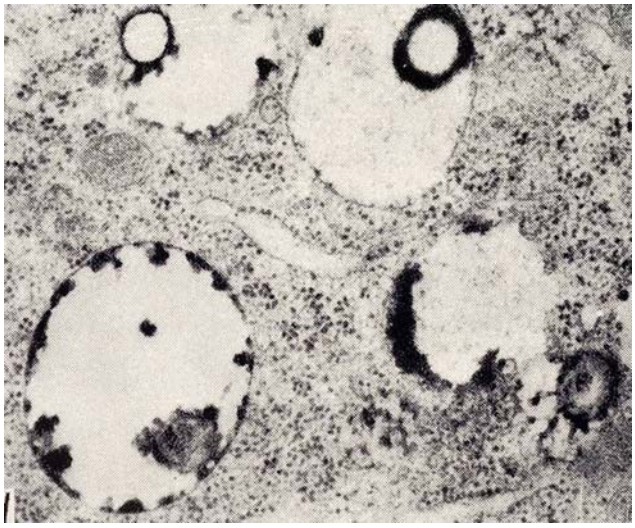


Fig. 4 Electron-dense calcium deposits accumulated at the inner surface of the membrane of a cytoplasmic vesicle in midgut epithelial cells of a feeding fifth instar larva of *Bombyx mori.* Reprinted from [21]. Copyright (1974) with permission from Elsevier

other terms have also been used to describe these bodies. For simplicity, and in recognition of the fact that irrespective of their component metal ions all granules contain electron-dense organic material, I will principally use the term *electron-dense granule* in this review. Furthermore, I will present only a brief summary of what is known about them: the field is large. Several reviews have been written on the subject [24, 25, 43], and a number of more recent papers contain many references to previous works [16, 22, 37, 44, 45].

Granular allsorts

Simkiss [25] divided granules into types A and B. Type A exhibit high purity, have sculptured, skeletal structures, are crystallographically well organized, contain an organic matrix and are formed in vesicles associated with the Golgi apparatus. Type B, which are typically spherical and have a concentric layered structure, are usually located in the same part of the cell. Their organic matrix consists of lipid and glycoprotein, while the inorganic component, which is poorly crystalline or amorphous, is rich in calcium, phosphate, CO_3^{2-} and magnesium. They also tend to contain trace amounts of low molecular mass species such as organic phosphates. Type C granules, members of a third group classified by Hopkin [46], are crystalline or flocculent and contain large amounts of iron. A fourth, type D granule, described by Ahearn et al. [37], is extracellular and composed principally of calcium carbonate. Given the dissimilarities in the properties of these granule types, as well as their widespread occurrence throughout a huge range of organisms, broad generalizations are difficult. Nonetheless, it is possible to summarize their features briefly: the information presented in Table 2 was compiled from Brown [24] and Simkiss [25]. As would also be expected from their widespread phylogenetic origins and different compositions, the physical features of metal granules are also highly variable.

Figure 5 shows a collection of transmission electron microscopic (TEM) images of granules derived from a disparate collection of organisms. A large and varied assortment of shapes and sizes is immediately obvious. Many (e.g., panels 3, 4, 6, 8, 16 and 22) are obviously laminated, consisting of alternating electron dense (organic) and pervious (mineral) layers. Others (e.g., panels 2 and 19) seem to consist principally of organic material, in contrast to those from *Bombyx* (panel 1), which contain small islands of dark material in what otherwise appear to be empty vacuoles. Some have only a few layers, while others have many. A few (panels 24 and 27) are surrounded by fine whiskers of electron-dense matter. The morphology of the granules can differ widely, even when present in the same tissue sample (panels, 21 and 26). Such large differences obviously reflect their heterogeneous origins. However, their appearances also depend upon factors such as fixation techniques, metal ion contamination in the surrounding environment, the stage of the animal's life cycle (e.g., ecdysis, reproduction) and nutritional state [24], as well as their functions, all of which contribute to the difficulty of generalization. But there is another simple reason for the variegated granular morphology, particularly in the same tissue sample—cut! Figure 6 shows laminar patterns that can result from slicing a sphere constructed of many concentric layers at different planes. They range from small, apparently solid circles, to larger multi-ringed structures—a heterogeneous miscellany evident in the granules shown in panel 6, which were observed in a single section of the ileum of a cockroach after exposure to mercury [16].

Table 2 Properties of metal-containing granules

1. Often spherical and laminated
2. Common to all the major phyla
3. Associated with lysosomal function
4. Between ~0.1 and 100 μm in diameter
5. Occur both intracellularly and extracellularly
6. Principally amorphous CaP (as apatite) or $CaCO_3$
7. Also Mg, Se, Pb, Cd, Zn, U, Ba, Sr, Mn, Na, K, Si, S, Hg
8. Usually in digestive, storage and excretory organs—kidney!
9. Structure and size dependent on diet, age, species, environment

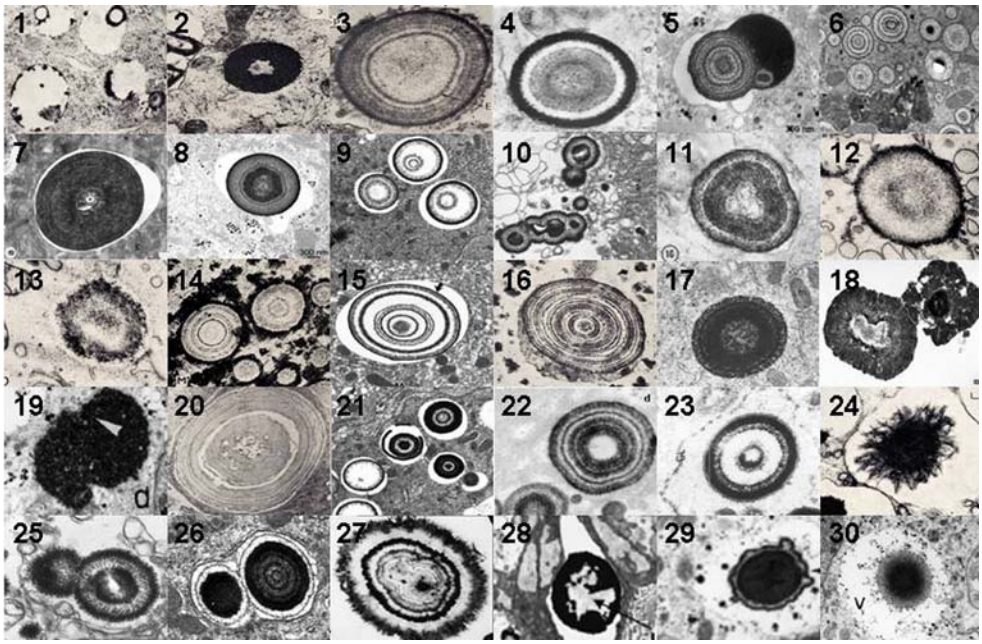


Fig. 5 Composite diagram showing the large variability in the appearance of transverse sections of granules obtained by transmission electron microscopy. *Panel 1*: *Bombyx mori:* Reprinted from *Tissue and Cell* [21], copyright (1974) with permission from Elsevier. *Panel 2*: Mineral concretions from the midgut of the insect *Cercopis sanguinea* [29]. Part of Fig. 9 reproduced from *The Journal of Cell Biology, 1968, 37: 316–328*. Copyright 1968 The Rockefeller University Press. *Panel 3*: Calcium granules from the cytoplasm of the trematode *Cyathocotyle bushiensis* [105]. Part of Fig. 9 reproduced with the kind permission of the *Journal of Parasitology*. *Panel 4*: Mineral concretions from the midgut of the insect *Cercopis sanguinea* [29]. Part of Fig. 11 reproduced from *The Journal of Cell Biology, 1968, 37: 316–328*. 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Copyright© Wiley-Liss. Part of Fig. 1 reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. *Panel 10*: Luminal mineral and purine concretions from the proximal segment of the ant midgut [16]. Copyright© Wiley-Liss. Part of Fig. 10 reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. *Panel 11*: Mineral concretions from the midgut of the insect *Cercopis sanguinea* [29]. Figure 16 reproduced from *The Journal of Cell Biology, 1968, 37: 316–328*. Copyright 1968 The Rockefeller University Press. *Panels 12 and 13*: Calcifying granules/calcium spherites in the hepatopancreas of the snail *Helix pomatia* L. Springer and Zeitschrift fuer Zellforschung und Mikroskopische Anatomie 108, 1970, 501–505, Electron microscope studies of the intracellular origin and formation of calcifying granules and calcium spherites in the hepatopancreas of the snail *Helix pomatia*, Abolinš-Krogis A, Figs. 6 and 7: With kind permission of Springer Science and Business Media. *Panel 14*: Calcium phosphate granules in the hepatopancreas of the Blue Crab, *Callinectes sapidus* [30]. Reproduced from *The Journal of Cell Biology, 1974, 61: 316–326*. Copyright 1974 The Rockefeller University Press. *Panel 15*: Intracellular spherocrystal from the ant midgut [16]. Copyright© Wiley-Liss. Part of Fig. 2 reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. *Panel 16*: Mineral concretions from the midgut of the insect *Cercopis sanguinea* [29]. Figure 20 reproduced from *The Journal of Cell Biology, 1968, 37: 316–328*. Copyright 1968 The Rockefeller University Press. *Panel 17*: Mineral concretions from the midgut of the insect *Cercopis sanguinea* [29]. Figure 10 reproduced from *The Journal of Cell Biology, 1968, 37: 316–328*. Copyright 1968 The Rockefeller University Press. *Panel 18*: Luminal spherocrystals from the proximal segment of the Malpighian tubule of the cricket [16]. Copyright © Wiley-Liss. Figure 8 reprinted with permission of Wiley-Liss, Inc, a subsidiary of John Wiley & Sons, Inc. *Panel 19*: Electron-dense granules from the midgut of the centipede *Lithophorbius forficatus*. Part of Fig. 3b reprinted from [44]. Copyright (1998) with permission from Elsevier. *Panel 20*: Calcareous corpuscles in the cat tapeworm *Taenia taeniaeformis.* Figure 10 reprinted from [39]. Copyright (1969) with permission from Elsevier. *Panel 21*: Spherocrystals from the ant midgut [16]. Copyright© Wiley-Liss. Part of Fig. 1 reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. *Panel 22*: Cadmium-containing granules from the kidney of the dolphin *Lagenorhyncus acutus*. Part of Fig. 2b reprinted from [31]. Copyright (2001) with permission from Elsevier. *Panel 23*: concretion bodies from the Malpighian tubules of *Rhodnius prolixus* [23]. Reproduced with permission of the Company of Biologists. *Panel 24*: mineral concretions in the mesentery of the collembolan insect *Pogonognathellus longicornis.* Zeitschrift fuer Morphologia der Tiere, 78, 1974, 93–109, Localisation, structure et genèse des concretions minérales dans le mésentéron des Collemboles Tomoceridae (Insecta, Collembola), Humbert W, Fig. 4a: With kind permission of Springer Science and Business Media. *Panel 25*: Luminal mineral and purine concretions from the proximal segment of the ant midgut [16]. Copyright © Wiley-Liss. Part of Fig. 10 reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. *Panel 26*: Spherocrystals in the ant midgut [16]. Copyright© Wiley-Liss. Part of Fig. 6 reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. *Panels 27 and 28*: Lysosomal granules from nephrocytes of the pond snail *Lymnaea stagnalis* after exposure to Cd. Plates 3F and 2A reprinted from [22]. Copyright (2006) with permission from Elsevier. *Panel 29*: Electron-dense granules from the midgut of the centipede *Lithophorbius forficatus*. Part of Fig. 3c reprinted from [44]. Copyright (1998) with permission from Elsevier. *Panel 30*: Electron-dense granules from the midgut of the centipede *Lithophorbius forficatus*. Part of Fig. 3b reprinted from [44]. Copyright (1998) with permission from Elsevier

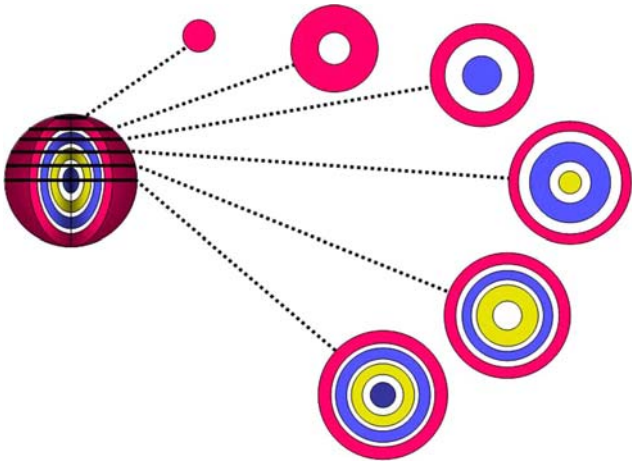

Fig. 6 Diagram showing the transverse granule patterns obtained by cutting sections through planes in a sphere consisting of concentric layers of materials with alternating high and low electron densities. After Brown [24]

Functions of electron-dense granules

Given their occurrence in a diverse range of organisms living in environments as widely apart as water and land, it is hardly surprising that the functions of granules can also differ markedly. The information presented in Table 3 was again derived principally from Brown [24] and Simkiss [25]. Granules provide a convenient means of excreting excess metal ions and of disposing of other unwanted materials. By sequestering ions in a solid, innocuous form, they also reduce their free concentration within the cytoplasm and thereby, regulate the intracellular or interstitial osmotic pressure. Simkiss [25] distinguished the functions of type A and B granules, the former fulfilling primarily a structural or skeletal role and the latter having more dynamic functions, including acting as mops for removal of toxic ions from the environment, storage of expensive ions required at later stages of the life cycle, and excretion of those excess to immediate need and which (such as in the case of *Bombyx*) cannot all be accommodated in an animal's organs.

Table 3 Functions of metal-containing granules

1. Waste disposal
2. Osmoregulation
3. Excretion of excess ions
4. Calcium storage and mobilization
5. Phosphate storage and mobilization
6. Carbonate storage and mobilization
7. Skeletal function (shell, integument)
8. Detoxification of poisonous heavy metals

One of the more fascinating examples of function is the role of extracellular granules in the growth of land and sea dwelling arthropods—particularly the insects and crustaceans, whose integuments and shells must be shed periodically to allow the animals' soft tissues to grow. The mechanical strength of these protective chitinous structures, examples of which occur in the terrestrial crustacean isopods *Porcellio scaber* [47, 48] and *Orchestia cavimana* [49], owes much to their mineral content—usually calcium carbonate. Because arthropods depend upon the rigidity and strength of their exoskeletons for support and protection, they are particularly vulnerable in the teneral state during the period between the shedding of the exuvia and the hardening of the new cuticle. It is therefore imperative that formation and fortification of the new integument occur quickly, a process that requires immediate access to large amounts of calcium and other components of the reinforcing granules. This need cannot be satisfied quickly from dietary intake. Instead, the granules are dismantled, and the constituent calcium released and reclaimed for the manufacture of new granules for strengthening the regenerated skin [47]. As will be discussed in more detail later, granule disintegration is assisted by storage of the calcium salt in an amorphous form, which is considerably more soluble, and hence more rapidly mobilized, than crystalline mineral [47, 49].

A universal phenomenon?

The granules shown in Fig. 5 are representative of those occurring in organisms spanning an enormous range of phylogenetic complexity—from trematodes (panel 3) to snails (panels 27 and 28) to crabs (panel 14) to dolphins (panel 22). However, their occurrence seems to extend to organisms even further down the evolutionary scale. They appear to be identical to acidocalcisomes, which are electron-dense granular structures rich in calcium and phosphate, whose functions include storage of phosphorus and various metal ions, metabolism of polyphosphate, maintenance of intracellular pH, osmoregulation and calcium homeostasis [50]. Thin sections of acidocalcisomes reveal varied internal patterns suggestive of solid electron-dense spheres, laminated bull's-eyes and largely empty vacuoles containing particles of electron-dense material—just like those shown in a number of panels in Fig. 5 [50, 51]. Although studied most extensively in trypanosomes, which are flagellate eukaryotic parasites, acidocalcisomes have been detected in organisms as widely divergent as bacteria and mammals. Human platelets were reported only relatively recently to contain electron-dense granules reminiscent of acidocalcisomes [52]. However, 40 years ago, similar structures consisting of composites of organic material mixed with Mg, carbonate and amorphous CaP were

observed in rat liver mitochondria incubated under conditions favouring maximum accumulation of calcium and phosphate [53].

It is clear then that intra- and extracellular electron-dense granules are distributed throughout phylogeny. Moreover, irrespective of the name by which they are known by their disciples in the various fields in which they are studied, they have similar structures and functions. And they occur in humans. But what does all of this have to do with kidney stones?

Granules and kidney stones

Only *connect!*

(EM Forster 1879–1970)

It may come as a surprise that electron-dense granules from other organisms bear a close similitude, both in general size and morphology, to particles more usually associated with the formation of human kidney stones. Using a combination of freeze-fracturing, selective demineralization and high resolution field emission electron microscopy it has been elegantly shown that the interior of the extracellular granules associated with ecdysis in crustaceans contains concentric, spherical alternating layers of amorphous mineral and organic material, transverse sections of which show alternating dark and light internal laminations [47, 48] similar to those of many images presented in Fig. 5. Such features are common in granules having calcium as a major constituent [24]. Figure 7a shows the three-dimensional structures of extracellular granules from the common woodlouse [48], which closely mimic those of the spherular CaP particles present in decalcified CaOx human kidney stones depicted in Fig. 7b. Indeed, closely-packed $CaCO_3$ particles in the transition zone between the homogeneous and proximal spherular layers of *Porcellio scaber* [48] are virtually identical in cross-section to the CaP spherules from human stones [54]: both exhibit polygonal outlines and internal layered structures. The same basic structure is also to be found in CaP particles in kidney stones [55] and in the urine of hyperoxaluric rats [56].

Thus, calcium-containing granules have similar two- and three-dimensional structures, whether from living organisms or kidney stones, or from artificially induced nephrocalcinosis, which implies that they are a natural, universal phenomenon in pro- and eukaryotes. However, they also bear an uncanny resemblance to another group of particles, whose propagation and function—indeed, very *quintessence*, continue to be the object of debate and scepticism in the scientific literature.

Excursus: the vexed issue of nanobacteria

In 1997, "nanobacteria", a sub-group of proteobacteria with extraordinary properties were reported to be present in

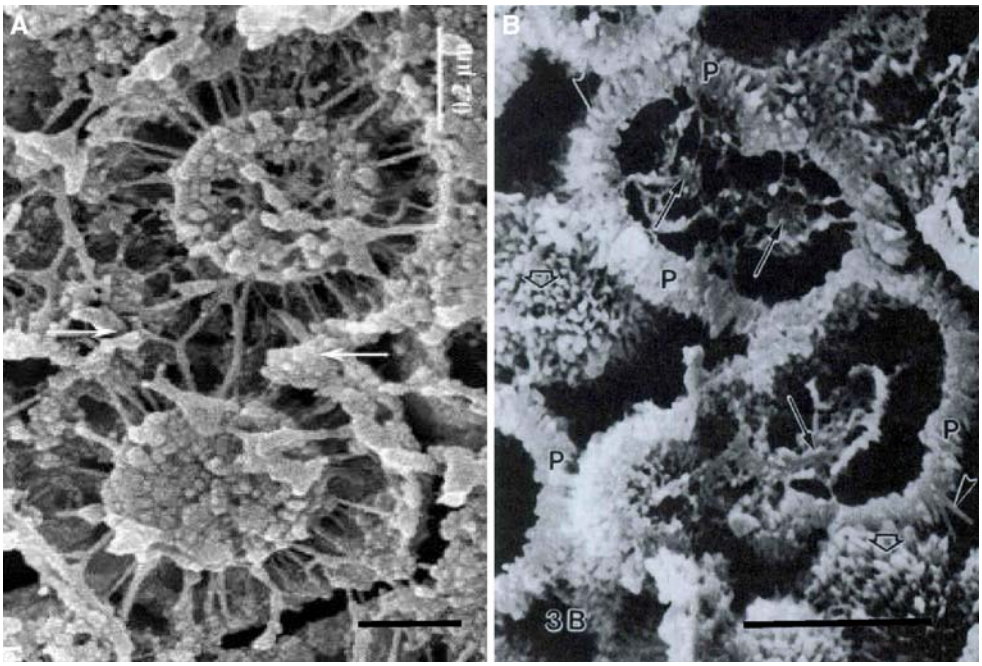

Fig. 7 **a** Decalcified $CaCO_3$ particles from the anterior sternites of the common woodlouse *Porcellio scaber*. Bar is 0.2 µm. Part of Fig. 2 reprinted from [48]. Copyright (2005) with permission from Elsevier. **b** Decalcified CaP particles from a human CaOx kidney stone. Bar is 10 µm. Figure 3b is reproduced from Khan [54], published in the *Journal of Urology*. Copyright (1997) with permission from Elsevier

human and bovine blood [57]. Nanobacteria have been reported to act as initiating centres for the precipitation of apatite [58] in an ever-increasing and mind-boggling list of human pathologies associated with ectopic calcification—from atherosclerosis to gallstones, prostatitis to Alzheimer's disease, cholecystitis to periodontal disease, and cancer to polycystic kidney disease [59]. Of greatest relevance to this review is their putative association with urolithiasis, which has been predicated principally on their isolation from human kidney stones [58, 60, 61] and their accumulation in the kidney and urine after injection into rabbits [62]. Kidney stone formation has also been reported in two papers to occur following injection of nanobacteria into rats [63, 64]. It should be noted, however, that in both instances data were obtained from only three experimental animals and controls were injected only with the suspension medium. Nevertheless, on the basis of these observations and the apparent sensitivity of the particles to tetracycline and citrate [65], as well as to a range of other antimicrobial agents [66], the use of anti-nanobacterial therapy has recently been advocated for the prevention of stone recurrence [67]. But is this justified?

Pathological agents, physiological functionaries or inanimate oddities?

Nanobacteria are controversial critters. The assertion that they are distinct living entities was seriously questioned by Drancourt et al. [68] who, though able to isolate nanoparticles from kidney stones employing methods identical to those used by the Kajander and Çiftçioglu group, could not propagate them in culture or confirm their status as living bacteria. Cisar et al. [69] successfully produced nanoparticles with identical physical features from "cultures" of fetal bovine serum, saliva and dental plaque filtrates, but concluded that they were products of nucleation of apatite by non-vital macromolecules via mechanisms similar to those used in the assembly of biominerals. Irrespective of whether so-called nanobacteria are dead or alive, there is one feature of them that seems consistent: so far, at least, they have been connected principally with pathologies involving disordered mineralization in humans. It is exceedingly puzzling that, at least to my knowledge, nowhere in the large and expanding literature devoted to nanobacteria has there been even a single reference to the longstanding, large body of literature attesting to the existence of calcium-containing granules in other organisms—despite the fact that their physical and biochemical characteristics are so similar.

Though individual nanobacteria vary in size from only 80 to 100 nm [61], they are housed in apatite "igloos" [61], "fortresses" [60], "shelters" and "dwellings" [58] of up to several microns in diameter. The calcified structures consist of laminated spheres which, on TEM examination, comprise electron-dense and light layers [58, 61], presumably corresponding to alternating deposits of macromolecules and mineral, which appear identical in size, composition and morphology to spherical apatite particles observed in human kidney stones [59, 60, 61]. However, they also bear a striking resemblance to electron-dense granules from other organisms. Figure 8 shows a TEM image of an ultrathin section of a calcified nanobacterial particle, reproduced from Vali et al. [70]: the strong similarity to the adjacent lysosomal granule derived from nephrocytes of the pond snail *Lymnaea stagnalis* after exposure to cadmium [22] is immediately obvious. Panel 24 in Fig. 5 shows a granule from the mesentery of the collembolan insect *Pogonognathellus longicornis* [28], which is remarkably similar to the image presented in Fig. 2c of Kajander and Çiftçioglu [58]—from the dense central portion to the surrounding whisker-like fringe. Transverse sections of granules from the proximal segment of the malpighian tubules of the ant (Fig. 10 in [16]), examples of which are shown in panel 25 of Fig. 5, are very like that of the nanobacterial igloo presented in Fig. 1c of Kajander et al. [61]. The resemblance also extends beyond two dimensions. Figure 1b of Çiftçioglu et al. [60] shows a fractured calcified nanobacterium whose three-dimensional and interior structure is very similar to that of intact and demineralised calcium carbonate spherules from *Porcellio scaber* [48].

Given their similar compositions, structures and dimensions, we may wonder whether calcified nanobacteria are different from those ubiquitous little structures in other organisms, which perform a variety of essential functions [24, 25], and which are most unlikely to be associated only with disease. Materials scientists and biomineralogists have long been aware of the diversity of crystalline and amorphous mineral forms that can be produced by self-assembly of nanoparticles produced from mixtures of simple inorganic ions, proteins and synthetic peptides [71], and mesocrystals are a prime example. Meso-crystals, which are hybrids of inorganic minerals ($CaCO_3$ among them) and macromolecules, represent a rapidly developing and exciting area of scientific endeavour. Constructed not by classical successive deposition of simple ions, but by stacking of individual modular nano-blocks similar in size and shape to nanobacteria, their microscopic structures look very like nanobacterial igloos [72, 73]. Similar nanometer-sized particles, linked like beads along nucleating chains of what appear to be collagen microfibrils, have also been shown to be precursors in the formation of osteogenic hydroxyapatite crystals [74]. Available evidence suggests that organic polymeric templates first induce the formation of these nanoparticles, which then coalesce into the crystal-like structures so familiar to biomineralogists [75]. It appears common, therefore, for the formation of biogenic crystals

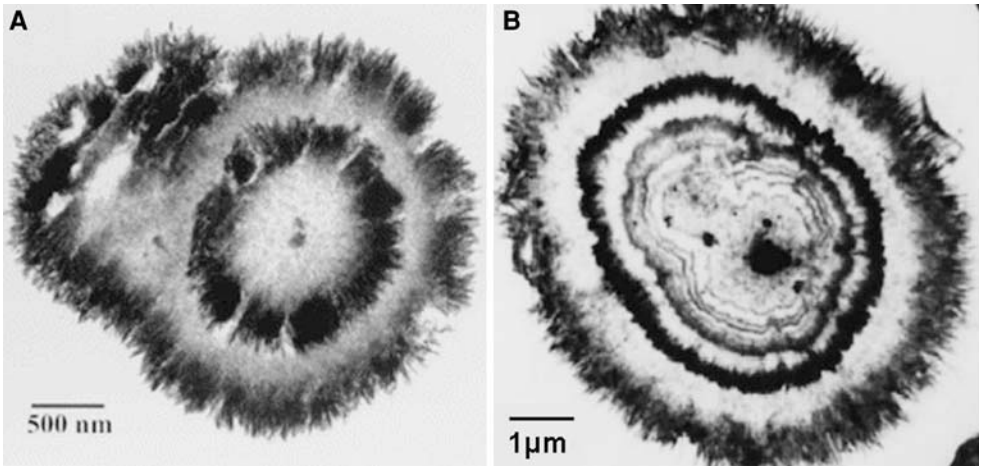

Fig. 8 **a** TEM image of an ultrathin section of a monolayer of semi-spherical calcified nanobacteria obtained from a serum-free culture, showing the internal structure. Figure 10b reproduced from [70], Copyright (2001) with permission from Elsevier. **b** Lysosomal granule from nephrocytes of the pond snail *Lymnaea stagnalis* after exposure to Cd. Plate 2A reprinted from [22]. Copyright (2006) with permission from Elsevier

to involve an interaction between nanoparticles and organic macromolecules. But this is not confined to bone, to biominerals generally, or even to living systems.

We have demonstrated (Fig. 9) sub-crystalline ovoid particles of similar size and morphology to nanobacteria, steeped in a macromolecular mud and packed inside protease-treated CaOx monohydrate crystals precipitated spontaneously from sterile human urine [18, 19]. It is probable that these intracrystalline particles, which are of the order of 50–100 nm in size, are examples of the nano-blocks alluded to previously, which assemble into meso-crystals that become coated with more refined CaOx mineral as macromolecules are depleted from the surrounding urine. The mineral coat gives the final product the outward appearance of a typical coffin-shaped single CaOx urinary crystal. We have also shown that similar nano-sized particles are present within CaOx crystals isolated from plants (Fig. 9). There is, therefore, convincing evidence that the salubrious formation of calcium biominerals—both in vitro and in vivo, involves the formation of nanoparticles, which undergo meso-scale transformation to produce single crystal superstructures comprising nanoparticles embedded in an organic phase [73]. We should thus at least consider the possibility that some, if not all, "nanobacteria" associated with pathological calcification might be nanoparticles formed spontaneously under conditions of high physiological supersaturation. Perhaps as research on the mechanisms of biomineralization, and especially meso-crystal formation advances, the true nature of these tiny maverick particles will be revealed once and for all.

I included this excursus on nanobacteria not just because they have been implicated in stone formation, and not to become entangled in the continuing controversy about whether they are living or inanimate. Neither was my intention to contribute to the debate about their putative involvement in what seems to be an alarming number of human ailments. Rather, it was to alert the stone community to the existence of a huge literature concerned with the study of remarkably similar, presumably inanimate and certainly not infectious, electron-dense granules occurring in innumerable, *healthy* organisms populating our planet. The functions of these granules, as well as the factors that affect their deposition and degradation may well have ramifications for other disciplines concerned with errors of mineralization, not the least of which is human urolithiasis.

Intrarenal calcium deposits and Randall's plaque

Interest in the location of crystalline material within the human kidney extends back at least to the 1800s, beginning with Henlé's discovery of microscopic nephrocalcinosis, primarily in the collecting tubules, but with frequent extension into the interstitium [76]—an observation that was later confirmed by Beer [77] and Stout et al. [78]. Other studies occurred sporadically throughout the 20th century, all of them entailing examination of healthy and diseased kidneys obtained at autopsy, using a variety of techniques to process the renal tissue for analysis. And they all reported similar findings. Anderson and MacDonald [79] studied 168 diseased and normal kidneys and detected microscopic calcareous deposits, which they referred to as "flecks of calcareous material" and "droplets", in the parenchyma of almost all specimens "anywhere in the kidney except within the lumen of the tubule". They proposed that the droplets passed through "ingestion", "coalescing" and amorphous" stages to form areas of Randall's plaque, which they regarded as the result of aggregation of micro-

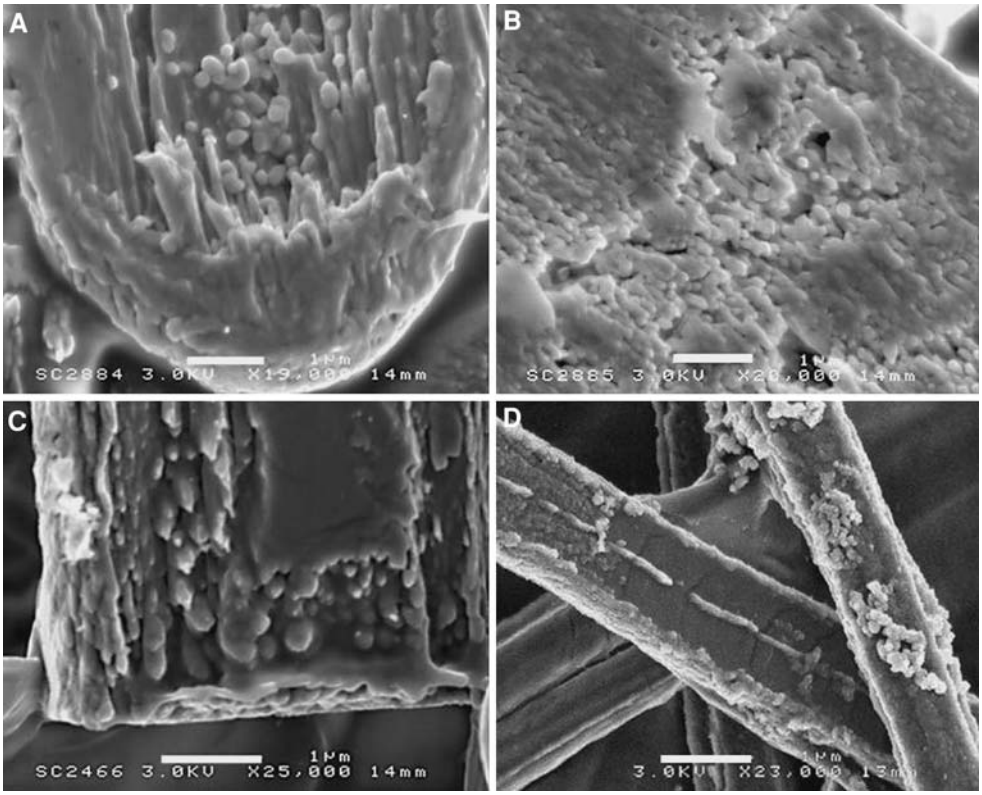

Fig. 9 Field emission scanning electron micrographs of calcium oxalate monohydrate crystals from human urine and plants. Crystals shown in **a** and **b**, which were precipitated from centrifuged and filtered human urine and treated with protease, show discrete, closely packed sub-crystalline particles. **a** Individual particles appear to be mixed with projections that may comprise stacks or sheets of similar particles linked in a linear fashion. **b** Exposed crystal interior shows highly ordered chains of particles interspersed with organic material. **c** Protease-treated styloid crystal isolated from the leaves of *Agapanthus* growing in the author's garden, showing the presence of numerous small particles resembling those shown in **a** and **b**. **d** Lateral view of protease-treated raphide crystals isolated from the flesh of *Ananas* (pineapple). Organic material appears to delineate the two halves of the larger twinned crystal, and again, clumps of small nanosized particles are clearly evident. Images in **a** and **b** are reprinted from [18] with permission from Mary Ann Liebert, Inc.

calculi that he had described, and *not* a consequence of primary degenerative changes in the papilla. Their conclusion was, therefore, that microscopic calculi occur in almost everyone as a result of phagocytic ingestion of calcium reabsorbed by the kidney tubules. In other words, the deposition of intrarenal calcareous material is a *normal process*, a view shared by Carr [80] who demonstrated calcific deposits in almost all of 209 kidneys from patients older than 9 years. From a high-resolution radiographic study of 61 kidneys, Bruwer [81] hypothesized that primary renal calculi result from the migration of parenchymal calcific deposits to the surface of the renal papillae. Although the presence of calcium deposits within the parenchyma was a consistent observation in those studies, one investigation [82] detected intratubular crystals in 32 of 500 patients, only 14 of which showed anatomical evidence of renal disease. Thus, calcareous deposits seem to have been observed everywhere in the kidney, including the lumina of the tubules. It is possible therefore, that their observed location in any particular published study depended upon the technique used to process the kidney and visualize the deposits. There are a number of plausible reasons why crystals might be lost during processing of a kidney [83] or remain undetected, not the least being that complete study of a kidney with ten pyramids could require the examination of as many as 10,000 sections [79]! It is also possible, though perhaps less probable, that they might be precipitated posthumously during cold storage prior to histological sectioning of a kidney that was originally crystal-free. However, in drawing conclusions about the origin of renal calculi from the early autopsy studies discussed above, perhaps the most crucial issue is whether or not the kidney donors had been stone formers when alive; yet in every case, the kidneys were removed at routine autopsy from individuals with no documented history of stone formation.

It would be fair to say that almost all of the aforementioned studies, as well as many others that have not been cited, were prompted by observations reported in a series of papers published during the late 1930s by Alexander Randall, who in 1937 described the results of *post mortem*

examinations of kidneys performed with the object of tracking down the papillary lesions that he believed held the key to the beginning of stone formation. Having studied 27 kidneys without discovering any lesions, he then observed a sub-epithelial cream-coloured area near the tip of one papilla, "so innocent in appearance and so abnormal" compared to his previous observations and which, upon microscopic examination appeared to be a plaque of calcium deposited within the interstitium of the papilla. He found similar plaques in 17% of 429 pairs of kidneys. Randall was confident that his studies had conclusively proved his hypothesis that renal stone formation is simply a symptom of some form of pre-existing papillary damage upon which induction of calculogenesis depends [84], despite the fact that in many cases plaque was present even when stones were not. In 1940, by which time Randall's associate Paul Leberman had examined kidneys from a total of 1,154 autopsies, Randall reported that 15% of 678 of those kidneys contained plaques or "milk patches" close to the papillary tips [85]. He also recognized a second type of papillary pathology, characterized by deposition of urinary salts within the tubules, which is noteworthy, given the view that intratubular crystals are observed only rarely [83]. Randall claimed to have demonstrated that the calcium salt deposits within the papilla were "an inert replacement manifestation", but if they were near the papillary tip, or growing, constituted the stone nidus [85]. One can only surmise what he meant by "inert replacement manifestation", along with his earlier description of plaque [84] as "a natural reparative process to some form of tubule damage, the occurrence of which is in much higher incidence than the actual frequency of renal stone". But it is possible that he was expressing the view that the calcium salt deposits were harmless—a point to which we shall later return.

But wait… now there's more!

Randall's hypothesis that stones are a product of an underlying pathological lesion prompted intense interest in the possible mechanisms of stone formation and generated a flurry of further investigation that lasted into the 1970s. However, although his theory was supported by some observations, it could not explain the genesis of all stones or explain their occurrence in kidneys of individuals without them [80]. So, as often happens in scientific research, Randall's plaques were pushed to the back of the scientific wardrobe until the emergence of more sophisticated instrumentation—principally digital flexible nephroscopy—during the late 20th century, when they once again became fashionable. For the first time, it became possible to look at and obtain samples of renal tissue from *living* people and also to compare patterns of plaque distribution in stone formers and healthy individuals.

The modern study of Randall's plaques was pioneered by Low and Stoller [86], who demonstrated endoscopically the presence of papillary plaque in three quarters of 57 patients undergoing ureteroscopic or percutaneous stone removal. By comparison, plaques were observed in three (43%) of seven individuals with no record of stone disease. That work was the bell-wether for a longer series of papers by Evan et al. [87], who performed light, infrared, X-ray diffraction and TEM examinations of renal biopsy tissue from 4 subjects with no history of stones and 15 CaOx stone patients with raised urinary calcium levels, but normal oxalate excretion. As expected, 4 additional stone formers who had undergone intestinal bypass surgery for obesity were normocalciuric, but excreted high amounts of oxalate. Plaque was absent from the healthy subjects, but present in all the CaOx stone formers, where it appeared as small, poorly crystallized deposits of hydroxyapatite originating from the basement membranes of the thin loops of Henlé and spreading thence through the interstitium to beneath the tubular epithelium. Bypass patients lacked plaque, but instead had intratubular deposits of well-crystallized hydroxyapatite. Those studies have since been extended to show that the area of plaque on a particular papilla is related to an individual's stone type [88], urinary calcium concentration [89] and number of stones [90], and inversely related to urine volume [89]. Although those findings have largely confirmed Randall's early observations, and certainly indicate that plaque is associated with the formation of renal stones, they still do not support Randall's view that a papillary lesion is *necessarily* the starting point for calculogenesis: not all papillary stones show features consistent with their having formed on plaque [91]; stones form in the absence of plaque, and plaque is found in people who have no history of stone disease.

An extreme normality?

To my mind at least, the most exciting feature of the recent work by Evan et al. is that it has re-focused attention on the microscopic structure and composition of Randall's plaque. Figure 10 shows another collage of TEM images of electron-dense granules. The remarkable similarities of these granules to those shown in Fig. 5 are so manifestly obvious that an unknowing observer would immediately conclude that the images are simply additional examples of the same phenomenon. Indeed they are; but unlike those presented in Fig. 5, the granules presented in Fig. 10 were obtained from humans. Anyone who has seen the cover of the urolithiasis research meeting held in Indianapolis in November 2006 will recognize Fig. 10, which is a composite of pictures of particles ranging in size from 0.05 to 0.4 μm in diameter, which were observed in biopsies taken from kidneys of patients with CaOx stones—principally from the basement

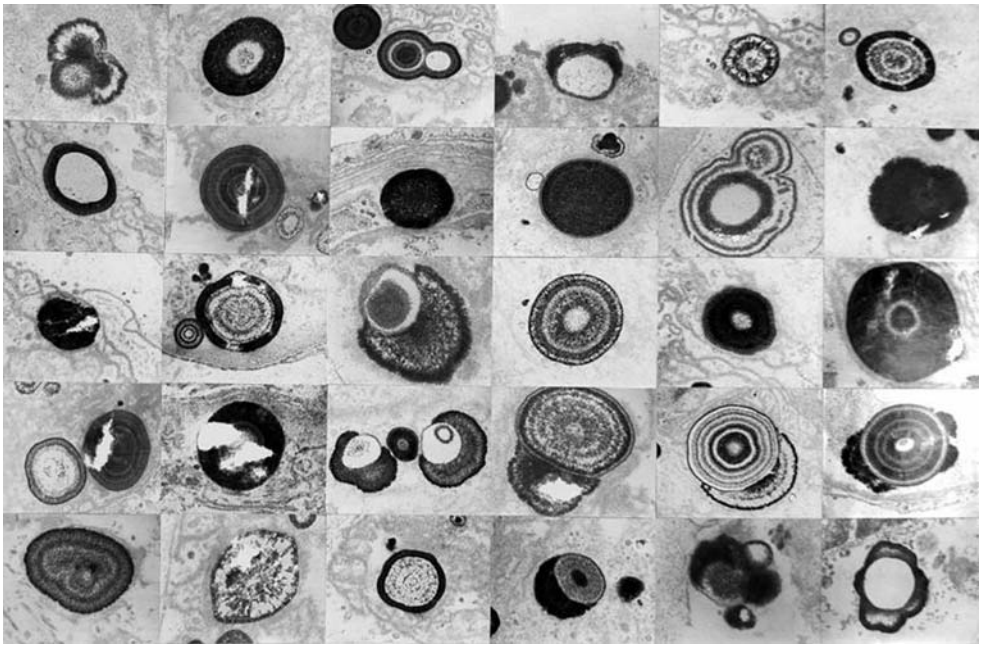

Fig. 10 Transmission electron micrographs of particles present in biopsies of Randall's plaques obtained principally from the basement membranes of the thin limbs of the loops of Henlé in kidneys of patients with CaOx stones. The particles range in size from 0.05 to 0.4 μm in diameter. Reprinted with permission from Evan AP, Lingeman JE, Williams JC (eds), Renal Stone Disease. 1st Annual International Urolithiasis Research Symposium, AIP Conference Proceedings, vol 900, pxiii, 2007, American Institute of Physics. Original image kindly provided by Dr. AP Evan

membranes of the thin limbs of the loops of Henlé. In fact, they are particles of Randall's plaque [87, 92].

Figure 8 is surely the largest collection of images of the lamellar particles comprising Randall's plaque ever assembled. But it is not the first. Using TEM, Weller et al. [93] showed dense mineral deposits around the loops of the thin limb of Henlé, as well as in the tubular basement membranes and surrounding interstitial collagen, in the renal papillae of all kidneys from three normocalciuric people without stones. Figure 8 of that paper shows a single laminated deposit abutting the basement membrane of a collecting duct. Khan et al. [55] examined the papillary tip of a patient with intratubular CaOx stones and observed in the interstitium electron-dense deposits, including many laminated spherules ranging in size from 120 to 150 nm, along with other irregularly shaped bodies up to 90 μm in diameter that probably represented collections of coalesced spherulites. The TEM image of one spherical laminated electron-dense body contained needle-like crystals of hydroxyapatite. Using energy dispersive X-ray analysis, they demonstrated that the mineral content of the deposits consisted exclusively of hydroxyapatite. However, the most comprehensive earlier illustration of laminated particles was presented by Haggitt and Pitcock [94] in a paper reporting a study of a series of 100 randomly selected autopsied patients whose kidneys were examined by TEM for gross and microscopic evidence of calcifications. Of the 100 kidneys examined, 23 had Randall's plaque, 7 had stones and 4 had plaques and stones; the remaining 66 had neither. But in *every single case*, medullary calcium deposits were observed as electron-dense bodies occupying the basement membranes of collecting ducts and the interstitium. The deposits, which are reproduced in Fig. 11, are indistinguishable from those shown in Fig. 10 and in other papers published by the Indianapolis group [87, 88, 95]. As had Anderson and McDonald [79] 25 years before, and Khan et al. [55] around a decade later, Haggitt and Pitcock observed a strong tendency for clusters of the concretions to migrate into a subepithelial location in the renal papillae, that is, to form Randall's plaques, which then tended to erode through the epithelium and initiate the development of a free calculus.

Perhaps the most crucial fact to emerge from my rummagings through the literary attic of Randall's plaque was that a number of authors consistently observed calcium-staining or electron-dense deposits in virtually *all* adult kidneys examined, particularly those from people without stones [79, 80, 94]. Although this seems to contrast with the findings from the Indianapolis group, who reported the presence of only minuscule amounts of plaque in kidneys from non stone formers [87, 89, 90], this may perhaps result from their examination of only four control kidneys. Their papers also do not report TEM analyses of the normal kidneys, which might have revealed electron-dense particles in areas remote from plaque. Of course, it could be argued that the observation of electron-dense granules in all

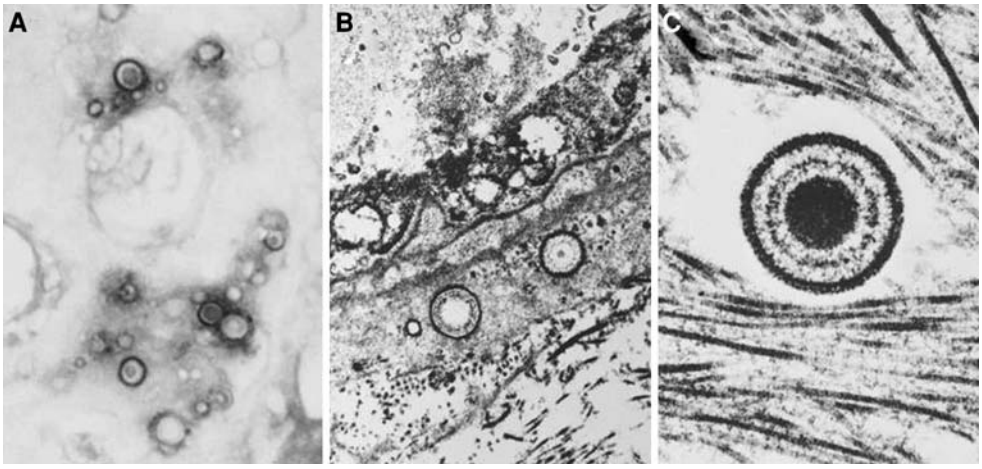

Fig. 11 Transmission electron micrographs of particles observed in autopsy sections of kidneys from patients with no history of stone disease. **a** Spherules in the interstitium adjacent to collecting ducts (relative magnification ×1,000); **b** laminated electron dense bodies in the basement membrane of a collecting duct (relative magnification ×21,000); **c** higher power view of a single laminated electron dense body in the interstitium (relative magnification ×54,000). **a, b** and **c** depict selected portions of Figs. 4, 6 and 5, respectively, of Haggitt and Pitcock [94], reproduced with permission from the *Journal of Urology*. Copyright Elsevier (1971). Images were reduced for original publication. The figure was constructed by cropping the published images without altering the magnifications

kidneys [79, 80, 94] might have been a *post mortem* or experimental artifact, but the very reproducibility of the observation would strongly suggest that is unlikely. I must therefore draw the inescapable conclusion that plaque particles are *normal*—just as are the electron-dense granules in other organisms perched upon just about every branch and twig of the phylogenetic tree. Furthermore, much as I might like it to be, I must hastily add that this opinion is not original: I share it with Anderson and MacDonald [79], Carr [80], and Haggitt and Pitcock [94], who expressed their views many years ago. Assuming they and I are correct, the economy of Nature requires that the particles must have some sort of function in humans, else they would not have persisted through millennia of evolution. Casting aside our self-indulgent humanistic tendency to believe ourselves somehow "special" amongst the occupants of this planet, we are left with the logical assumption that the functions of electron-dense granules in our organs are no different from those in the equivalent body parts of other living creatures.

With the obvious exception of structural roles in shells and integuments, it is not difficult to imagine how electron-dense granules could perform some of the other functions listed in Table 3—all of which are related to the regulation of calcium homeostasis, directly, or by controlling the ambient concentration of two of calcium's physiological counter-ions, phosphate and carbonate. The probability that granules fulfil a similar role in the human kidney is bolstered by their similar physical structures and mineral compositions [55, 87, 94] to those of other organisms, which, as discussed earlier, are typically composed of amorphous $CaCO_3$ and CaP. Furthermore, human plaque granules are poorly crystallized [87], which suggests that they may be amorphous. The importance of amorphous $CaCO_3$ in biomineralization [96] and calcium storage [49] is well recognized, as is the likely involvement of amorphous CaP in the mineralization of bone [97]. In addition to its mechanical advantages and ability to transform easily into a crystalline phase, amorphous calcium salts are more soluble, thus allowing more rapid mobilization of calcium ions during periods of high demand, such as during ecdysis [96].

Calcium-rich granules in the human kidney may therefore play a role in calcium homeostasis by acting as a sink for excess calcium during times of plenty and a readily accessible source of the ion during episodes of famine. Since large deposits of plaque are more common in calcium stone formers [90] and related to higher rates of calcium excretion [87, 89], it is reasonable to speculate that over-excretion of calcium overwhelms the kidney's capacity to store excess calcium in the form of granules, which then accumulate towards the papillary tip where they somehow encourage the precipitation and entrapment of stone crystals within the collecting ducts. On the other hand, the absence of plaque from bypass patients almost certainly reflects a normal excretion of quantities of calcium easily accommodated by the kidney's storage granules: their problem is one of over-excretion of oxalate instead. It seems entirely logical therefore, that Randall's plaque particles, like urinary CaOx crystals, are not products of specific aberrant metabolism, but examples of healthy biomineralization. Unfortunately, however, like all healthy biomineralization, it sometimes goes awry [75], leaving stone researchers to find out why. But *how?*

A pertinence of questions

In the proceedings of the 2006 Indianapolis Urolithiasis Symposium Professor Fred Coe [98] asked a battery of highly pertinent research questions about Randall's plaque, the stone–plaque junction, and the plugging of the inner medullary collecting ducts with apatite deposits:

> Does plaque cause erosion and apoptosis at the papillary epithelium?
> What is plaque matrix composed of?
> Do HAP crystals affect H^+ secretion by IMCD cells?
> Where is plaque made?
> Is plaque affected by urine calcium, volume and pH?
> How is the epithelial barrier breached?
> How does plaque form?
> Do HAP crystals affect NH_4^+ transporters in IMCD cells?
> Do urine ions crystallize on plaque?
> Can we study the mechanisms of HAP attachment?
> What leads to IMCD injury by biological apatite?
> What is the stone matrix at the attachment site?
> Will plaque particles form in cultured cell systems?

It is improbable that solutions to all of these questions or the many others that Professor Coe posed will be found in the foreseeable future, but the last one, at least, has already been answered. Spherical, laminated particles of CaP have been produced on plastic culture dishes and in pseudocysts formed in soft agar culture of Madin Darby Canine Kidney (MDCK) cells after 21 days in continuous culture [99, 100]. More recently, the formation of CaP "flat mineralizing structures" up to 40 μm in size and consisting of aggregates of small round particles of CaP, was observed in conditioned media from a number of cultured cell lines, including ATDC5 and MDCK cells [101]. Mass spectrometric analysis of the structures showed them to contain specific proteins derived from the cells, from serum and from the extracellular matrix. It is worth noting that in the presence of non-conditioned culture medium the small round particles did not agglomerate into the large mineralizing structures. High power TEM images were not presented, but it is possible that the individual CaP particles may be the same as the nanobacteria-like structures observed by Cisar et al. [69]. The ability of cultured cells to induce the formation of apatite structures may therefore provide a convenient model for studying factors affecting biomineral and plaque formation.

Stooping to conquer

Professor Coe asked many other questions, all very relevant to stones, but not specifically to this review. And because it suits my developing argument, I wish to single out just three of them:

> Can we obtain an animal model of IMCD and BD plugging?
> Can animal models of attached stones on apatite plaque be produced?
> Can one produce an animal model of human renal apatite plaque?

Animal models have been the mainstay of most research related to human disease—for obvious reasons, both ethical and practical, and certainly a great deal of what we know about stones and their formation has come from animal studies. However, these three important questions, like all the others [98], are about Randall's plaque. While there is little doubt that Randall's plaque plays a major role in the formation of *some* urinary stones, calculi are not just lumps of plaque. The majority of them consist of cemented clusters of CaOx crystals—and *they* form in urine, not in the renal interstitium. Future stone research cannot ignore the many factors that affect crystal formation and retention within the kidney, and in keeping with my conviction that urinary crystals and plaque particles are normal components of healthy calcium homeostasis, I will now add a fourth question to the animal model theme:

> Is there an animal model of normal CaOx crystal formation?

It is moot whether answers will ever be found to the first two of these four questions, but my response to the last two is a resounding *Yes!* As part of calcium homeostasis, numerous organisms form CaOx crystals, as well as CaP particles with identical physical characteristics and probably analogous functions, as those comprising Randall's plaque. Indeed, any one of the organisms that produced the electron-dense granules illustrated in Fig. 5 can be regarded as just one of the huge menu of potential models of plaque formation out there in Nature just waiting to be explored. Many have already yielded a veritable encyclopaedia of knowledge about calcium granules, including their structure, movement, composition, macromolecular content, synthesis and resorption. That knowledge could help us discover the basic mechanisms and events surrounding the role of plaque in human urolithiasis. The fact that all of us pass crystals [102]—even if only rarely—and all of us form interstitial calcium-rich granules [79, 80, 94], yet only about 10% of us form stones, strongly suggests that our mechanisms for controlling calcium homeostasis are unlikely to differ substantially from those used by our earthly fellow travellers. There are, therefore, many possible biomimetic models that could be exploited to unravel the processes involved in the formation of CaOx crystals, apatite granules and kidney stones. But which one? Well,

why not *Bombyx mori*? As part of its normal metamorphosis, this clever moth manufactures CaP granules. It also lays down in its kidney-like Malpighian tubules CaOx crystals that look remarkably like human urinary crystals, which it *resorbs*. Ridiculous? Not at all. The following quotation comes from a prominent Australian physician, medical scientist and immunologist, Sir Gustav Nossal [103].

> The new principles of biology which create whole movements of consequent research and discovery almost always come from studies done on laboratory animals or smaller life forms.

And he's right. Figure 12 shows a long list of scientific discoveries that have proved to be of incalculable benefit in the comprehension of human metabolism and physiology, and thence, in the treatment and cure of human disease. And lest it be thought that some members of the list are perhaps not quite as groundbreaking as *I* might consider them to be, it is salient to point out that all of them were regarded as being of sufficient import to be awarded a Nobel Prize for physiology or medicine. And were those prizes awarded for scientific investigation performed on human subjects? Of course not! Except for investigations involving only trivial degrees of invasiveness or low pharmacological risk, it is simply not possible to perform studies of disease processes, mechanisms and treatments in human beings, as some investigators have learnt to their peril and condemnation [104]. We have gleaned practically all we know about genetics, biochemistry and biophysical principles, upon which the understanding and treatment of human ailments are founded, from the study of literally *hundreds* of cultured cell lines, but more especially from animals and organisms to whom we usually attach the derogatory tag of *lower form of life*. Presented in Fig. 13 is a list of just a few of our more modest cousins that have been used in studies for which the researchers were awarded Nobel prizes in physiology or medicine, as well as in other invaluable research that did not make it to the top of the Nobel committee's list. They include modest little organisms like viruses, bacteria and yeasts, algae, amoebae, corals and worms, snails, sea slugs and sea squirts. There are also more elevated creatures—mammals, even plants and insects. You see, the proposal that we learn how to balance our calcium input and output from a lowly insect like *Bombyx mori* is perhaps not quite as preposterous as it might first have seemed.

Back to the future of stone research

Like most research on human pathologies, the study of urolithiasis has proceeded tentatively and haltingly. There have been no monumental discoveries that have laid bare all the

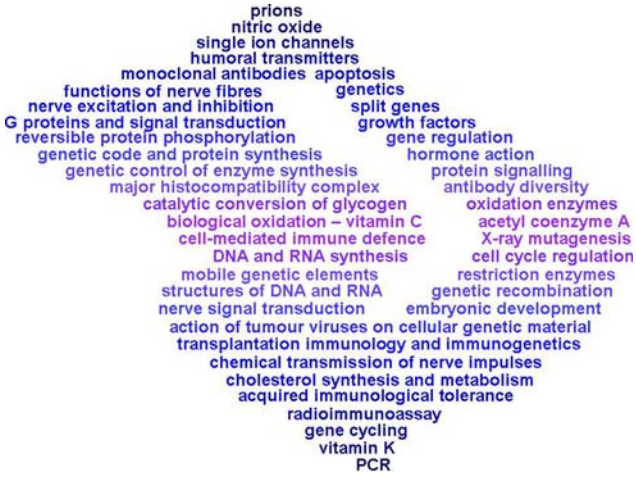

Fig. 12 Scientific discoveries awarded Nobel prizes for physiology or medicine (http://www.nobelprizes.com/nobel/medicine/medicine.html), which were not based on the study of human subjects

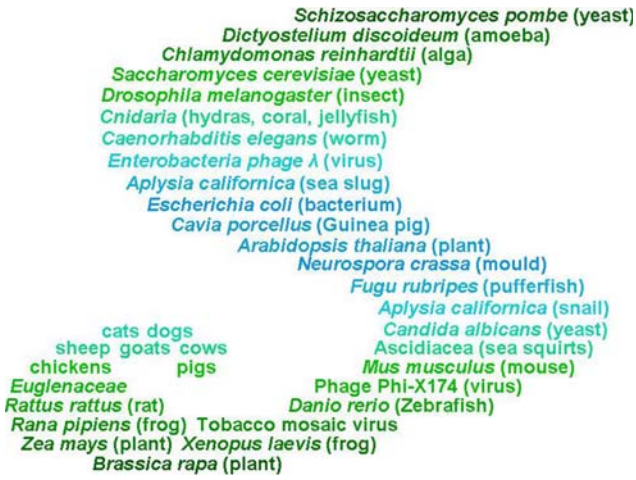

Fig. 13 A small selection of organisms used in Nobel prize-winning research studies

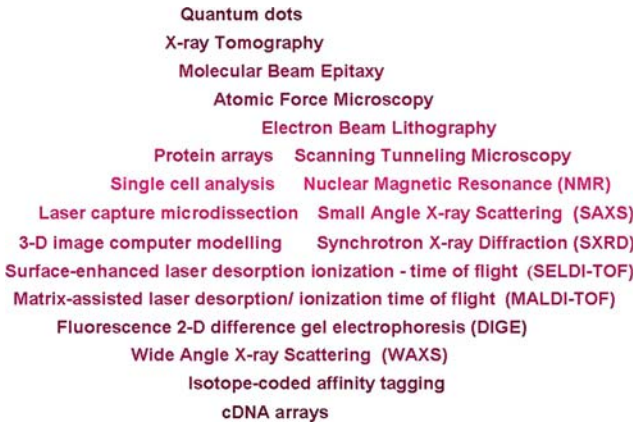

Fig. 14 The toolkit for the future: techniques recently been used in stone research, or which could provide valuable basic information in the future

mechanisms involved in the progression from supersaturation to crystal to stone and nor are there likely to be. In this review I have focused, quite purposely, on basic scientific phenomena of potential relevance to stone research, because I believe that they, rather than clinical investigation, hold the key to the fundamental understanding of human urolithiasis. But that does not mean that I regard clinical research as unimportant or irrelevant. My brief was to discuss the future of stone research, and instead of presuming to venture uninformed suggestions to my colleagues about precise areas of study they should undertake, I have chosen to express my thoughts in the form of ten commandments. These are offered more as suggestions for *approaching* the investigation of stone disease; it is not a catalogue of studies that I believe should be undertaken. They began as nine, because I thought ten would be rather obvious, but I added one (number 9) following a wise suggestion from Professor Roger Sutton.

1. *Persevere with the clinical research*. We do not currently have the ability to cure or prevent stone disease, and it is therefore vital that we continue to find surgical and pharmacological forms of treatment. But it is equally important that we redress the historical imbalance between basic and clinical research in order to discover *why* stones form: cure and prevention will come only from an understanding of the basic mechanisms of disease pathology.
2. *Look at the world around us*. Learn from healthy biomineralization systems. They got it *right!*
3. *Shed the blinkers and read the papers from other disciplines*. There are abundant, relevant concepts and facts out there that we can garner and harness for our work.
4. *Use the toolkit of the future* (Fig. 14). Or stick to the same old methods and techniques. And stagnate.
5. *Befriend the experts who use the tools of the future and know how to analyse and interpret the data they produce*. The tools are complex and far too sophisticated for most of us to understand.
6. *Cherish the model—animal and inanimate*. For stone research will live by it forever. But recognise its limitations: it is still just a model, **not** the actual disease.
7. *Do not be slaves to the current fashion.* Fashions come and go; ignore the hemline and keep sight of the whole outfit.
8. *Consolidate the known before moving on. Really* read (not just the abstracts!) what our colleagues have written and are writing—it may all have been done before.
9. *Foster collaboration between scientists and clinicians*. Communicate, or run the risk of performing clinically irrelevant or scientifically questionable work.
10. *Rummage through the attic.* The average period between the time a discovery is made to the time it is awarded a Nobel prize in Science, Physiology or Medicine is approximately 20 years—old does not equate with obsolete or unimportant. The interstitial calcium granules discovered and photographed by Haggitt and Pitcock way back in 1971 are indistinguishable from those in the splendid collection recently assembled by Evan et al.. The difference is perhaps that their significance was not fully appreciated in 1971, or simply that other aspects of stone disease and research became more exciting. Certainly, at that time their occurrence in other organisms was not appreciated. And so, the fact that the human kidney contains apatite particles was consigned to the attic. To be sure, the name of Randall was often mentioned, but the early observations by Carr, Anderson, MacDonald, Haggitt and Pitcock seemed to have suffered the same fate as the gem and flower of Grey's famous poem—

Full many a gem of purest ray serene
The dark unfathom'd caves of ocean bear:
Full many a flower is born to blush unseen
And waste its sweetness on the desert air
Thomas Gray (1716–1771)
Elegy Written in a Country Churchyard

—until the turn of this century, when the peculiar little particles in Randall's plaque were rediscovered. Now we know that they are virtually identical in size, composition and construction to the vast array of granules cleverly used by inestimable throngs of living organisms throughout the earth to dispose of their waste, construct their skeletons or control the ebb and flow of calcium and other essential and toxic ions—all without forming pathological concretions. And future stone research? Find out how they do it, of course!

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