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A simple technique for studying struvite crystal growth in vitro

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Summary. Struvite urolithiasis forms as a consequence of a urinary tract infection by urease-producing species of bacteria such as *Proteus mirabilis.* Ammonia, produced by the enzymatic hydrolysis of urea, elevates urine pH causing a supersaturation and precipitation of Mg^{++} as struvite (NH_4MgPO_4). Calcium often precipitates as well, forming the mineral carbonate-apatite ($Ca_{10}(PO_4)_6CO_3$). We have developed a procedure based on direct observation by light microscopy whereby struvite crystal growth can be quickly monitored in response to chemical changes in urine. As struvite crystals assume a characteristic shape or crystal habit based on their growth rate, the effect of urine chemistry and the action of various crystallization or urease inhibitors on struvite formation can be quickly shown. In addition preliminary effects of alkaline pH, or the presence of toxic compounds on bacteria can also be shown through their loss of motility.

Key words: Struvite – Urolithiasis – *Proteus mirabilis* – Crystallization – Light microscopy – Urease

Struvite urolithiasis arises as a result of urinary tract colonisation and infection by urease-producing bacteria such as *Proteus mirabilis* [12, 18, 20]. Ammonia, resulting from the urease-catalyzed hydrolysis of urea, elevates urine pH causing the formation of struvite (NH_4MgPO_4), carbonate-apatite ($Ca_{10}(PO_4)_6CO_3$) [13] due to the reduced solubility of Mg^{++} and Ca^{++} and increasing presence of PO_4^{3-} and CO_3^{2-} at neutral or alkaline pH [14]. Although the importance of urease, and urease-producing bacteria in the pathogenesis of these calculi has been well established, normal urine often is quite resistant to crystallization due to the presence of one or more crystallization inhibitors [15, 25].

Crystallization studies are becoming increasingly important as we attempt to understand the mechanisms of urolithiasis. Two major classes of compounds have been identified. These include the urinary glycosaminoglycans (chondroitin sulphates, and heparin sulphates) [3, 7, 10, 11, 24], and pyrophosphate [23, 24, 31]. Presumably these compounds act by chelating divalent cations such as Ca^{++}, making them unavailable for precipitation. Urinary proteins have also been implicated (H. Hedelin – personal communication). An increasing number of very elaborate techniques are being developed to study urinary crystallization processes [2, 4, 5, 16, 28, 31]. Essentially all of these techniques depend upon: 1) generating the crystals, and 2) evaluating the crystals formed by a variety of chemical or physical procedures.

Our new experimental strategy presented in this paper is actually a very old concept – direct observation. Using a light microscope equipped with phase contrast and dark field optics, we can follow the growth of struvite crystals in vitro. Since struvite crystals assume a characteristic shape or crystal habit which is dependant on growth rate [1], this technique can be used to screen potential crystallization inhibitors for their effect on struvite formation.

Material and methods

The urease-producing strain of *Proteus mirabilis*, designated as strain 2573, was isolated from a patient with a struvite urinary calculus, and maintained on a slant of tryptic soy agar (Difco Laboratories, Detroit, MI, USA). The artificial urine and protocol used in struvite production was essentially our modification [21] of that described by Griffith et al. [13].

A continuous culture flask containing 800 ml of filter-sterilized artificial urine was inoculated with *P. mirabilis* [21]. Sterile artificial urine was added to the flask at a rate of 60 ml/h providing a dilution rate of $0.075\ h^{-1}$. The volume of the flask and the dilution rate were chosen to approximate the bladder volume and urine production in human males. Samples were removed at 0 h, 0.5 h, 1 h and thereafter hourly until 7 h, and monitored for pH, culture optical density, bacterial cell counts, urease activity, and ammonia concentration. In addition, samples were also taken prior to inoculation, as well as 24 and 48 h after inoculation. Examination by light microscopy was performed by placing a drop of the artificial urine on a clean microscope slide, covering with a glass cover slip, and examining unstained with a Leitz Aristoplan light microscope (Ernst Leitz Wetzlar GmbH, Wetzlar, FRG) using either the phase contrast or dark field mode. Photomicrographs were taken using the 35 mm

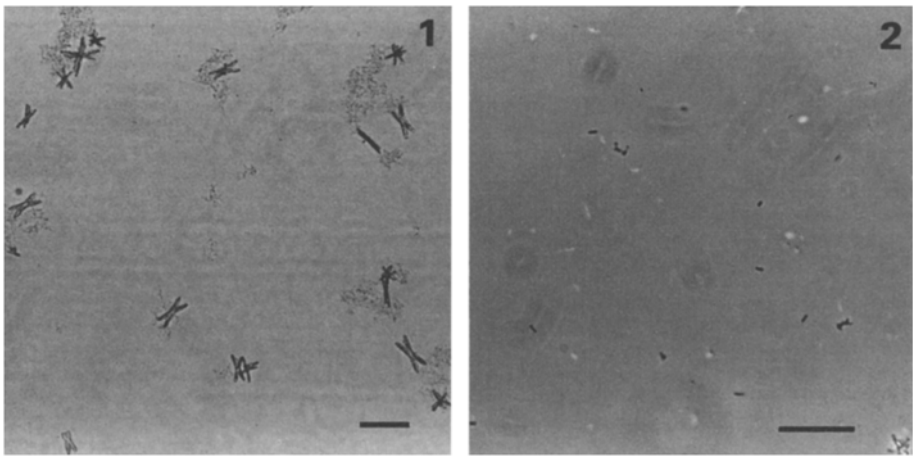


Fig. 1. Phase contrast light micrograph of the *Proteus mirabilis* inoculum in artificial urine (pH 9.0) showing large numbers of dendritic, X-shaped struvite crystals (indicative of rapid crystal growth [1]) in the vicinity of the bacteria. Bar = 100 μm

Fig. 2. When this inoculum is introduced into fresh artificial urine (pH 6.0) struvite crystals rapidly dissolve and the bacteria become motile. Bar = 25 μm

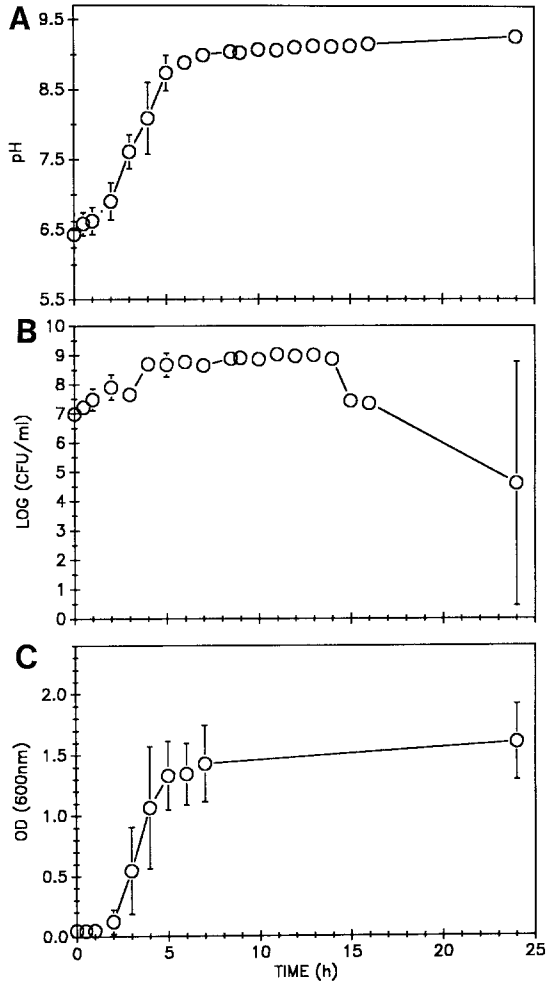


Fig. 3. A pH, B CFU, C Turbidity due to *P. mirabilis* growth in artificial urine

camera attached to the microscope using Kodak ASA 400 Tri-X pan film (Kodak Canada Inc., Toronto, ON, Canada).

Culture urease activity and ammonia concentration were monitored by removing culture samples, and assaying for ammonia concentration or production due to urease activity using the phenol-hypochlorite ammonia determination procedure of Weatherburn [30] as modified by Cook [9]. Urease activity was expressed as (μg NH_3 produced) (ml culture)$^{-1}$ (min)$^{-1}$.

In order to estimate the density of crystals and *P. mirabilis* cells, artificial urine culture samples were removed and their optical density measured at 600 nm.

P. mirabilis growth was also monitored by aseptically removing culture samples, serially diluting in phosphate buffered saline (PBS) 5 mM K_2HPO_4, 4.5 mM KH_2PO_4, 150 mM NaCl, pH 7.2), plating onto nutrient agar (Difco), and incubating overnight at 37°C.

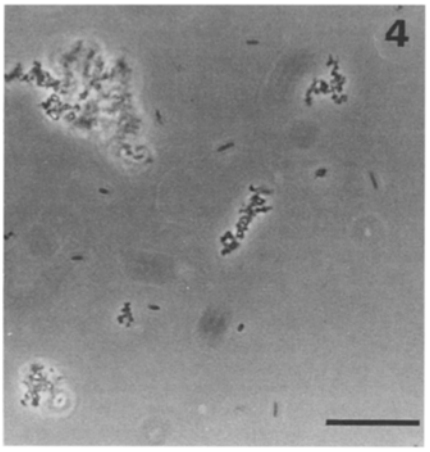
For X-ray diffraction analysis, crystals were harvested by low speed centrifugation (200–300 g), washed twice on 0.05 m Tris(hydroxymethyl) amino methane (Tris) buffer pH 8.6 and dried under partial vacuum in a dessicator as previously described [8].

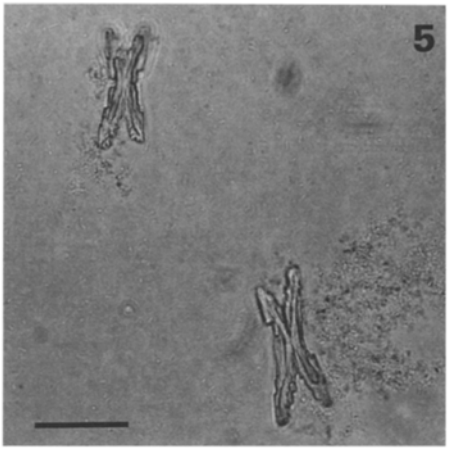
Results

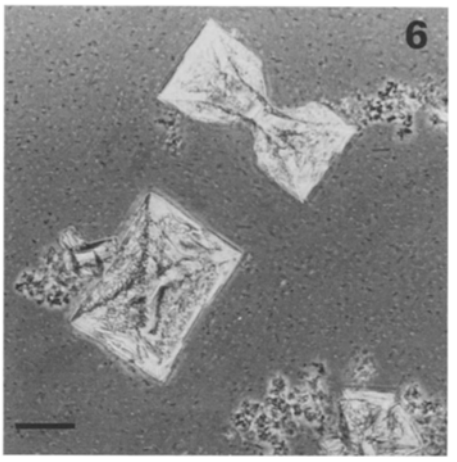
Microscopical examination of the inoculum containing approximately 4×10^8 CFU/ml *P. mirabilis*, revealed the presence of bacterial cells and a large number of struvite crystals (Fig. 1) due to the alkaline pH. The bacteria were largely non-motile, with motion being largely limited to Brownian motion [29]. The few cells that were motile moved in an erratic spinning or tumbling manner [6, 19]. Within 5–10 minutes of inoculation into fresh artificial urine, most of these crystals had dissolved, and the vast majority of bacteria became motile, exhibiting both swimming and tumbling forms of motility (Fig. 2). Subsequent growth of *P. mirabilis* in artificial urine produced a rapid rise in pH, optical density, and cell numbers (Fig. 3). Urease activity and elevated ammonia levels due to *P. mirabilis* were also present (data not shown).

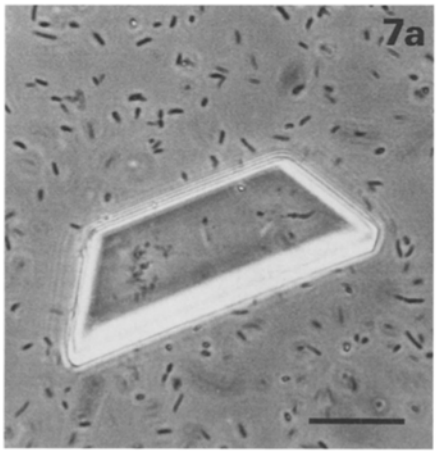
Struvite production was marked initially by the appearance of increasing quantities of amorphous precipitate (Fig. 4), as the pH rose above 7. This was then followed within 1–2 h by crystal development (Fig. 5–8). Quite often the first crystals to develop (especially when the urine was not agitated) were X-shaped (Fig. 5). After several hours, these X-shaped crystals developed a more tabular appearance (Fig. 6). In other cases, only trapezoidal or octahedral crystals were seen (Fig. 7). Powder X-ray diffraction analysis confirmed that all of these crystal morphologies were struvite (Fig. 8). X-ray diffraction also confirmed that under our experimental conditions, no carbonate-apatite was produced.

Upon introduction into fresh artificial urine, *P. mirabilis* cells became rapidly motile, exhibiting mainly swim-









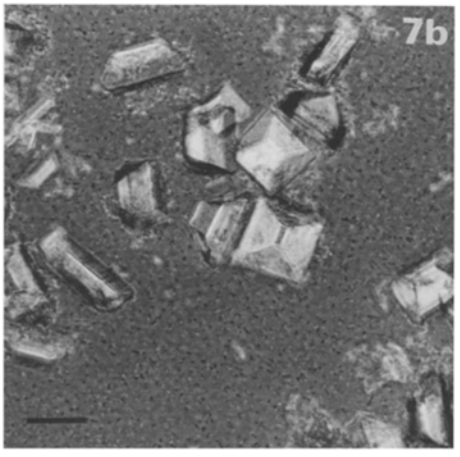


Fig. 4. The onset of *P. mirabilis*-induced struvite production is initially characterized by the appearance of an amorphous magnesium precipitate. Bar = 25 μm

Fig. 5. Dendritic, X-shaped struvite crystals indicative of rapid crystal growth and high Mg^{2+} and NH_4^+ concentrations [1]. Quite often struvite crystals were associated with the amorphous precipitate. Bar = 50 μm

Fig. 6. As growth slows, the X-shaped struvite crystals take on a more tabular appearance. Bar = 50 μm

Fig. 7. **a** Very slow growing struvite crystals are typically trapezoidal of **b** octahedral in shape, since they grow more or less evenly along all crystal planes. Bar = 20 μm (**a**), 50 μm (**b**)

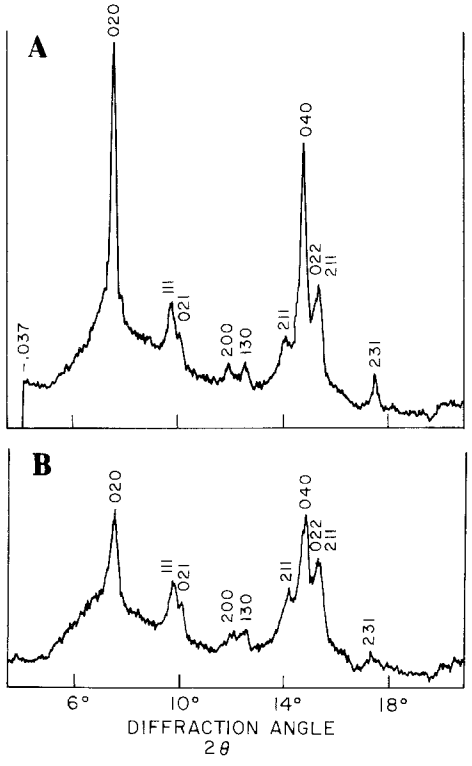


Fig. 8. **A** Powder X-ray diffraction of X-shaped crystals and **B** octahedral crystals. The various struvite crystal planes (identified by numbers) are all present. No carbonate-apatite crystals were identified by this technique

ming, although a limited amount of tumbling motility also occurred [6]. As the pH and ammonia concentration increased, the number of motile organisms decreased quite markedly. At high pH, the vast majority of cells agin exhibited Brownian motion [29], and the few cells that were motile became very erratic and exhibited predominately tumbling motility [6]. We attribute this decrease and alteration of motility as evidence that the organisms were undergoing stress due to the alkaline pH and/or high ammonia levels. This was confirmed in that cell numbers of *P. mirabilis* often declined quite markedly by 24 h (Fig. 3). After 48 h, no *P. mirabilis* were detected by viable plate counts.

Discussion

Struvite urolithiasis has been documented since the time of Hippocrates. Early workers in the 20th century described the association of ureolytic bacteria with these calculi [20], but is has only been since the work of Griffith et al. [13] and Hedelin et al. [14] that the chemistry of struvite precipitation has been studied in any detail.

Precipitation occurs in a solution (such as urine) when it becomes supersaturated with respect to one or more ionic components. Supersaturation may occur for a variety of reasons, however in the case of struvite production, it is the appearance of large quantities of NH_4^+

and alkaline pH resulting from bacterial urease activity [14, 20] that reduce the solubility of Mg^{++}. If solubility is greatly exceeded, an amorphous precipitate rapidly forms. These amorphous materials form more readily that crystals because they do not possess an ordered structure. Nevertheless, they are less stable than crystals, so assuming appropriate chemical conditions, the molecules in this amorphous precipitate will rearrange into an ordered array, which upon growth manifests itself as a crystal. Crystal nucleation, namely the formation of the first few ordered crystal atoms, is a difficult process, and for thermodynamic reasons, usually begins on a solid substrate [17]. In this case, the initial amorphous precipitate, and/or bacterial components, provides this nucleation site (Fig. 4). Most of the crystals observed were associated to some extent with the amorphous precipitate. From this one would suspect that this solid material may have been at lease partially responsible for crystal nucleation. The eventual formation of the mineral, struvite, due to Mg^{++} supersaturation, is a result of the high pH, and presence of NH_4^+ and PO_4^{3-} [14]. Crystals exhibit characteristic shapes and X-ray diffraction characteristics ultimately derived from their molecular packing, however variations in growth rates may result in a change in morphology (crystal habit) due to their preferential development along one or two crystal planes [17].

The crystal habit of struvite has been shown to vary quite markedly according to the rate of growth. Very high growth rates induce the appearance of dendritic or X-shaped crystals because of elongation along one or two crystal planes. As growth rates slow down and become more evently distributed among the various crystal planes, tabular crystals, and finally trapezoidal and octahedral crystals develop [1]. These three crystal habits were seen in Fig. 5.–7 respectively.

P. mirabilis, are very motile organisms by virtue of their numerous flagella. Motility arises when the flagella are rotated by the bacterial cell, a process which either drives the cell forward in a straight line (swimming), or reorients the cell (tumbling) [6]. This process allows bacteria to travel as well as change direction. Under normal circumstances, periods of swimming are periodically interrupted by periods of tumbling. When bacteria are in the presence of or moving towards an attractant (often a nutrient source), their frequency of swimming increases, and tumbling decreases. The converse is true if a repellant (often a harmful compound) is present. The energy for motility arises as a direct result of cell physiology (membrane proton motive force) [6], therefore any stress on the bacterium that would disrupt cell physiology, might manifest itself as a loss of motility. We observed that bacterial motility declined as the pH exceeded 7, and that the limited motility seen was mainly an end over end tumbling. As alkaline pH is a known repellant [26], we would interpret the increased tumbling to this phenomenon, and the greatly reduced overall motility as evidence of physiological stress.

One must recall that struvite calculi are formed in situ by struvite mineralization within an organic matrix [22]. Crystals freely suspended in urine, such as those observed in this experiment, are usually lost upon voiding. While keeping this in mind, the direct effects of potential crystallization inhibitors on struvite mineralization might be most apparent on struvite formed within the artificial urine solution where the exposure to the inhibitor would be greatest. This technique at present is therefore best used for preliminary screening of potential crystallization inhibitors.

Direct observation, particularly with phase constrast light microscopy, offers a distinct advantage, in that unstained biological materials can be instantly examined. In addition, it has been widely used to study crystal surface characteristics [27]. This allows one to monitor developments as they occur over the duration of an experiment. In this case, we were able to observe the development of struvite crystallization, and see the rate of crystal growth as reflected in crystal habit. In addition, we could also detect preliminary evidence of physiological stress on *P. mirabilis* (reflected by a change and loss of motility) almost 24 hours before a decline in bacterial numbers occurred due to alkaline pH and high ammonia levels. We are currently using this technique of direct observation by phase contrast light microscopy, in conjunction with several other techniques to study the effects of various compounds (antibiotics, crystallization inhibitors, etc.) on struvite formation.

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