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Potential contribution of optional urease-positive bacteria to idiopathic urinary calcium stone formation

II. Microlith formation kinetics in a fermenter model of the urinary tract infected by optional urease-positive microorganisms

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Abstract We investigated the effects of weak to moderate urease hydrolysis by optional urease-positive microorganisms in an artificial urine model enriched with calcium phosphate and calcium oxalate in respect of calcium stone formation. The incubation experiments were performed using a discontinuously running fermenter device to simulate the urinary system. The kinetics of cell division rates, pH and ammonium ion production were measured and correlated to crystallite appearance in the incubation medium. Qualitative analyses of the sediments revealed apatite. Investigations using light microscopy and scanning electron microscopy (SEM) confirmed the matrix effect of bacterial glycoproteins. It was shown that initiation of calcium oxalate stone formation is in all probability equally determined by matrix effects and by heteronuclear crystallization if the urinary tract is infected by optional urease-positive bacteria. When urinary inorganic phosphate is present, calcium phosphate nidi are always initially formed, and may subsequently be coated by calcium oxalate.

Key words Calcium stones · Microlith formation · Urinary tract model · Optional urease-positive infection

Besides their crystalline components, all urinary calculi are known to contain about 3–5% of high molecular weight organic substance known as "matrix" [6]. This is either produced by the secretion from the cells lining the urinary passages [3] or it may be of bacterial origin [8]. The role of matrix in the pathogenesis of stone formation is based on two possible mineralization mechanisms: (1) dead or living bacteria act as a heterogeneous nidus for mineral deposition or (2) bacterial exopolysaccharides (glycocalix) floating in the ambient urine become concentrated with Ca^{2+} and Mg^{2+} by anion exchange mechanisms, resulting in mineralization.

In a previous study [13] we isolated certain organisms from the urines of non-struvite stone patients. These bacteria were commonly regarded as being unable to hydrolyse urea. However, all of the strains were found to express the enzyme urease when challenged by a need, such as inorganic nitrogen depletion in the medium. Thus the theory that bacterial surface material alone can act as a matrix for calcium stone formation has to be modified to include the possibility of struvite nucleation occurring due to the presence of these organisms causing some hydrolysation of urea.

We investigated whether the weak to moderate urea-splitting potency of optional urease-positive (*oup*) microorganisms in a complex artificial urine model enriched with calcium oxalate and calcium phosphate and subjected to flow and stasis states would induce microlith formation such as struvite stone initiation, or whether precipitations of poorly soluble calcium salts of phosphoric acid (apatites) and the formation of calcium oxalate as a whole be the basis of stone genesis.

Material and methods

To perform incubation experiments with *oup* urinary pathogens in an artificial urine (AUR), we employed a fermenter device analogous to the "mixed suspension mixed product removal crystallizer" (MSMPRC) as established by Finlayson [5]. This operated in a discontinuous mode in so far as we altered the medium and the flow during a run. The incubation medium was a complex urine model developed on the basis of the well-known "Christensen urea

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medium" [4]. By the addition of inorganic ions (according to Miller et al. [10]), it was equalized to a natural standard urine. During flow phases, Ca^{2+}, Mg^{2+} and oxalate anions were added separately, and thus the basic medium was enriched with calcium oxalate (CaOx) and calcium phosphate (CaP) by chemical reaction (Table 1). According to the flow options and the mean AUR ion strength of $I = 0.25$, the solubility product of hydroxyl apatite (HAP) was achieved within 8–10 min, and saturation with CaOx was achieved after 25–30 min (calculated on the basis of data given by Raflaub [12]). Phases of batch incubation simulated stasis conditions and a day-night rhythm of micturition in the urinary tract. Preceding all incubations with microorganisms, the AUR was repeatedly examined in respect of its physicochemical stability during sterile runs.

The *oup* bacteria were isolated from the urines of stone patients as described elsewhere [13]. The following strains were investigated in duplicate runs: *Enterococcus faecalis* (type I), *Enterococcus faecalis* (type II), *Enterobacter cloacae* and *Escherichia coli*. *Proteus mirabilis*, which characteristically synthesizes urease, was matched as a control.

Each incubation cycle consisted of four phases: (a) a 72-h precultured bacteria suspension to adapt microorganisms from survival state of the strain collection to an exponential growth phase (PRE). The resulting suspension (standardized to an optical density $OD_{600\,nm} = 0.3$) was used to inoculate the basic medium BM residing in the fermenter (vol = 300 ml). (b) The system was run in the batch mode for 24 h in order to enrich the bacterial culture (B1). (c) From the 2nd to 5th days the reservoirs A and B were connected at a flow rate of 6 ml/h for 8 h each day, interrupted by 16 h of the batch incubation mode (F/B). (d) This period was followed by an uninterrupted incubation in the batch mode (B2) until there was sufficient material (crystallites, cells, mucins, sediment) for preparative analyses. The system was continuously supervised during phases a–d by macroscopic and light microscopic inspection for documentation of spontaneous or induced microlith formation, growth rate and any contamination by extraneous organisms. During phases (c) the OD (expressing the cell division rate), the pH and the NH_4^+ concentration of the medium were measured at the onset of flow, after 4 h of flow and at the end of flow. The same measurements were performed once a day during phase (d). After harvesting the fermenter contents, sediments and submarine objects colonized by bacteria were prepared for analysis by infrared spectral photometry (IR), X-ray diffractometry (XR) and scanning electron microscopy investigations (SEM).

Table 1 Composition of the artificial urine AUR

Component	Basic medium BM (mM)	Reservoirs[a]	
		A (mM)	B
Bio-gelytone (g/l)	1.00	2.00	
Glucose	5.55	11.10	
Urea	333.00	666.00	
Oxalate dianion		1.51	
Phenol red	0.03	0.06	
Na^+	213.76	502.60	
K^+	125.15	252.30	
Mg^{2+}			16.23
Ca^{2+}			8.93
Cl	278.30	556.60	17.86
HPO_4^{2+}		33.11	
$H_2PO_4^-$	14.70	35.22	
SO_4^{2-}	23.44	46.89	16.23
	Total = AUR		

[a] Connected to the fermenter during flow phases

Results

The incubation of the urine model enriched with CaOx and CaP in an MSMPRC with alternating flow and batch culture (F/B) phases successfully simulated the state of an infected urinary tract either during a partial change of the fermenter contents, representing moderate emptying and refilling of the bladder, or during the batch culture periods, representing reversible and total stasis conditions. The incubation modes F/B and B2 showed significant differences particularly in respect of the gram-negative rods when looking at the data for OD, ammonium ion concentration and pH. No *oup* bacteria strain induced microlith formation before onset of the B2 mode, i.e. no crystallization event was ever observed during F/B periods, whereas the urease-positive(*up*) control organism *Proteus mirabilis* triggered crystal formation early in the 2nd day of incubation.

The optical density remained constant at between 0.2 and 0.3 throughout the total incubation time when the AUR was infected by cocci. Cultivation of gram-negative enterobacterial rods raised the OD from 0.3 up to 1.0 when the B2 phase had started. However, the moment of crystal appearance could not be correlated to a distinct OD value (Fig. 1).

The ammonium ion concentration of the AUR, being the criterion of urease activity of the current infective strain, was subjected to the known regulation mechanisms of feedback [11] and therefore sometimes fluctuated considerably. Concerning cocci, it increased to half of the maximum during the F/B periods. Like the OD, it did not alter, on average, during the subsequent incubation time. The rods hydrolysed minor amounts of urea during the F/B periods, but made up for this by a steep increase in NH_4^+ production at the beginning of the B2 phase. By the 2nd day of incubation, the buffer capacity of the AUR was saturated with NH_4^+ by *Proteus* in a blasting reaction. Also with respect to the ammonium ion concentration, there was no absolute threshold value for the appearance of crystallites (Fig. 2).

The initial pH value of both the sterile AUR and of the reservoirs A and B was 6.6. For sustainment of cell growth, the AUR contained some lactose, the fermentation of which resulted in the secretion of organic acids by bacteria. After inoculation, and obviously depending on cell titre, AUR acidity was enhanced significantly during the first F/B period (up to one unit in coccal infection). Ammonium ion production and partial exchange of the incubates during the F/B periods stabilized the AUR pH at the level of weak acidity. Whenever the pH approached neutrality or passed it, the first crystallites appeared in the AUR suspension. Thus, the pH of the medium was the ruling factor for crystal nucleation (Fig. 3). This finding was strengthened by sterile runs in which the pH was the only changing parameter, increasing above neutral by chemical

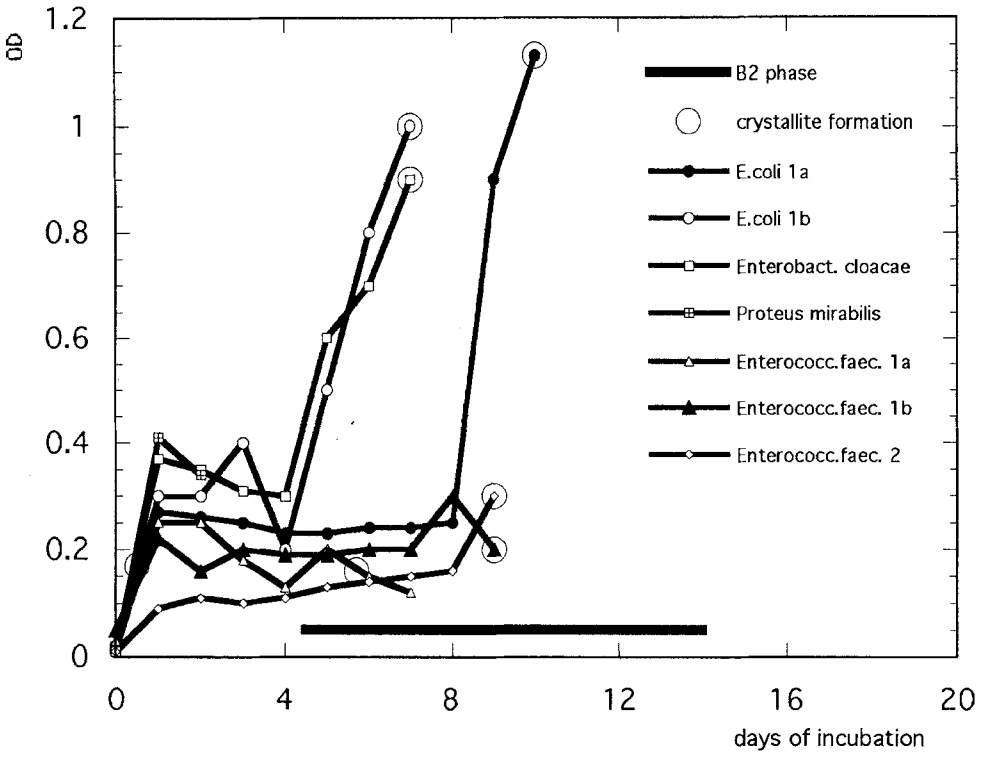

Fig. 1 Kinetics of optical density (*OD*) during incubation of the urine model with uropathogenic bacteria. *B2 phase* – second batch culturing phase

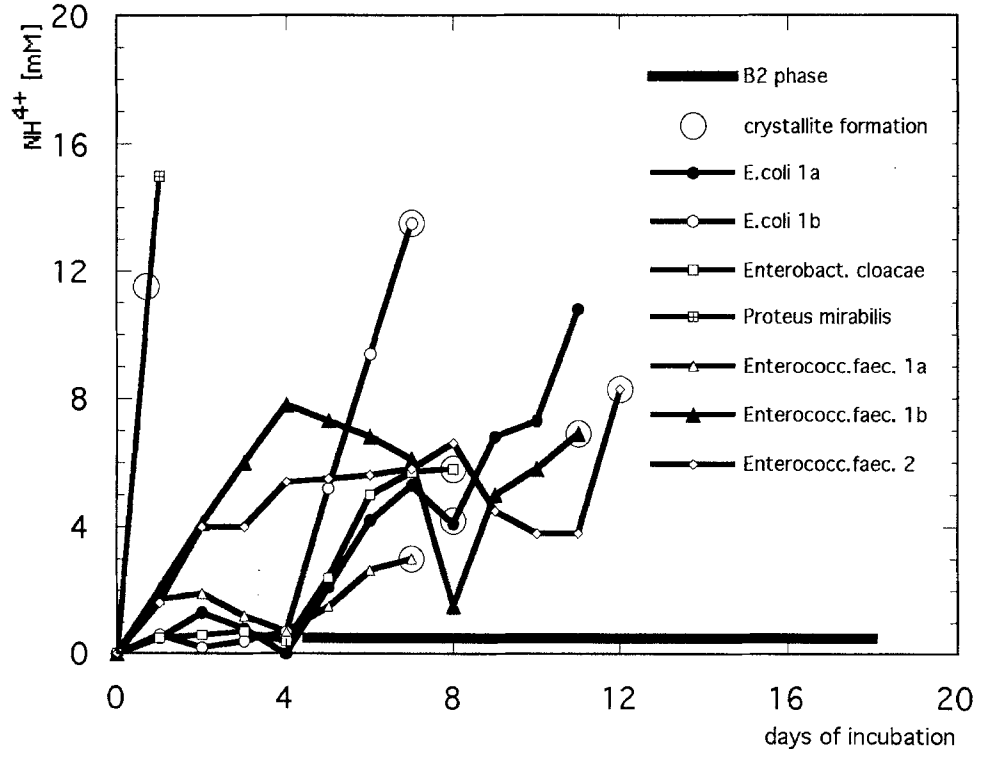

Fig. 2 Kinetics of NH_4^+ production during incubation of the urine model with uropathogenic bacteria. *B2 phase* – second batch culturing phase

means. Also in these experiments, CaP precipitated in the range of pH 7. Light microscopy revealed that crystallite formation occurred almost exclusively in the areas of mucinous bacterial aggregates, or crystallite growth centres were related to mucinous material (Fig. 4a, b). Crystallites occasionally found floating free in the medium seemed to be preparation artefacts.

SEM photographs (Fig. 5) exhibited globules or spherolites grown up on the surface of the bacterial cells; cells were partly or totally embedded in mineralized

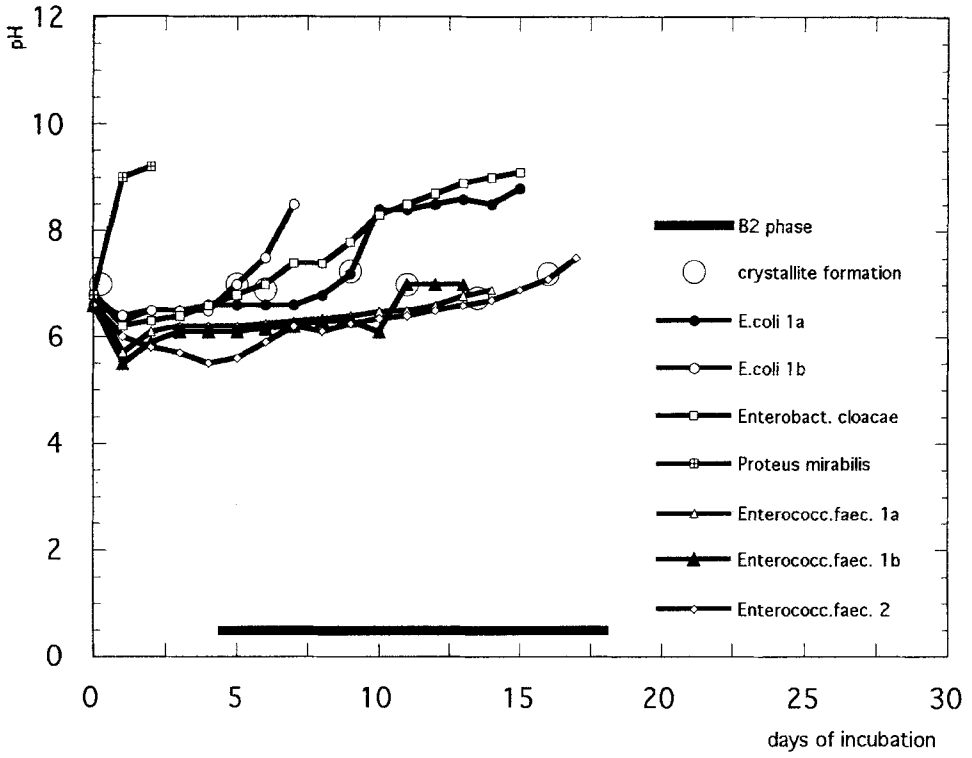

Fig. 3 Kinetics of pH during incubation of the urine model with uropathogenic bacteria. *B2 phase* – second batch culturing phase

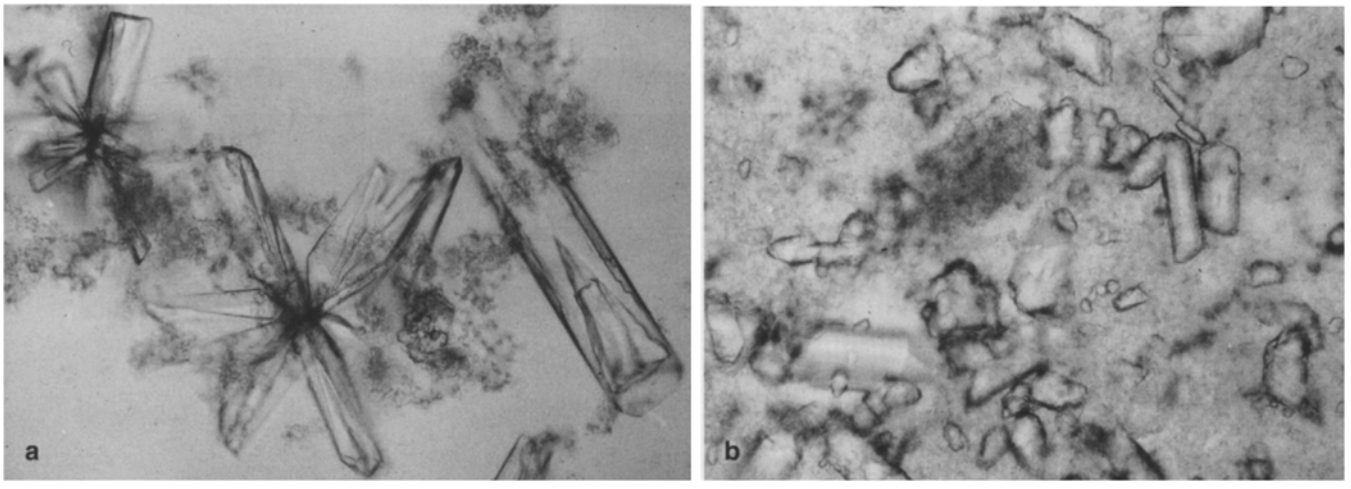

Fig. 4a, b Calcium phosphate (brushite) crystallites from infected artificial urine (*AUR*). **a** Joined to slimy bacterial clots, **b** stuffing bacterial mucus aggregations. (The shapes of bacterial cells are scarcely visible caused by light scattering from the mucus and as the crystals are focused upon. For view of bacteria see Fig. 5.) Light microscopy, ×400

material; and slimy threads were encrusted by calcium salts. There was an evident calcified basic texture, an organic "matrix" composed of bacterial surfaces and bridging capsular slime. Energy-dispersive X-ray atomic absorption analysis revealed the encrustations to be amorphous calcium phosphates. The morphology of the individual crystals as identified by phase contrast light microscopy was similar to that of brushite. Struvite sometimes appeared. Calcium oxalates (weddelite, whevellite) could not be identified with certainty. After preparation, XR and IR analysis always revealed apatite, which is the transformation product of unstable brushite [10].

Discussion

In our fermentation experiments with artificial urine (AUR), we imitated the basic conditions of infectious calcium concrement formation by infecting the MSMPRC urinary tract model with *oup* microorganisms

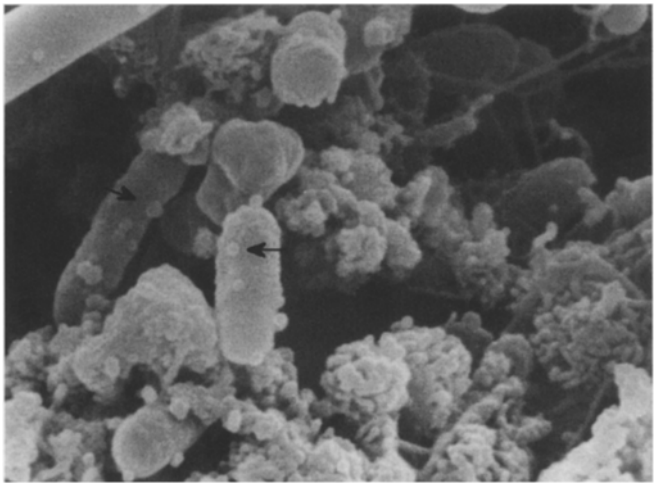

Fig. 5 Calcified bacterial cells and slime threads isolated from infected artificial urine (*AUR*). *Arrows* indicate calcium phosphate (apatite) spherolites. SEM, × 15000

from the urine of calcium stone patients. We found that: (1) the *oup*, i.e. the weakly urea-splitting microorganisms, like the *cup* uropathogens, were able to alkalinize the model urine at least under stasis conditions, the investigated cocci in a rather slow manner. (2) However, on the basis of our results, the moderate urea splitting of the *oup* bacteria would not be sufficient to generate struvite (infection) microliths in a normally flushed urinary system. (3) We were able to demonstrate that bacterial cell surfaces and capsular slimes, chemically representing glycoproteins and mucoproteins, provided a matrix capable of being easily encrusted by minerals from the model urine. The encrustations and spherolites were selectively formed by CaPs, as CaP salt formation (brushite, apatite) suppressed the formation of calcium oxalates (weddellite, whewellite), though the latter should precipitate in an acid milieu ($pK_{CaOx} = 1.46$ and 4.4; $pK_{CaP} = 2.0$ and 7.2). However, the solubility product (SP) of CaP (1.1×10^{-56} in urines) was incomparably lower than that of CaOx (1.7×10^{-9}) [7]. Thus it is obvious that CaP nucleation is favoured in comparison to CaOx nucleation as long as soluble inorganic phosphate is present in urine. In this process, a CaP matrix or CaP crystallites are initially formed and we assume it provides nucleation centres for further heterogeneous Ca salt (CaP, CaOx) deposition. This correlates well with the frequent finding of amorphous CaPs and apatite spherolites in the centres of calcium stones [1, 2, 9]. Our results suggest that, for the initiation of CaOx stone formation, neutralization up to alkalinization of the urine (partially and/or temporarily) started by urease activity may be of some importance, as the SP of CaP diminishes drastically near pH 7, and as a result of this rapid heterocrystalline nucleation can occur. We should also bear in mind that the buffer capacity of urine may be lowered significantly when phosphate is eliminated from the medium by CaP precipitation. The slight glucose supplementation of the AUR may not conform with normal urine. But as enterobacteria are known to use the carbohydrate moiety of glycoproteins and mucins for metabolism [14], we believe not to have deviated too much from natural conditions. (4) As the polyanionic macromolecules of the cell surface and the acid mucins of the capsular slimes can concentrate calcium and its counterions in its immediate vicinity, thus leading to local supersaturation, it becomes the preferred generation loci of calcium mineral precipitates (Figs. 4a, b, 5).

In consideration of our finding that none of the strains we had isolated from the urine of stone patients was really urease-negative, i.e. that under certain conditions would be able to split urea [13], the seemingly contradictory ideas about the formation of calculi by spontaneous crystallization in the course of supersaturation or by deposition of minerals onto a matrix by anion exchange can be combined. It is arguable whether these *oup* strains in vivo under urinary tract conditions will produce significant amounts of ammonium ions for increasing urine pH. But as a hypothesis for further investigation it may likewise be assumed that *oup* bacterial metabolism in some micro-environments which are cut off from urine flow and thus from active substrate supply (diverticles, mucosal crevices, bacterial colonies entrapped in solid glycocalices or mucus plaques) can cause local precipitation of struvite or CaP crystal seeds for the beginning of an additional heterogeneous nucleation. Entrapped in the network of the macromolecular jelly of the bacterial glycocalices (and/or highly viscous mucosal secretions in vivo), microlith growth can begin.

This in vitro study tried to simulate chemical, thermodynamic and kinetic parameters in the urine. In vivo some renal tubular cell metabolism will also influence early events of stone formation and further growth. Hence we need more investigations to bridge the gap between heterogeneous struvite or CaP nucleation and CaOx stone formation.

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