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Intracellular Crystal Formation in Bacteria from Human Urines: A Contributing Factor in Urinary Calculi

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Summary. Understanding of the bacterial contribution to urinary calculi has been limited to those organisms capable of altering the urine through urease activity. Sterilized urines from stone forming and non-stone forming individuals were inoculated with bacteria having either strong, weak, or no urease activity. All organisms grown in unbuffered urines produced crystallization (calcite or apatite) as demonstrated by X-ray diffraction. Bacteria grown in conventional medium (Heart Infusion broth) did not demonstrate crystal formation. Unstained specimens revealed electron-dense deposits within bacteria grown in urine. Deposits were not present in organisms grown in conventional media. Analysis revealed increased levels of calcium within these deposits as compared to extracellular levels. These findings support the hypothesis that both urease producing and non-urease producing organisms may accumulate calcium crystals intracellularly and form nidi for calculus formation.

Key words: Calculus disease, Bacteria, X-ray diffraction.

INTRODUCTION

Of the known causes of urinary stone disease, infection is the most common with an associated frequency of 12 to 32% (12). Understanding of the bacterial role in urinary calculi, however, has been limited to those organisms capable of altering the physicochemical state of the urine via urease activity (13). This does not explain the presence of organisms with weak or absent urease activity in cultures obtained from a variety of urinary calculi (22).

Several investigators have noted the ability of a variety of micro-organisms to accumulate intracellular crystalline deposits when grown in a specialized medium (8, 9, 14). Keefe and Smith (15) proposed a similar occurrence based on X-ray powder diffraction of several genera of organisms that had been inoculated into sterile urine obtained from a known stone forming individual. However, neither direct visual nor physical (elemental analysis) evidence for this material being intracellular in origin was obtained.

The purpose of this study was to investigate bacterial mineralization in human urines obtained from both stone forming and non-stone forming individuals, using microorganisms having different degrees of urease activity. In addition, an Escherichia coli strain grown in Heart Infusion broth and in different urines was examined for the presence of intracellular calcium deposits with electron microscopy and by elemental analysis. This particular organism was chosen for its absence of urease activity and its frequent occurrence in human urinary tract infections.

MATERIALS AND METHODS

Unbuffered Urines

Freshly voided urine was obtained from stone forming and non-stone forming individuals. Urinary calcium concentrations were determined by atomic adsorption spectrophotometry (Perkins-Elmer 303, Perkins-Elmer Corp., Norwalk, Conn.). Urinary pH was also measured. The urines were filtered through Whatman filter paper number one (Whatman Inc., Clifton, N. J.) to remove large particles, then filtered through a millipore Swinnex Apparatus (Millipore Corp.,

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Bedford, Ma.) containing a filter of 8 micrometer pore size to remove smaller particles. Finally, a sterile Swinnex apparatus containing a filter of 0.22 micrometer pore size was used to sterilize the urine. Urines were dispensed in 25 ml aliquots into sterile 50 ml centrifuge tubes. Heart infusion broth was prepared and filter-sterilized in the same manner as above to serve as a control medium.

Buffered Urines

Urines buffered at pH 7.4 with 0.15 M N-tris [hydroxymethyl] methyl-2-aminoethane sulphonic acid (TES) were prepared by addition of the buffer prior to the filtration process described above. The urinary pH in each instance was elevated to the pKa of the buffer (7.4) with the addition of 6 M NaOH.

Bacteria

Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa and Escherichia coli were isolated from patients with urinary tract infections and identified by our clinical laboratory using conventional techniques. Assessment of urease activity was performed using Christensen's medium (2).

Bacteria were grown on Heart Infusion agar for 24 hrs, collected and washed three times with 0.85% sterile saline. A 1 x 10^7 per ml suspension of each bacterial genus was prepared and one ml aliquots were dispensed into sterile vials and stored at -70°C. Before use, the bacterial dilution was thawed and inoculated into urines prepared as previously described using sterile techniques. Controls for bacterial inoculations consisted of sterile saline.

Bacterial Incubation and Recovery

Buffered urines, unbuffered urines and control media were inoculated as described above and incubated at 37°C. Uninoculated urines were also incubated. After two weeks incubation the urine pH was determined and the bacteria were harvested by centrifugation at 1400 g for 15 min at 5°C. The resulting bacterial pellet was then resuspended in distilled water and centrifuged three more times. Washed pellets were prepared for either X-ray powder diffraction or electron microscopy.

X-ray Powder Diffraction

Bacterial pellets were air dried and then ashed over a bunsen burner flame in aluminum planchets. X-ray powder analysis was performed by means of a Debye-Scherrer powder camera with a nickel filtered Cu-K alpha energy source operated at 30 kV and 20 ma. Powder diffraction patterns obtained were identified by the Hannawalt method of comparing "d" values and line intensities to known standards of calcite, apatite and other crystalline compounds (16).

Electron Microscopy and Elemental Analysis

Bacterial pellets were fixed in 2.5% gluteraldehyde buffered with 0.1 M sodium cacodylate. Those bacteria selected for electron microscopy (E. coli from one stone former, two non-stone formers, and control medium) were post fixed in 1% osmium tetroxide buffered with 0.1 M sodium cacodylate. After 24 h, the cells were dehydrated with ethanol-water (20, 40, 60, 75, 95, 100% ethanol) at 15 min intervals (5). Specimens were embedded in a low viscosity epoxy resin (21). Thick (approximately 0.5 μm) sections were prepared and examined using scanning transmission electron microscopy (STEM) techniques at 100 kV employing a TEMSCAN 100-CX (JEOL Inc., Medford, Ma.). Intracellular and extracellular elemental analysis was performed with an energy dispersive spectrometer (EDS) (EDAX 707-B, EDAX INT., Prairie View, Ill.). Computer analysis of EDS peak to background ratios was used to show the range of calcium levels between organisms grown in the various urines. Chloride levels were noted to be relatively constant among bacteria and extracellular areas of different urines and control groups. This provided a standard by which a semiquantitative interpretation of the EDS analysis could be made as described by Dempsey et al. (6). By assigning the chloride content a relative value of 1.0 and determining the calcium/chloride ratios for the organisms and extracellular areas, a comparison of bacterial calcium levels and extracellular calcium levels could be made. Unstained specimens, as well as specimens stained with uranyl acetate and lead citrate, were examined.

RESULTS

Urease Activity

The organisms used in this study had a wide range of urease activity. Proteus mirabilis demonstrated rapid alkalinization of Christensen's medium with a positive colour change (yellow to purple) in 2 h. Klebsiella pneumoniae and Pseudomonas aeruginosa had intermediate activity with colour changes noted 24 h and 48 h after inoculation respectively. Escherichia coli demonstrated no alkalinization of the medium even after two weeks incubation.

Crystallization in Urines

All organisms inoculated in unbuffered or buffered urines produced crystallization during the two week incubation period (Table 1). Crystallization was noted to occur in the form of apatite or calcite as demonstrated by X-ray diffractometry. Examples of the calcite and apatite diffraction patterns are shown in Fig. 1. Calcite crystals were not apparent in buffered urines (Table 1). The ability to form crystals was dependent on the buffered urine in which the bacteria were inoculated. Bacteria grown in urines demonstrated to have higher calcium concentrations by atomic adsorption spectrophotometry retained the ability to produce apatite crystals. Those urines having lower calcium concentrations did not support crystallization. Bacteria grown in Heart Infusion broth did not produce apatite or calcite. Spontaneous precipitation of crystals did not occur in control urines (Table 1).

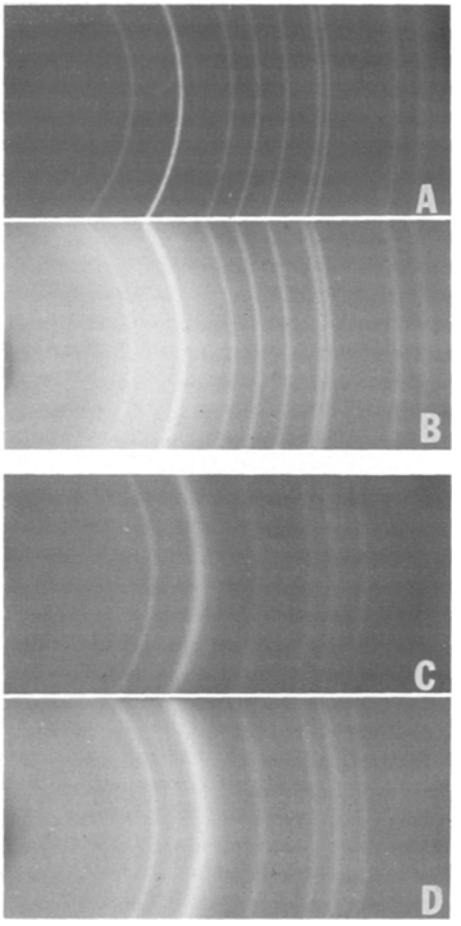


Fig. 1 A-D. Examples of X-ray powder diffraction patterns. A Standard for calcite. B Bacterial ash (Proteus mirabilis) from subject CC demonstrating calcite. C Standard for apatite. D Bacterial ash (Escherichia coli) from subject DD demonstrating apatite

Table 1. Crystalline material formed by bacteria grown in urines[a]

Code[b]	Initial Ca[c] in mg/dl	Control[d]	E. coli		K. pneumoniae		P. aeruginosa		P. mirabilis	
			Unbuffered	Buffered[e]	Unbuffered	Buffered	Unbuffered	Buffered	Unbuffered	Buffered
AA	12.1	neg[f]	apatite	neg	apatite	apatite	apatite	neg	apatite	apatite
BB	8.5	neg	apatite	neg	apatite	neg	apatite	neg	apatite	apatite
CC	25.2	neg	calcite	apatite	calcite	apatite	calcite	apatite	calcite	apatite
DD	15.5	neg	apatite	apatite	apatite	apatite	apatite	neg	apatite	apatite
EE	2.4	neg	apatite	neg	apatite	neg	neg	neg	apatite	neg
FF	3.7	neg	apatite	neg	apatite	neg	apatite	neg	apatite	neg
HI	0.5	neg	neg	nd[g]	neg	nd	neg	nd	neg	nd

[a] crystallization determined by X-ray powder diffraction

[b] AA- No history of urinary tract stone, no history of urinary tract infection
BB- No history of urinary tract stone, positive history for urinary tract infection as a child
CC- No history of urinary tract stone, no history of urinary tract infection
DD- Active stone disease (history of stone passage in past 2 years), no radiological evidence of residual stone, positive history for urinary tract infection
EE- Active stone disease (history of stone passage in past 2 years), positive radiological evidence of residual stone, no history of urinary tract infection
FF- Inactive stone disease (no evidence of stone disease in past 5 years), no radiological evidence of residual stone, no history of urinary tract infection

[c] mg/dl as determined by atomic adsorption spectrophotometry

[d] urines inoculated with 1.0 ml sterile saline

[e] urines buffered with 0.15 M TES

[f] neg means that both calcite and apatite crystals were not detected

[g] nd means not done

Pre- and post-incubation pH levels varied slightly in unbuffered urines inoculated with non-urease forming organisms (-0.3 to +0.7 pH units) and were equivalent to variations noted in control urines inoculated with sterile saline (-0.5 to +0.5 pH units). Bacteria with urease activity produced a wide range of urinary pH levels (-0.2 to +4.0 pH units) with Proteus mirabilis producing marked alkalinity in urine (+2.2 to +4.0 pH units) (Table 2). Heart Infusion broth inoculated with bacteria showed minimal pH alteration (-0.2 to +0.1 pH units).

The formation of crystals was not related to urinary pH in buffered urines and thus, crystal formation in this instance, appears independent of urease related pH alterations (Table 1). Apatite was evident in buffered urine at pH 7.4 inoculated with bacteria having intermediate or no urease activity. Levels of pH varied minimally (-0.2 to +0.2 pH units) with these bacteria. In contrast, P. mirabilis did not produce crystallization in all instances, even in the presence of unabated urease activity (as evidenced by the rise in urinary pH, +0.9 to 1.6 pH units) (Table 2).

Electron Microscopy and EDAX Analysis

Escherichia coli grown in urines from subjects EE, CC, AA (one stone former and two non-stone formers, respectively) as well as E. coli grown in Heart Infusion broth (HI) were examined using transmission electron microscopy. Most individual bacteria grown in urines had electron dense deposits intracellularly (Fig. 2A) that were not observed in similarly prepared specimens grown in Heart Infusion broth (Fig. 2B). Electron dense regions were noted in E. coli grown in Heart Infusion broth only when sectioned samples were counter-stained with heavy metal stains.

Analysis of intracellular electron dense areas by EDS in 2 to 3 bacteria from each of the above cultures demonstrated increased levels of calcium (Fig. 3A) as compared to extracellular regions (areas in epoxy resin sections without bacteria) (Fig. 3B). A statistically significant difference existed ($p < 0.05$) between the calcium levels in individual bacteria and the extracellular levels. A wide range of calcium levels existed between organisms grown in different urines (Fig. 4) and appeared related to the calcium concentrations of the urines as previously demonstrated (Table 1). The difference, however, was not statistically significant ($p > 0.05$).

A difference in the calcium levels between individual organisms grown in the same urines was also apparent. For example, in a single sample of urine from CC, one bacterium had a calcium/chloride ratio of 0.31, while another organism had a 0.08 ratio (Fig. 4). This indicates that each bacterium accumulated different amounts of calcium.

Bacterial calcium levels detected by EDS analysis of Heart Infusion-grown organisms were not statistically different when compared to urine-

Table 2. Alteration of pH in buffered and unbuffered urines inoculated with bacteria

Code[a]	Control urine, initial pH[b]		Control urine, final pH[c]		E. coli		K. pneumoniae		P. aeruginosa		P. mirabilis	
	Unbuffered	Buffered	Unbuffered	Buffered	Un-buffered	Buffered	Un-buffered	Buffered	Un-buffered	Buffered	Un-buffered	Buffered
AA	5.2	7.4	5.2	7.4	5.2	7.4	5.6	7.4	6.4	7.4	9.1	9.0
BB	6.2	7.4	6.1	7.2	6.6	7.2	8.8	7.3	7.3	7.2	9.0	9.0
CC	6.7	7.4	7.2	7.4	6.8	7.3	7.5	7.4	6.9	7.3	8.9	8.9
DD	6.7	7.4	6.2	7.3	6.4	7.2	7.3	7.3	8.8	7.3	9.0	9.0
EE	7.0	7.4	7.2	7.4	7.5	7.3	7.1	7.6	7.4	7.2	9.0	8.3
FF	5.2	7.4	5.5	7.4	5.9	7.4	6.0	7.4	7.8	7.4	9.2	9.0
HI	6.8	7.4	6.8	nd[d]	6.8	nd	6.6	nd	6.9	nd	6.7	n.d

[a] see legend b in Table 1

[b] pH of urine either unbuffered or buffered with TES prior to inoculation

[c] pH of uninoculated urine after two weeks incubation at 37°C

[d] nd means not done

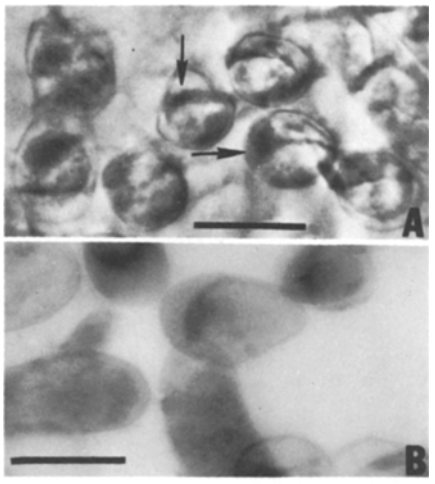
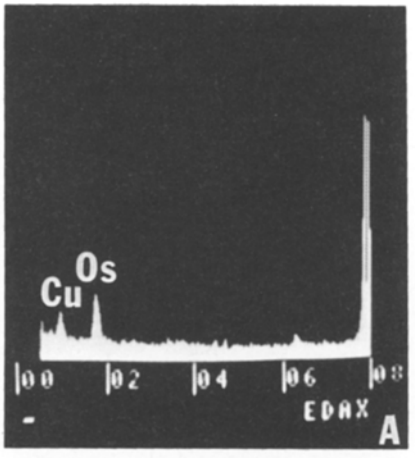


Fig. 2 A, B. STEM of thick (0.5 μm) section of Escherichia coli (unstained specimens). A Multiple bacteria with electron dense intracellular deposits (arrows) after 2 weeks incubation in human urine (AA) - Bar represents one micrometer. B Multiple bacteria lacking electron dense deposits after two weeks incubation in Heart Infusion broth - Bar represents one micrometer



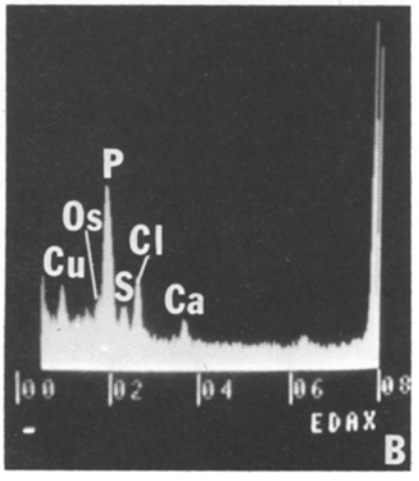


Fig. 3 A, B. Spectra produced by energy dispersive spectrometric analysis (EDS). Ordinate represents counts per second, Abcissa represents energy in kV. Ca = calcium, Os = osmium, Cl = chloride, P = phosphorus, S = sulphur, Cu = copper (from specimen grid). A EDS analysis (1000 sec) of an extracellular region demonstrating negligible calcium levels. B EDS analysis (1000 sec) of an intracellular electron dense region

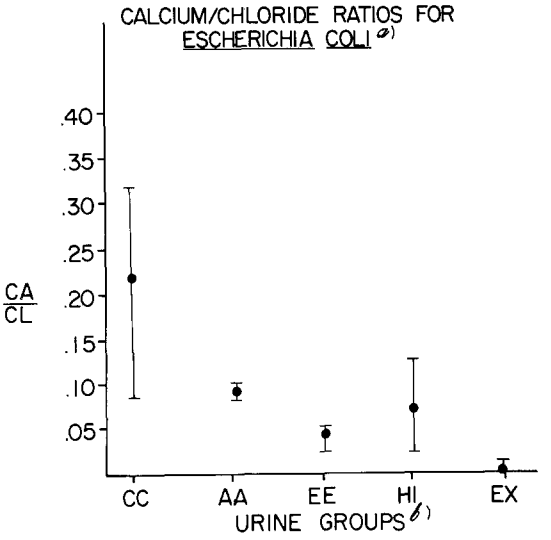


Fig. 4 A, B. A Calcium/chloride ratios determined by computer analysis of EDS data. B CC, AA, EE, HI are urine groups and control media described in Table 1 - EX represents extracellular areas scanned for EDS analysis

grown bacteria. However, the electron microscopic appearance, and the absence of crystallization on X-ray diffractometry (Fig. 2B and Table 1) in Heart Infusion-grown organisms, indicates that calcium can be concentrated by bacteria even in conventional growth medium, but not in the form of calcite or apatite. These crystal forms were demonstrated only in urine-grown bacteria.

DISCUSSION

The theory of bacteria-related calcification is not a new one. Its contribution to urinary tract calculi was proposed in 1923 (17). One of the first suggestions of bacterial calcification occurred in 1926 when a calcium phosphate precipitating factor was obtained from lysed cells of Leptothrix buccalis (later designated Bacterionema matruchotti) (1). Ennever restudied this organism in the 1960's and demonstrated intracellular crystal formation (7). A survey of microorganisms (9, 14) demonstrated crystalline formation in a variety of bacteria as well as a fungus (11).

In our study, bacteria-related crystal formation occurred in unbuffered urines from stone forming and non-stone forming individuals and occurred irrespective of bacterial urease activity. The addition of TES buffer was done in an effort to stabilize the physicochemical state of the urines and to exclude pH alteration as the cause of apatite and calcite formation. In the presence of buffer, crystallization was still noted to occur in urines of stone forming and non-stone forming individuals and appeared related to the urinary calcium concentration noted prior to the addition of buffer. Spontaneous precipitation of calcium salts was not observed in control urines inoculated with sterile saline. In buffered urines with low calcium concentration in which Proteus mirabilis had been inoculated, the urease activity was unabated, but apatite and calcite did not form (Tables 1 and 2). In contrast, weak and non-urease producing organisms formed apatite even with stabilization of urinary pH in urines with high calcium concentrations. In the absence of spontaneous precipitation of calcium salts, stabilization of the urinary pH by buffering, and the repeated washing of bacteria to remove extracellular material, the apatite and calcite crystals appear to be of intracellular origin.

Electron microscopy and EDS analysis of Escherichia coli grown in urines was done to confirm the intracellularity of crystallization. The extracellular regions examined microscopically demonstrated no evidence of crystalline forms. EDS analysis of these regions confirmed either the very low or undetectable concentration of calcium in the surrounding media. In contrast, intracellular electron dense deposits were seen in E. coli grown in urines. EDS analysis demonstrated significantly elevated calcium levels in these deposits as compared to surrounding levels.

Analysis of individual bacteria grown in Heart Infusion broth demonstrated intracellular calcium. Increased intracellular calcium in bacteria grown in HI broth was not unexpected, since growth media represents a good source of calcium (10). EDS is an elemental analysis, and demonstrated calcium in bacteria grown in HI broth, regardless of its form. However, X-ray diffractometry reveals this calcium not to be of an apatite or calcite nature. The reason for this is not entirely clear, but we suggest that urine represents a modified metastable solution of calcium, phosphate and carbonate similar to the specialized media developed by Ennever (7). Similar ratios apparently do not exist in conventional growth media. In addition, our data show that bacteria can form calcite and apatite crystals in urine from both stone forming and non-stone forming individuals. This suggests that any individual may be susceptible to calculi formation in the presence of subclinical or clinical bacteriuria.

In the previously noted studies by Ennever, the earliest detectable intracellular apatite was seen after 3 days incubation with E. coli grown in specialized medium (10) and after longer periods with Candida albicans (11). This could be associated with increased intracellular calcium levels that occur in aging, damaged or dead organisms (18-20). The fact that there is no statistical difference in our study between the mean calcium/chloride ratios of groups of organisms grown in different urines and organisms grown in similar urines is consistent with the fact that bacteria of different ages have different calcium levels.

The possibility that microorganisms can form intracellular deposits of calcite, or more interestingly apatite, when inoculated in urine provides a plausible explanation for the occurrence of weak and non-urease producing organisms in urinary calculi. Apatite is a frequent association crystal in most urinary calculi and often composes a "nucleation" (nidus) crystal (12). Calcium carbonate (calcite) does not occur in urinary calculi in a pure crystalline form, but is present in infected calculi as carbonate-apatite crystals (13). Further, the infrequent presence of calcite detected in this study is possibly the result of conversion of apatite to calcite by the heat of the bunsen burner (15). Nevertheless, the presence of either crystal is significant. Although a major contribution of bacteria to stone disease appears to be alteration of urinary physicochemistry via urease activity, the demonstration of intracellular crystals of apatite and calcite suggests that bacteria themselves may contribute to the formation of calculi. Nidi could result by the destruction of organisms and crystal deposition (15) or by attachment of calcified bacteria to urinary tract structures (3, 4).

Active infection need not be a contributing factor to stone disease initiated by intracellular bacterial crystallization. Subclinical bacteriuria with subsequent entrapment of organisms and exposure to a constant flow of urine could result in accumulation of crystaline material such as apatite and form a nidus for calculus formation. In this manner, bacteria could contribute to stone formation in a variety of disorders in which the mechanisms are fairly well established such as varous hypercalcaemic states. More importantly, in those disorders in which mechanisms are not yet established and form the large category of idiopathic stone disease, our findings suggest that mineralized bacteria could serve as initiators of stone disease.

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