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# An In Vitro Ultrastructural Study of Infectious Kidney Stone Genesis

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**A ureolytic strain of *Proteus mirabilis*, isolated from a patient with infectious kidney stones, produced struvite ($MgNH_4PO_4 \cdot 6\ H_2O$) and apatite [$Ca_{10}(PO_4)_6CO_3$] crystals in vitro when grown in artificial urine. Surface-attached crystals were encased in a slime-like layer. Scanning electron microscopy revealed that surfaces submerged in the artificial urine were colonized by *P. mirabilis*. Bacteria-associated crystals appeared soon after colonization and eventually became coated with an amorphous substance. Energy-dispersive X-ray analysis of these crystals revealed the presence of Mg, Ca, and P which are major components of struvite and apatite. Transmission electron microscopy of surface scrapings revealed that the glycocalyx of *P. mirabilis* contained a large number of crystals. Based on these observations and previous work, a theory for infectious renal calculogenesis is proposed. The kidney is initially colonized by invading ureolytic pathogens. These pathogens secrete copious amounts of glycocalyx which facilitates adhesion of the organisms to the kidney, provides protection for these bacteria, and serves to bind struvite and apatite crystals that result from bacterial urease activity. Growth of these calcified microcolonies into mature stones is characterized by continued bacterial growth, incorporation of urinary mucoproteins into the matrix along with bacterial glycocalyx, and a continued deposition of struvite and apatite crystals due to the high pH. The mature stone, in effect, represents an enlarged "fossilized" bacterial microcolony.**

Struvite, a crystalline substance composed of magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6\ H_2O$), is found in 15 to 20% of urinary calculi (7, 8). Ureolytic microflora such as *Proteus*, *Pseudomonas*, *Klebsiella*, or *Staphylococcus* organisms are often isolated from surgically excised struvite calculi (6, 7, 17). Isolation of non-ureolytic strains of *Streptococcus faecalis* has also been reported (27), but it has been shown that *S. faecalis* will often lose its ureolytic capability upon subculturing, due to the loss of a urease-coding plasmid (2).

Direct morphological examination of struvite stones by scanning electron microscopy (SEM) has revealed the presence of bacteria within these stones (27). Nickel et al. (18; J. C. Nickel, G. Reid, A. W. Bruce, and J. W. Costerton, Urology, in press) have suggested that these infecting bacteria play a major role in the structure of these stones. This is accomplished by the production of extracellular bacterial glycocalyx which serves to cement struvite and apatite [$Ca_{10}(PO_4)_6CO_3$] crystals into the stone matrix.

In vitro studies by King and Boyce (12) and Griffith et al. (9) have shown the importance of ureolytic bacteria and urease in the generation of struvite- and apatite-containing stones. Griffith et al. (9) also demonstrated in vitro how lowered urine pH and urease inhibitors could reverse stone formation. Clinically, the inhibition of urease and acidification of urine has been somewhat successful in preventing subsequent calculogenesis after surgery (17). Bacteria contained within residual stone fragments left over from surgery have been shown to be extremely resistant to antibiotics (21). The high recurrence rate of this infection (22), probably due to the presence of these bacterial "seeds," would suggest that conventional treatments are still inadequate.

Successful control of infection stones will require a thorough understanding of their etiology. As part of our investigation, we have examined the initial events in the in vitro production of infection stones.

(A preliminary report of this work was presented at the 34th Annual Meeting of the Canadian Society of Microbiologists, 10–14 June 1984, Kingston, Ontario, Canada [abstract no. IN4].)

## MATERIALS AND METHODS

**Culture and growth conditions.** A ureolytic strain of *Proteus mirabilis*, designated as strain FH-1, was isolated from a patient with a urinary calculus and maintained on a slant of tryptic soy agar (Difco Laboratories, Detroit, Mich.). Before experimentation, three successive 10-h subcultures were made in the artificial urine described by Griffith et al. (9) supplemented with 1% tryptic soy broth (Difco). The artificial urine contained the following (in grams per liter): $CaCl_2 \cdot 2\ H_2O$, 0.651; $MgCl_2 \cdot 6\ H_2O$, 0.651; NaCl, 4.6; $Na_2SO_4$, 2.3; sodium citrate, 0.65; sodium oxalate, 0.02; $KH_2PO_4$, 2.8; KCl, 1.6; $NH_4Cl$, 1.0; urea 25.0, creatine, 1.1; and tryptic soy broth (Difco), 10. pH was adjusted to 5.8, and the artificial urine filter was sterilized with a Millipore membrane sterilization unit having a pore size of 0.45 μm. All other apparatus and glassware were sterilized by autoclaving.

**In vitro stone production.** A continuous culture flask was set up as outlined in Fig. 1. The culture flask was aseptically filled with 800 ml of artificial urine and inoculated with a 1% inoculum of a 10-h culture of *P. mirabilis*. Agitation and aeration were accomplished by gently bubbling air through an aeration tube. A cotton plug in the aeration tube controlled bacterial contamination. Waste urine and air were removed through a side tube mounted on the flask. Fresh artificial urine was added to the flask at a rate of 60 ml/h, providing a dilution rate of 0.075 $h^{-1}$. pH and culture optical density were monitored hourly until 8 h. Measurements were also taken at 12 and 24 h, at which point the experiments were terminated. Several substances were suspended be-

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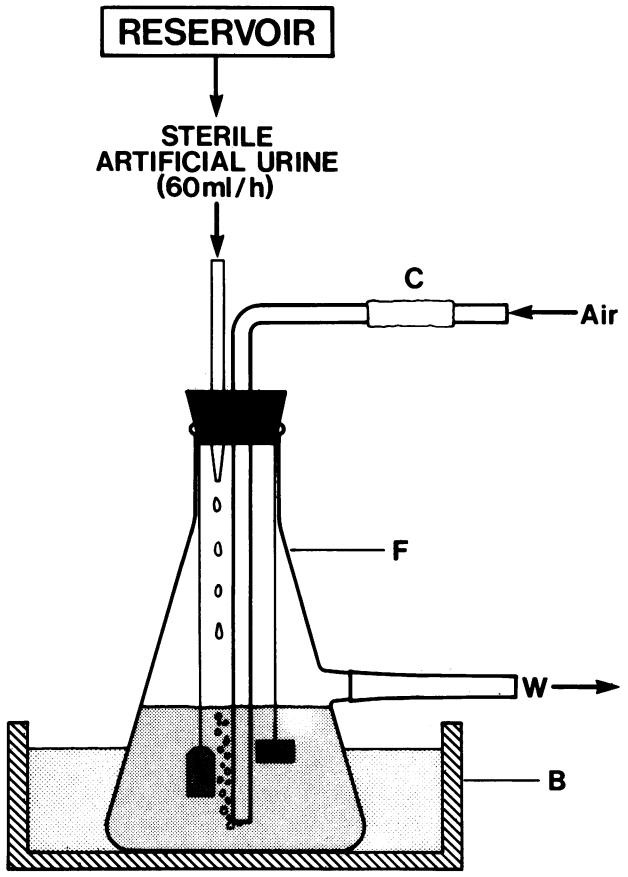


FIG. 1. Schematic diagram of continuous culture flask used for in vitro stone production by *P. mirabilis*. A modified side arm flask (F) was aseptically filled with 800 ml of artificial urine and inoculated with *P. mirabilis* as described in the text. Sterile artificial urine was added from a reservoir to the flask at a rate of 60 ml/h (dilution rate 0.075 $h^{-1}$). Surfaces (S) suspended in the artificial urine became coated with crystals after colonization with *P. mirabilis* and could be removed for examination by SEM and TEM. Agitation and aeration were accomplished by gently bubbling air through an aeration tube which contained a cotton plug (C) to control bacterial contamination. Waste urine and air were removed through a rubber tube (W) mounted on the side of the flask. Temperature was maintained at 37°C by suspending the flask in a water bath (B).

neath the surface of the culture medium to act as surfaces for bacterial colonization. These substances included Spurr (23) resin blocks, urinary catheter material, glass rods, and nichrome wire. These surfaces were sterilized by autoclaving before addition to the culture medium. Surfaces were removed at 4, 8, 12, and 24 h after inoculation for microscopy. All in vitro experiments were carried out in duplicate.

**TEM.** For transmission electron microscopic (TEM) processing, surfaces were fixed overnight at 4°C in 5% glutaraldehyde buffered in 0.1 M cacodylate buffer (pH 7.2). All buffers used in TEM processing contained 0.15% (wt/vol) ruthenium red. Scrapings taken from these surfaces were enrobed in 4% agar (Difco) and processed as previously described (16). Thin sections of these scrapings were stained with uranyl acetate and lead citrate (20), reinforced with evaporated carbon, and examined with a Hitachi H600 TEM having an accelerating voltage of 50 kV.

**SEM.** Processing for SEM presented a problem in that struvite crystals tend to dissolve at neutral or acidic pH (9). We therefore adopted measures for processing that reduced crystal loss. Surfaces removed from the culture flask for SEM were either placed in a fixative solution consisting of 5% glutaraldehyde in cacodylate buffer (0.067 M, pH 7.2) and processed for SEM as previously described (16) or dipped in 0.1 M Tris buffer (pH 9.0) and allowed to air dry without fixation. SEM specimens were mounted on aluminum stubs and sputter coated with a palladium-gold mixture. They were examined with either a Hitachi SEM model S450 or else a Cambridge Stereoscan 150 Mark-2 SEM with an accelerating voltage of 20 kV and a Microspec model 711 energy-dispersive analysis of X-rays analytical attachment.

## RESULTS

*P. mirabilis* culture optical density and pH were seen to increase rapidly (Fig. 2). pH increased to between 8.5 and 9.5 by 4 h and remained at this level for the duration of the experiment. Optical density reached a maximum at 6 to 8 h and thereafter declined somewhat, possibly due to growth being inhibited by the elevated pH and cells being diluted by the addition of fresh media to the flask. Surface-attached white crystals became visible to the naked eye after the pH exceeded 8.5. These crystals appeared to be enclosed in a slimy mass as evidenced by their coherence when they were scraped from surfaces inside the culture flask.

Exposure of encrusted surfaces to solutions of neutral or acidic pH caused the attached crystals to loosen and eventually dissolve. Consequently, SEM preparation techniques had to be altered so as to give either good crystal preservation or else good bacterial preservation.

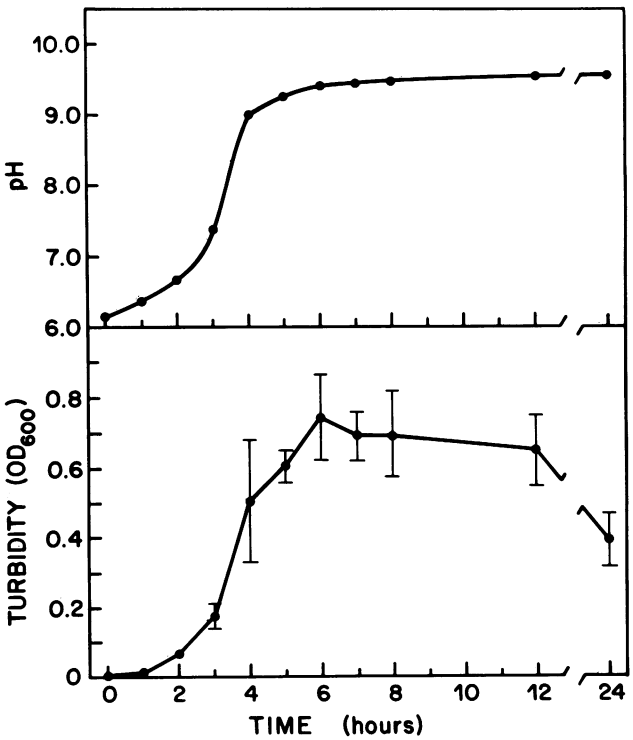


FIG. 2. Culture growth as measured by optical density (± standard deviation) and pH changes produced by *P. mirabilis* grown in artificial urine.

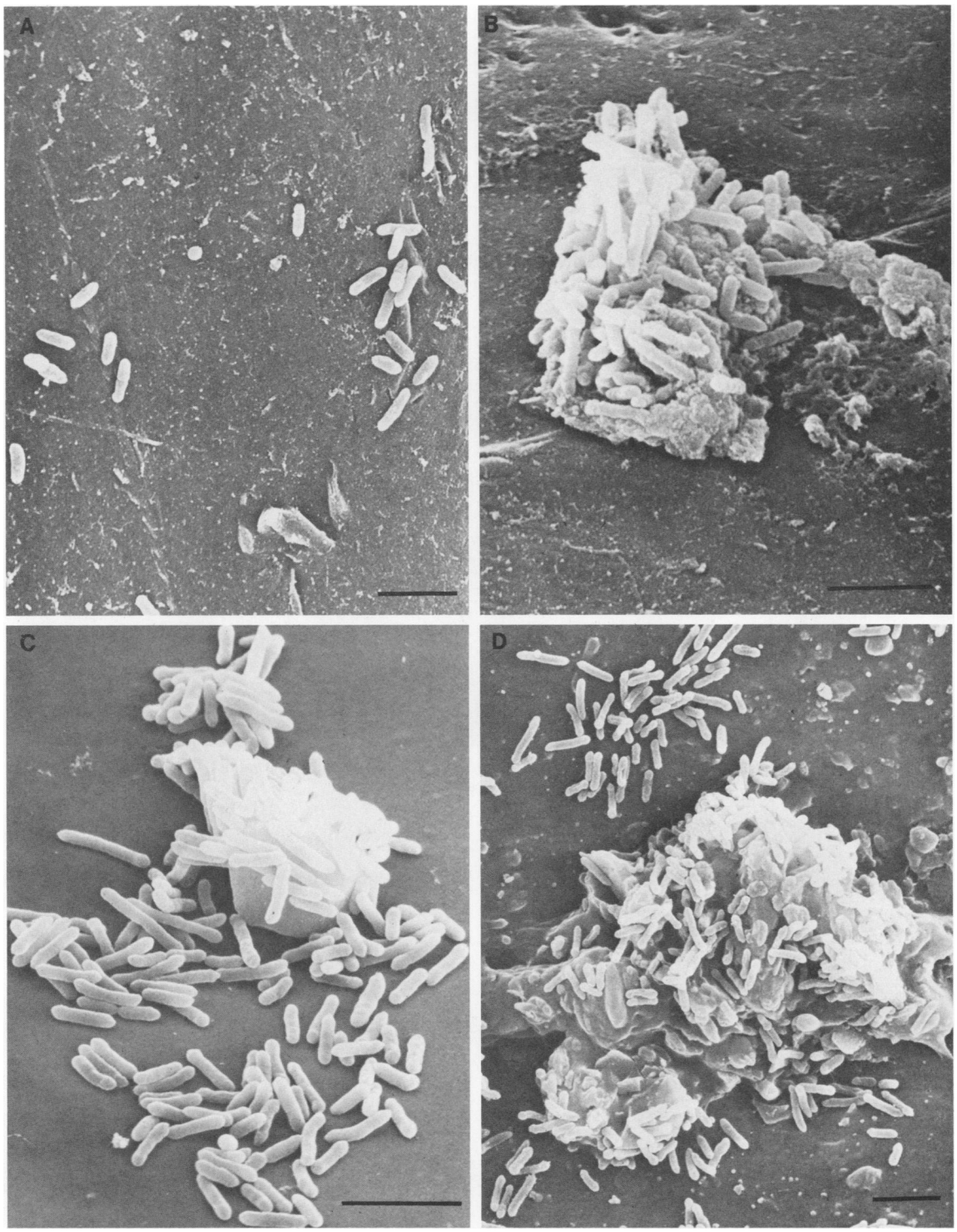


FIG. 3. Progressive colonization of *P. mirabilis* on submerged surfaces at 4 h (A), 8 h (B), 12 h (C), and 24 h (D) after inoculation as seen by SEM. Bar, 5 μm.

SEM examination of surfaces which had been suspended in the reaction flask revealed a progressive colonization of *P. mirabilis* (Fig. 3). Crystals seen in association with these bacteria morphologically resembled struvite and apatite (5). By 24 h, these crystals were covered by an amorphous substance (Fig. 4). Energy-dispersive X-ray analysis of these encrustations (Fig. 5) revealed the presence of Mg, Ca, and P, which would suggest the presence of struvite and apatite. The palladium used in the sputter coating of the specimens for SEM was also detected by energy-dispersive X-ray analysis. The nature of the submerged surfaces did not appear to affect colonization patterns of *P. mirabilis* or the appearance of crystals. Most SEM observations were performed on glass or catheter surfaces, as they gave less

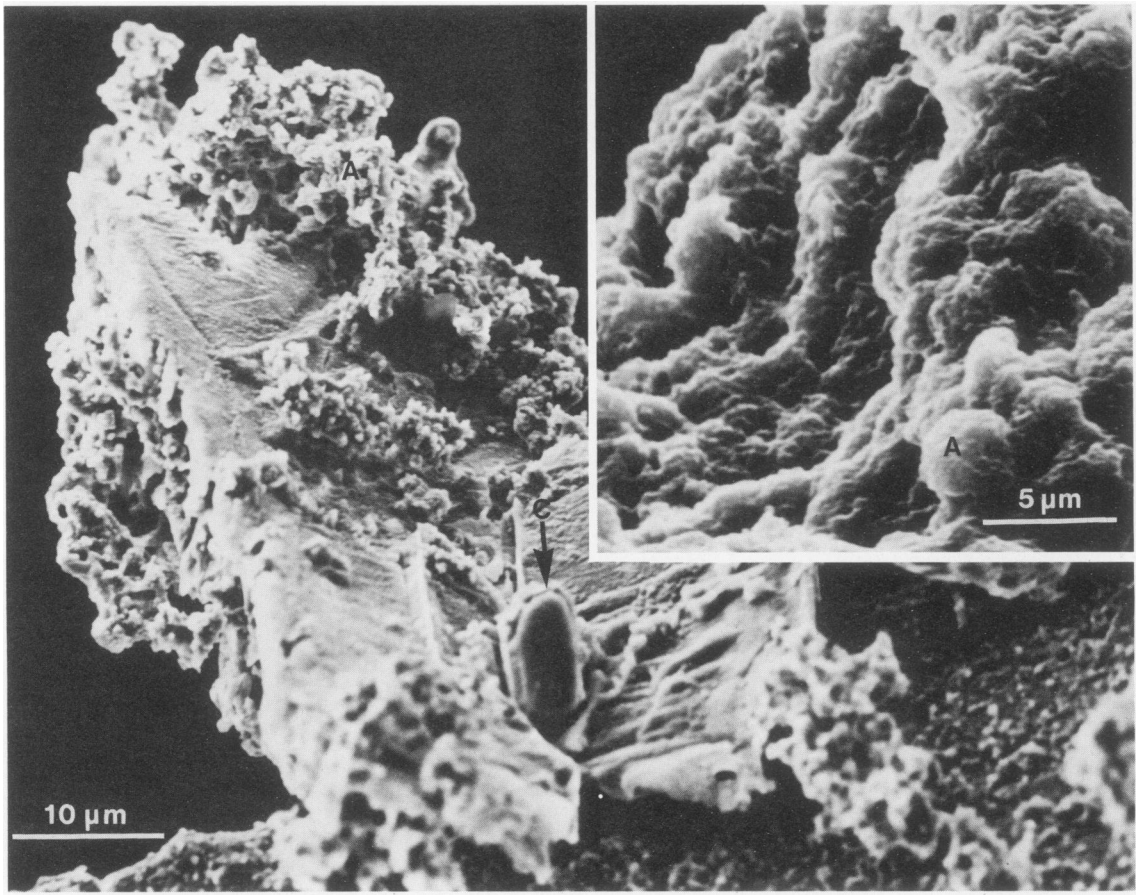


FIG. 4. When SEM processing for surfaces was altered to give good crystal preservation, crystals (C) appeared to be covered by an amorphous substance (A). This is especially evident at higher magnification (inset).

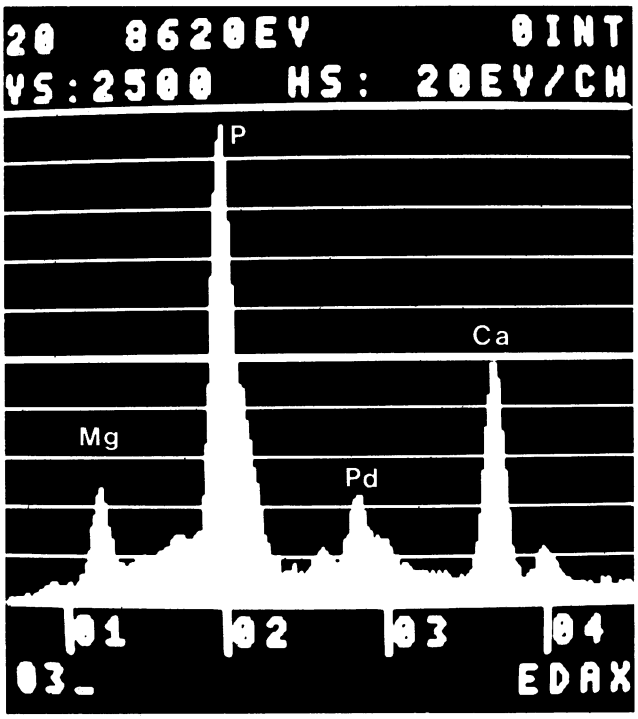


FIG. 5. Energy-dispersive X-ray analysis of crystal seen in Fig. 4, showing elements present.

interference with energy-dispersive X-ray analysis measurements.

TEM examinations of surface scrapings (Fig. 6) revealed cells of *P. mirabilis* to be surrounded by ruthenium red-staining, electron-dense amorphous material. Some of this material had condensed on electron-transparent foci at varying distances from the cells.

## DISCUSSION

The association of ureolytic bacteria with infectious kidney stones has been well established (6, 7, 11, 22). Urea hydrolysis creates an alkaline urine pH, causing a precipitation of struvite and apatite crystals (7, 9, 12). These crystals are entrapped in a matrix of heretofore indeterminate origin (8, 34, 35) which increases in size until severe renal damage may occur (7). Vermeulen and Goetz (29) noted that stone formation in an artificially induced bladder infection in rats (30) was enhanced by bacterial strains exhibiting a wide range of urease activity. They concluded that urease activity alone could not explain the pathogenicity of these strains.

A number of workers have studied this matrix. Observations that staphylococci were integrated into the matrix of infection stones led Hellström (11) to propose that these bacteria were implicated in matrix formation as well as crystallization. Boyce and Garvey (1) analyzed decalcified urinary calculi and found their matrices to consist largely of mucopolysaccharides and mucoproteins. Hexuronic or sialic acids, which are a constituent of many bacterial exopolysaccharides (25), were not detected. This may be explained in

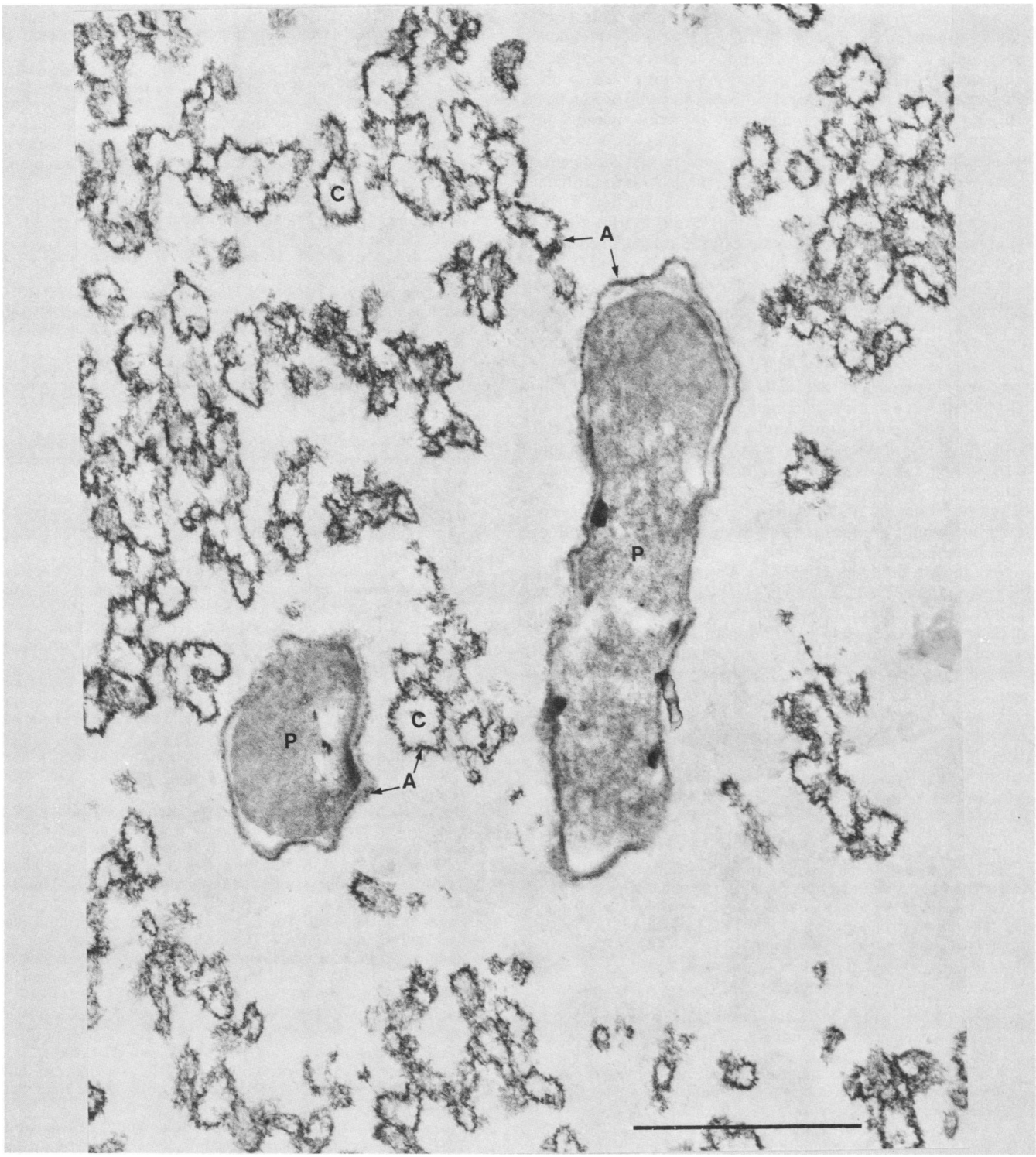


FIG. 6. Surface scrapings of submerged surfaces as visualized by TEM. Cells of *P. mirabilis* (P) are surrounded by electron-transparent crystals (C). Ruthenium red-staining amorphous material (A) has condensed on *P. mirabilis* cells and the surrounding crystals. Bar, 1.0 μm.

part by the presence of a renal enzyme, sialidase (15, 35), which alters these residues. Boyce and Garvey (1) proposed that matrix deposition was a necessary prerequisite to stone growth. In contrast to this, in vitro experiments by Vermeulen and co-workers (33) led to the hypothesis that matrix formation occurred solely as a consequence of crystallization processes (28, 31, 32). As crystal-free regions or "matrix" stones are often found at the periphery of struvite stones (35), it is likely that matrix formation precedes calcification.

For a bacterial species to exhibit virulence, it is often necessary for it to adhere to a tissue surface (3, 4).

Uropathogenic strains of *Escherichia coli* bind quite tenaciously to uroepithelial cells (10). The lower binding affinity of commensal intestinal *E. coli* strains to uroepithelial cells (26) may explain their comparatively low virulence in urinary tracts. Bacterial glycocalyx (3, 4) and pili production (10, 26) mediate bacterial adhesion in many pathogenic systems. Although *P. mirabilis* has been classified as nonencapsulated (13), there is recent evidence that slime production occurs during swarming (24) and growth in artificial urine (D. H. Schmiel, T. I. Ladd, J. C. Nickel, and J. W. Costerton, manuscript in preparation). As bacterial growth on conventional laboratory media can often lead to the loss of glycocalyx production which is otherwise present (3), it is conceivable that this organism could produce a glycocalyx in kidneys. This hypothesis is currently being investigated.

Crystal association with *P. mirabilis* is clearly revealed by TEM. *P. mirabilis* cells are seen to be surrounded by ruthenium red-stained material. Condensation of this material has occurred on the cells as well as on electron-transparent particles in the immediate vicinity. As ruthenium red stains polyanionic compounds, we interpret this material to be glycocalyx which has condensed on the cells and surrounding crystals. Bacterial glycocalyces, which may contain ca. 99% water, tend to condense markedly upon dehydration unless stabilized (14). Based on these observations, we would propose the following hypothesis for infectious renal calculogenesis.

Invading ureolytic pathogens such as *P. mirabilis* colonize kidney epithelial cells and form glycocalyx-enclosed microcolonies. The urease activity of these bacteria creates an alkaline urine, causing precipitation of struvite and apatite crystals. The glycocalyx serves to trap these crystals and other components present in the urine, such as mucoproteins, as well as to protect the pathogens from antibiotics (19) and host-mediated immune responses. As these struvite- and apatite-encrusted microcolonies enlarge into mature calculi, there is an increased incorporation of urinary mucoproteins into the matrix, along with continued bacterial growth, urease activity, and crystal deposition.

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