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Experimental technique simulating oxalocalcic renal stone generation

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Summary. A new technique simulating some of the conditions experienced by papillar and caliceal oxalocalcic stones during the early stages of their generation was developed. This technique enables the study of how conditions prevailing at calculogenesis, such as pH, composition of urine and presence of admixtures, influence the rate of formation and development, the crystalline texture and the composition of the concretion formed. Results achieved with this technique demonstrate that: (1) an appropriate substrate always gives rise to a crystalline concretion if it is in contact with supersaturated urine; (2) primary agglomeration plays a significant role in concretion development whereas secondary agglomeration is of minor importance; and (3) citrate and pyrophosphate exert a considerable influence on the shape and composition of particles constituting the concretion.

Key words: Calcalogenecis – Oxalocalcic renal stone – Calcium oxalate monohydrate – Calcium oxalate dihydrate – Stone development

The absence of a realistic concept of renal stone generation is, to a considerable extent, caused by the fact that this process cannot be observed directly in vivo and all hypotheses have to be based on results of in vitro experiments. The relevance of in vitro experiments to urolithiasis depends on the degree of correspondence between the experimental conditions and those prevailing in the stone-forming kidney.

In vitro methods must reproduce some of the stages of a real biological process. No experimental methods hitherto available has succeeded in modelling calculogenesis in its formidable complexity. In fact, each experimental method employed usually enables the study of only one particular stage of stone formation. For example, crystallizers (continuous, batch and semi-batch) allow study of the crystal growth stage. The majority of physicochemical studies on urolithiasis to date (e.g. [3, 7, 8]) have dealt with this stage, and undoubtedly their results are of importance for evaluating oxalocalcic stone growth. Nevertheless, recent data clearly indicate that heterogeneous nucleation of calcium oxalate on foreign particles retained at the boundary of the papilla and subsequent primary aggregation prevail during the early stages of stone formation and represent the principal stage in calcium oxalate urolithiasis.

The present paper describes a new technique enabling the study of this principal stage in urolithiasis under conditions experienced by a specific category of oxalocalcic calculi. Conditions characterized by coverage of crystals with a thin liquid film of urine supersaturated with respect of calcium oxalate correspond to those experienced by a significant number of kidney stones that initiate their formation attached to the papilla (not inside it, in the nephron). The technique can therefore contribute to a better understanding of the process of stone formation.

Materials and methods

Artificial urine supersaturated with respect to calcium oxalate was prepared by mixing equal volumes of solutions A and B. Solution A contained 11.02 g $Na_2SO_4 \cdot 10H_2O$, 1.46 g $MgSO_4 \cdot 7H_2O$, 4.64 g NH_4Cl, 12.13 g KCl, 1.41 g $CaCl_2 \cdot 3.5H_2O$ and 0.2 g mucin (from porcine stomach) in 1 l. Solution B contained 6.80 g $NaH_2PO_4 \cdot 2H_2O$, 2.19 g $Na_2HPO_4 \cdot 12H_2O$, 13.05 g NaCl, 0.29 g $Na_2C_2O_4$ and 1.0 g $NaC_6H_7O_7$ (sodium dihydrogen citrate) in 1 l if hyperoxaluric urine was being simulated and 2.65 g $NaH_2PO_4 \cdot 2H_2O$, 18.82 g $Na_2HPO_4 \cdot 12H_2O$, 13.05 g NaCl, 0.076 g $Na_2C_2O_4$ and 1.0 g $NaC_6H_7O_7$ in 1 l with pH adjusted to 6 by NaOH if normocalciuric-normooxaluric urine was being prepared. 0.1% of H_2O_2 was used as a disinfectant to prevent contamination with bacteria. Chemicals of reagent-grade purity were dissolved in deionized and redistilled water.

A schematic diagram of the experimental device used is shown in Fig. 1. Equal volumetric flows of solutions A and B were mixed in a T-shaped mixing chamber constructed in a Perspex block. A flow

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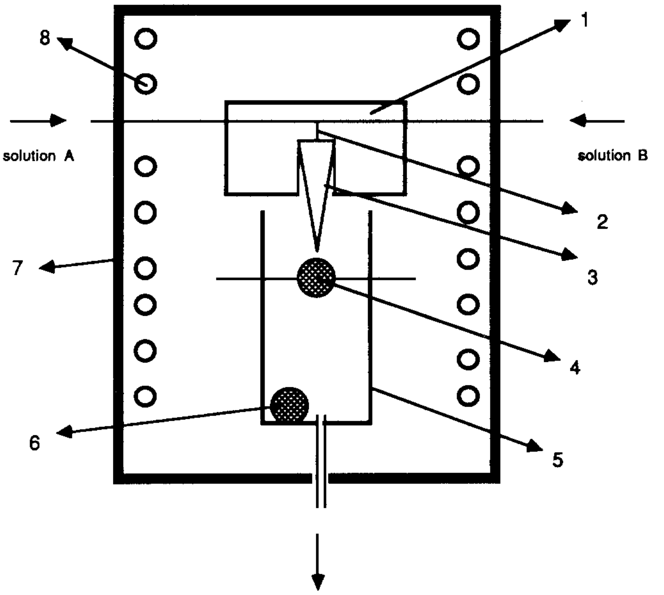


Fig. 1. A schematic representation of the experimental device. *1,* T-shaped mixing chamber; *2,* outlet channel; *3,* replaceable plastic conical tube; *4,* upper spherical substrate; *5,* container; *6,* lower spherical substrate; *7,* insulating box; *8,* coil

rate of 0.2 ml/min for each solution was maintained by a multichannel peristaltic pump. A replaceable conical tube connected to a 5-mm long outlet channel of 1 mm inner diameter drilled in the Perspex block delivered mixed solution, i.e. artificial urine, dropwise to the apex of a sphere serving as a substrate for calcium oxalate precipitation. This sphere of volcanic origin, 1 cm in diameter, consisted of a chemically inert material (complex silicate composed of 12% Si, 55% Ca, 14% Fe and 6% Al) with highly porous structure (Fig. 2). The sphere was supported in position just under the conical tube. The rate of addition of the artificial urine was fast enough to ensure frequent renewal of the liquid layer covering the surface of the sphere.

Artificial urine leaving the upper sphere was collected in a plastic container with total and effective volumes of 15 and 8 ml respectively. Another volcanic sphere, identical to the upper one, was placed free on the bottom of this container to serve as a substrate for calcium oxalate precipitation. The container was partially emptied every 20 min so that a major part of the lower sphere was periodically exposed to air for a limited time. The upper sphere was always above the liquid level in the container. Artificial urine periodically withdrawn from the container was collected in a bottle for 24 h under aseptic conditions and examined with light microscope for the presence of crystals.

The experimental device, i.e. the mixing chamber and the container, was placed in a closed insulating box ensuring 100% humidity of ambient air. A constant temperature was maintained by water circulating through a coil from the constant-temperature

Fig. 2. Surface structure of the volcanic sphere used as a substrate for crystal formation

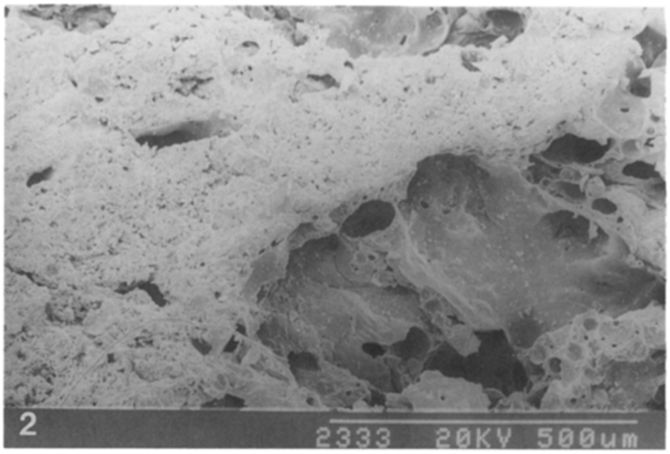


Fig. 3. Calcium oxalate monohydrate crystals that developed on the substrate from normocalciuric-normooxaluric urine

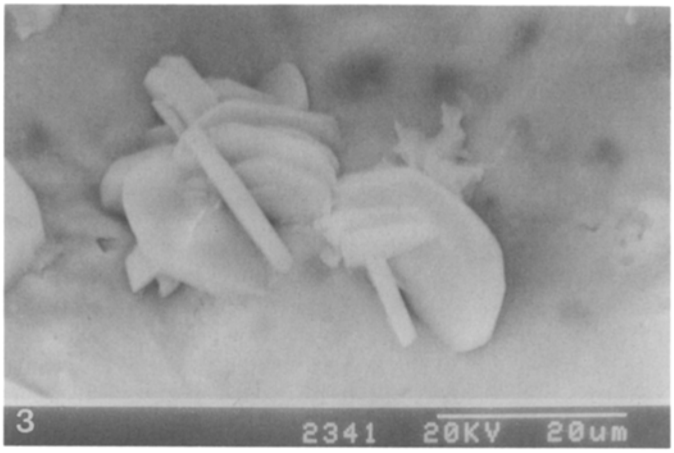
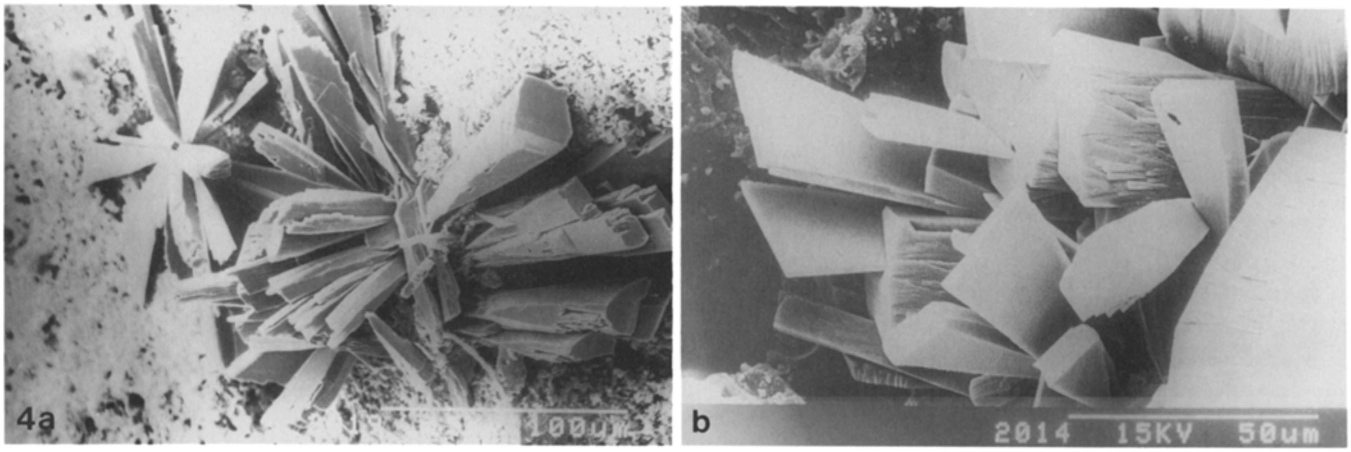
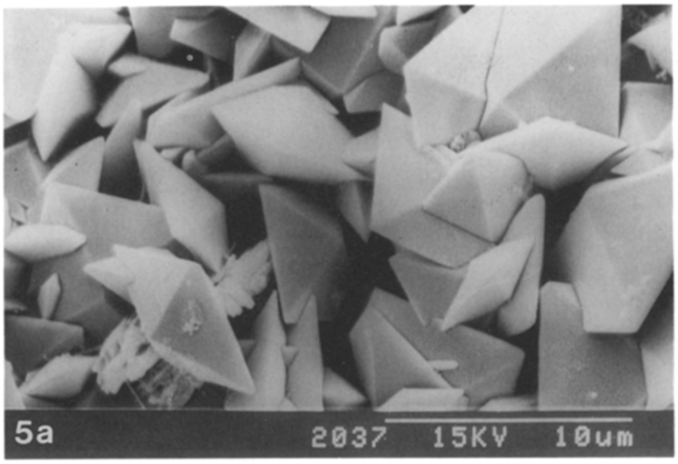
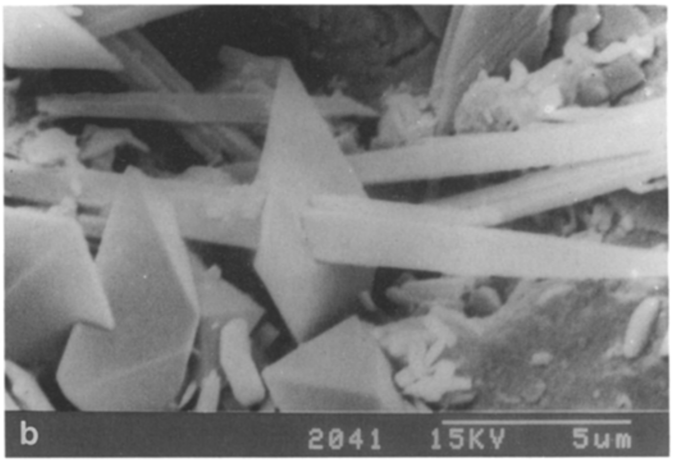
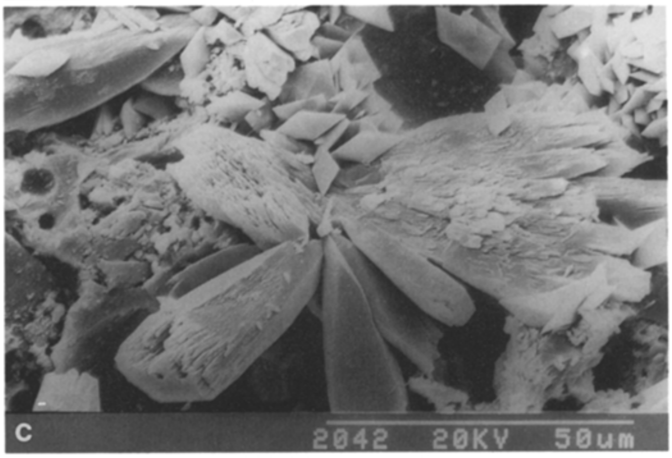


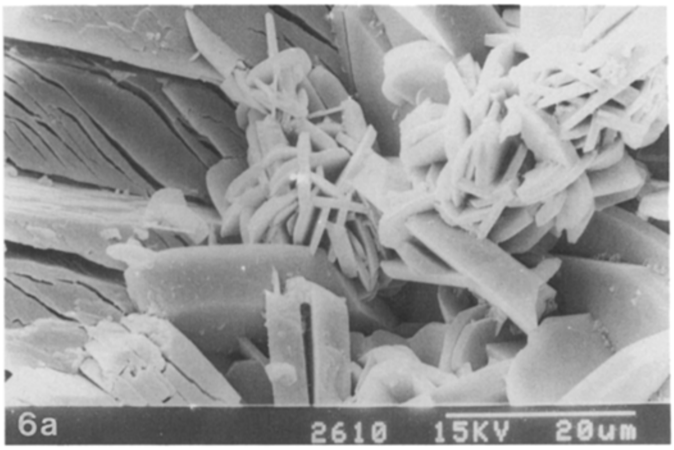
Fig. 4a, b. Columnar (**a**) and block-shaped (**b**) crystals of calcium phosphate formed from urine containing no citrate

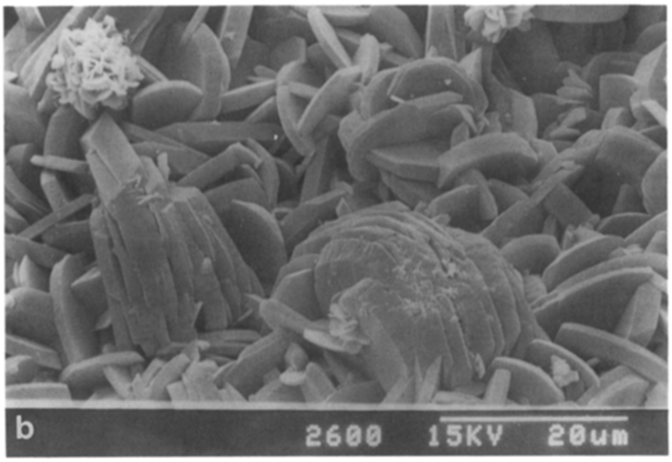












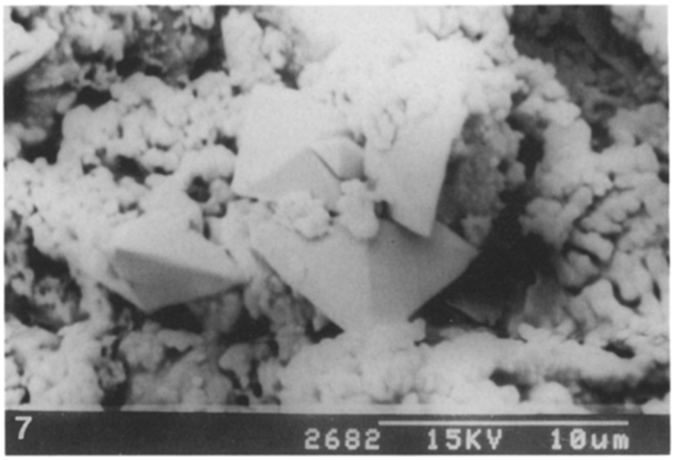


Fig. 5a–c. Calcium oxalate dihydrate (**a**), needle-like (**b**) and larger crystals (**c**) of calcium phosphate formed from urine without citrate but containing 40 ppm pyrophosphate

Fig. 6a, b. Calcium oxalate trihydrate and monohydrate crystals in close vicinity (**a**) and calcium oxalate monohydrate crystals (**b**) formed from hyperoxaluric urine at pH 5

Fig. 7. Calcium oxalate dihydrate crystals with particles of apparently non-crystalline matter formed from hyperoxaluric urine at pH 7.4

water bath in which the flasks containing feed solutions were immersed. All experiments were performed at 37°C.

The system was operated continuously for periods varying between 48 and 170 h. The plastic conical outlet tube was changed every 48 h to prevent its obstruction by the solid layer that gradually precipitated on its inner surface. The upper and lower spheres were removed at the end of the experiment, washed carefully with water, dried at room temperature in a desiccator and weighed. The amount of solid material precipitated on the substrate was estimated by the difference in the weights of the sphere before and after the experiment. The crystals on the substrate were observed using a scanning electron microscope equipped with an EDAX microanalytical device.

Results

No precipitate was formed on the surface of either substrate when oxalate-free solution B was used. Precipitate was detected within 24 and 48 h after the start of the experiment on the upper and lower substrate respectively, if solution B contained oxalate. The total mass of solid precipitated on the upper sphere during the experiment was usually about 10 times greater than that on the lower sphere.

The solid layer that developed on the substrate surface was of polycrystaline nature and consisted of innumerable crystals, mostly twinned or intergrown. The texture of this layer and the shape and size of its constituent crystals varied slightly without an obvious pattern between sites on the substrate surface. Crystals of different type and shape appeared both in intimate contact over a limited region and completely separated in certain areas. The overall structure of solid layers formed independently under identical conditions was, however, always similar and exhibited the same characteristic structural features but with a different frequency of occurrence. The structure of the crystalline coating that developed on the upper and lower spheres was similar under identical reaction conditions.

Typical plate-like crystals of calcium oxalate monohydrate (COM) were formed from normocalciuric-normooxaluric urine (Fig. 3). Columnar and block-shaped crystals of calcium phosphate appeared in addition to plate-like crystals of COM if no citrate was present in the urine (Fig. 4). Plate-like COM crystals were prevalent in the crystalline coating of the upper sphere whereas columnar and block-shaped crystals prevailed on the lower sphere. If normocalciuric-normooxaluric urine without citrate contained 40 ppm pyrophosphate, typical pyramids of calcium oxalate dihydrate (COD) represented the majority of crystals formed (Fig. 5a). These pyramids appeared infrequently with both needle-like crystals (Fig. 5b) and larger crystals of calcium phosphate that had a mostly uneven surface (Fig. 5c). Plate-like crystals of COM were not formed under these conditions.

A mixture of COM and calcium oxalate trihydrate (COT) crystals, often appearing in close proximity (Fig. 6a), was formed from hyperoxaluric urine at pH 5. COM crystals were twinned and intergrown (Fig. 6b). Adjusting the pH of this urine to 7.4 brought about the formation of typical COM and COD crystals together with small particles composed of apparently non-crystalline matter (Fig. 7).

Neither the fresh artifical urine leaving the container nor urine accumulated over 24 h contained any solid particles of a crystalline nature.

Discussion

The new technique described here enables a close simulation of some conditions prevailing in the kidney during the formation of papillar and caliceal stones. In the kidney urine reaching its final composition in collecting ducts emerges onto a papillar surface and enters the inner void space of the kidney. This fresh urine forms a continuously renewing thin film of liquid on the surface of a concretion attached to the papilla. Urine leaving the papilla is collected in the caliceal part of the kidney forming a "pond", and after certain time (the mean residence time) enters the lower urinary tract. This "pond" can occasionally be completely emptied as a consequence of body movement. Therefore, a stone situated in the calix experiences periods of full immersion in liquid urine followed by periods of either partial immersion or without any contact with urine.

In our experimental device the upper sphere is continuously wetted with fresh urine (formed just a few seconds previously by the mixing of two solutions) that forms a thin liquid layer on its surface. Hence, this substrate experiences conditions similar to those a papillar stone is exposed to. The lower sphere is exposed to conditions similar to those prevailing in the caliceal part of the kidney, since it is periodically either fully immersed or partially immersed in urine.

The volcanic spheres used in the experiments serve as a substrate for crystal formation. Crystals adherent to the substrate are then exposed to supersaturated urine, a situation identical to that experienced by crystals formed on living tissue in the kidney. Further development of these crystals is independent of the nature of the substrate on which the crystals are formed. Therefore, the early stages of crystal layer development must be, under identical conditions, similar in both cases. As the volcanic spheres used do not simulate a living tissue this experimental technique provides no information on stone initiation in the kidney. Our experiments show beyond doubt, however, that a solid precipitate is always formed on an appropriate substrate in contact with urine supersaturated with respect to calcium oxalate.

The polycrystalline nature of the layer formed on a substrate develops spontaneously as a result of irregularities in crystal growth: the process termed primary agglomeration [5]. Aggregation of crystals already present in a liquid, the process known as secondary agglomeration [11], plays virtually no role in polycrystalline layer formation. This is convincingly demonstrated by the development of a polycrystalline layer on the substrate despite the absence of crystals in the liquid. This shows that the widely accepted hypothesis postulating the principal role of secondary agglomeration in the development of oxalocalcic renal calculi [1, 9, 10] is largely unsubstantiated. Moreover, spontaneous polycrystalline layer formation combined with the observation that the COM crystals constituting this layer are mostly intergrown [2, 6] strongly emphasizes the significance of primary agglomeration in renal stone development under the conditions prevailing in the kidney.

Active admixtures modify the shape, size and composition of the crystals constituting the polycrystalline layer on the substrate. For example, pyrophosphate present in minute quantities in citrate-deficient urine suppresses formation of COM plates and favours formation of COD pyramids. A similar influence of pyrophosphate was detected in batch precipitation of calcium oxalate [4].

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