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# THE MATRIX OF URINARY TRACT STONES: PROTEIN COMPOSITION, ANTIGENICITY, AND ULTRASTRUCTURE

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## Abstract

We have extracted proteins from urinary tract stones by electrodialysis and have developed antisera to the core and the shell of a renal stone. The protein composition varies between stones but is identical in the core and the shell of the same stone. One stone antigen is present in the urine of normal individuals and stone formers, as well as in cholesterol gallstone extracts. Electron microscopy of the core of a urate-calcium oxalate stone before and after demineralization reveals a fibrillar structure associated with mineral deposits, as well as aggregates of crystals.

**Key Words**: Stone, core, shell, matrix, immunodiffusion, electrodialysis, electrophoresis, fibrils, crystals.

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## Introduction

The composition, structure and function of the matrix of kidney stones became topics of great interest in the 1950's spawning the studies of Boyce and associates, as well as others. It was determined in these early investigations that the protein moiety accounts for approximately 2% of the weight of renal stones. The major component of the matrix was reported to be a mucoprotein called substance A present in the urine of stone formers but absent from the urine of individuals without stones [2, 3]. Immunochemical techniques revealed the presence of Tamm-Horsfall protein in kidney stones [8], as well as a complex antigenic composition which varied according to the method of decalcification of the matrix [11, 12]. These investigators also reported the absence of hyaluronic acid and chondroitin sulfate in the acid mucopolysaccharides of stone matrix, as well as the absence of hexuronic acid and sialic acid [4, 14]. Hyaluronic acid and sialic acid, however, were subsequently detected [15, 17]. The lack of hydroxyproline further differentiated stone matrix from the matrix of bone and cartilage.

Subsequent amino acid analysis of ethylenediaminetetra-acetic acid (EDTA) extracts of stone matrix under alkaline conditions revealed the presence of $\gamma$-carboxyglutamic acid and o-phosphoserine thus providing mechanisms for calcium binding and stone formation [13]. However, amino acid analysis of whole powdered stone showed distinct differences from EDTA extracted material suggesting that some material is lost or resists solubilization with EDTA solutions [5, 19].

More recently, attention has refocused on the protein composition of the calcium oxalate stone matrix with the discovery of nephrocalcin, an inhibitor of calcium oxalate aggregation *in vitro*. Nephrocalcin is also found in normal urine but is reportedly deficient in $\gamma$-carboxyglutamic acid in the matrix of calcium oxalate stones, as well as the urine of stone formers [16]. Tamm-Horsfall protein has now been reinstated as an important inhibitor of calcium oxalate crystal aggregation *in vitro* having been described as a promoter by other investigators using different methodology [9, 18]. Albumin and transferrin are also on the list of proteins

extracted from the matrix of urinary tract stones [6]. A very complex protein pattern was reported by Sugimoto *et al.* who resolved EDTA extracts from four types of kidney stones by HPLC (high performance liquid chromatography). They obtained six fractions from every stone and each fraction could be further separated into three to six peaks on a gel permeation column [21]. A more drastic method of protein extraction was employed by Jones and Resnick who used a 10% acetic acid solution to partially solubilize matrix proteins which they subsequently studied by two-dimensional gel electrophoresis [10]. The role of these unidentified proteins, as well as that of albumin, transferrin, Tamm-Horsfall protein and nephrocalcin as constituents of the matrix remains unsettled and elucidation will ultimately require the study of biomineralization of the matrix itself.

In this report, we present results on the composition, structure and relationships of the matrix and its constituents which provide material and guidance for the further study of their role and that of the matrix in the formation of stones.

## Materials and Methods

Urinary tract stones were obtained at autopsy including a series of urate-calcium oxalate renal stones from an individual who had produced a large number of stones which could be conveniently separated into a core and a shell allowing the study of the embryonic stone. The stones were preserved in cold 0.15 M sodium chloride under the protection of sodium azide. Prior to protein extraction the stones were sponged, weighed and separated, when possible, into core and shell before pulverization. The proteins were extracted by electrodialysis in the ISCO electroeluter apparatus (Model 1750, Lincoln, Nebraska) attached to a refrigerated constant temperature circulator (VWR Scientific, Model 1145, Rochester, N.Y.) set to maintain the temperature at 10°C. The buffer was 0.03 M sodium citrate at pH 6.0 and electrodialysis was performed at 3 watts and 7 mA. The anode cup which retained the extracts has a membrane with 7,000 dalton cutoff and the replacement had a 3,500 cutoff. The extracted proteins were harvested daily from the anode cup and pooled until the readings at 278 nm become negligible. The extracts were either concentrated by electrodialysis or dialyzed against water and lyophilized. Gel electrophoresis was performed on 4-20% gradient polyacrylamide gels in tris-borate-EDTA buffer at pH 8.3. Bromophenol blue served as marker and Coomassie blue as the stain.

In one experiment, the shell of a urate-calcium oxalate stone was pulverized and one half was extracted with 5% EDTA solution changed daily for ten days and the other half was electrodialyzed. The pooled extracts were concentrated by electrodialysis prior to gel electrophoresis.

An antiserum was developed to the core and shell proteins of urate-calcium oxalate stones. Approximately 1.5 mg of lyophilized protein extract was dissolved in 0.15 M sodium chloride, thoroughly mixed with complete Freund's adjuvant and injected intradermally at multiple sites along the back of a rabbit. The procedure was repeated twice using incomplete Freund's adjuvant at two week intervals and subcutaneous injections. Blood was drawn two weeks after the last series of injections from the ear artery and the serum used for immunodiffusion in agar.

Electron microscopy was performed on the cores of three of the series of urate-calcium oxalate stones. The shell was peeled off and the core immobilized in a capsule and sectioned with a diamond knife on a Porter-Blum ultratome (Model 15350 UL Ivan Sorval, Newtown, Conn.). The sections were examined at 40 kV and 60 kV in a Siemens IA electron microscope after negative staining with uranyl acetate. Some sections were decalcified with 0.05% M EDTA, pH 7.0 for a period of two weeks before examination.

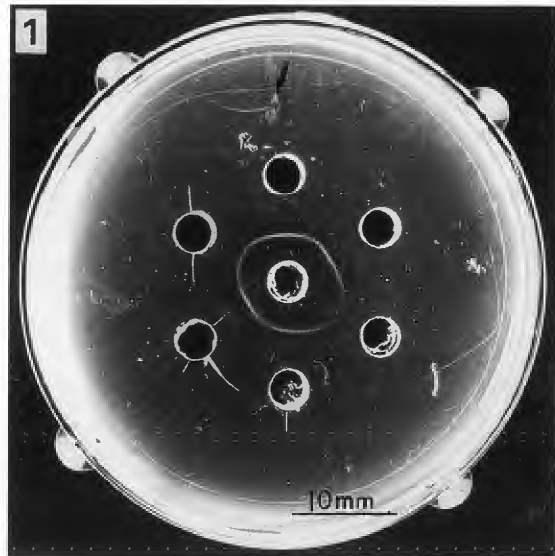
A strip of aorta obtained at autopsy was thoroughly washed with a saline solution and extracted in 0.045 M tris-glycine buffer at pH 7.5 in a Waring blender. After centrifugation, the supernatant solution was passed through an AcA44 column in the same buffer and the first three arbitrary fractions were concentrated and assayed in immunodiffusion against the antiserum to the shell protein extracts of the urate-calcium oxalate stone.

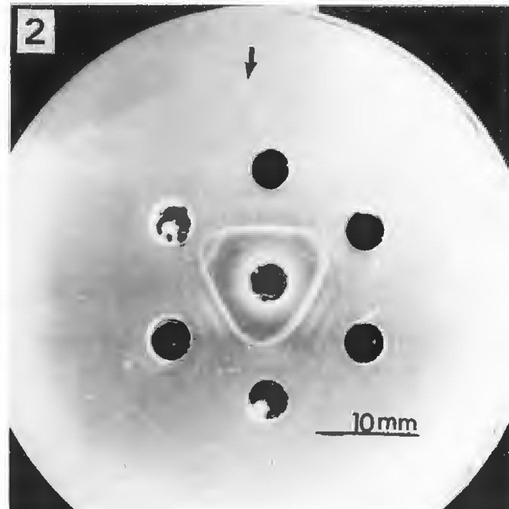
## Results

We have extracted 17 urate-calcium oxalate stones obtained from one individual and 21 stones recovered from different sites of the urinary tract of individual patients. While the pulverized stones are almost completely solubilized and the proteins extracted in soluble form by electrodialysis, a small residue remains in the cathode cup and a white paste accumulates in the anode cup and is particularly abundant after shell extraction of urate-calcium oxalate stones. Some of the residues were stained with Alcian blue for the presence of glycosaminoglycans yielding a positive reaction [17].

The relationships of matrix proteins are demonstrated by immunodiffusion in agar of antisera to the core or shell proteins of the urate-calcium oxalate stones as they develop lines of identity with protein(s) present in the urine of normal individuals as well as stone formers, in cholesterol gallstone extracts and the extracts of aorta (Figs. 1, 2, 3).

Gradient gel electrophoresis of some stone extracts solubilized by electrodialysis demonstrates both the similarity and diversity of the protein composition of urinary tract stones (Figs. 4, 5, 6). The shell and the core proteins of the urate-calcium oxalate stones have identical mobility and are the same in different stones of the same individual. Some bands are shared with the proteins of a prostate stone but a very different



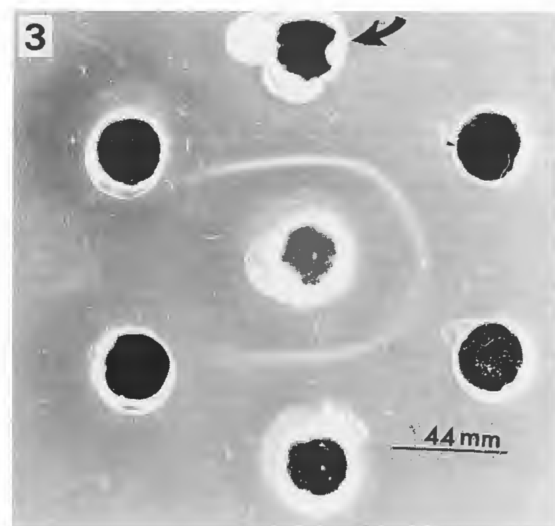




**Figure 1**. Immunodiffusion in agar designed to show reactions of identity between antiserum to core protein extracts of the urate-calcium oxalate stone and a widely distributed protein. Antiserum in center well, concentrated urine from normal individual in well 1 (arrow), and urine from stone formers in other wells.

**Figure 2**. Same antiserum in center well, concentrated urine from normal individual in well 1 (arrow), and shell extract of urate-calcium oxalate renal stone in well 6. Individual cholesterol gallstone extracts filled the other wells.

**Figure 3**. Immuno-diffusion of antiserum to shell proteins in the center well and purified urine protein in well 1 (arrow). Clockwise, wells 5 and 6 contain pooled human sera and AcA44 fractions of aorta extracted by homogenization in Tris-glycine buffer in wells 2, 3, 4.

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electrophoretic pattern is observed in another kidney stone and bladder stone. It is noteworthy that no band with the mobility of albumin is detected in many stone extracts, the protein patterns remain selective and no one protein is shared by all urinary tract stones.

The protein extracts which migrate in gel electrophoresis and diffuse in agar do not always account for the whole matrix of the urinary tract stones examined. The residues which remain in the cups after extensive electrodialysis complete the inventory. Examination by electron microscopy of the cathode cup residue of a urate-calcium oxalate stone reveals a uniform structure without distinctive features. In contrast, examination of the original core matrix of urate-calcium oxalate stones shows a fibrillar structure which may be heavily coated or selectively and unevenly sprinkled by dark deposits which are associated with the cell walls of the framework (Fig. 6). Not all walls participate in the stony architecture and some remain free of deposits. The filaments of the support structures are generally larger than the fibrils connecting the walls. Following decalcification with EDTA, most dark deposits are no longer seen and the remaining matrix appears as a web of thinner fibrils, suggesting a loss of protein(s) and alteration of the matrix (Fig. 8). Large, elongated crystals are present in all three cores examined (Fig. 9). They are usually seen in separate aggregates, but may be mixed with fibrils and free dark deposits.

## Discussion

The usual method of protein extraction from calcium containing stones employs EDTA solutions. However, the completeness of the extraction is usually not reported except by one group who specified that the

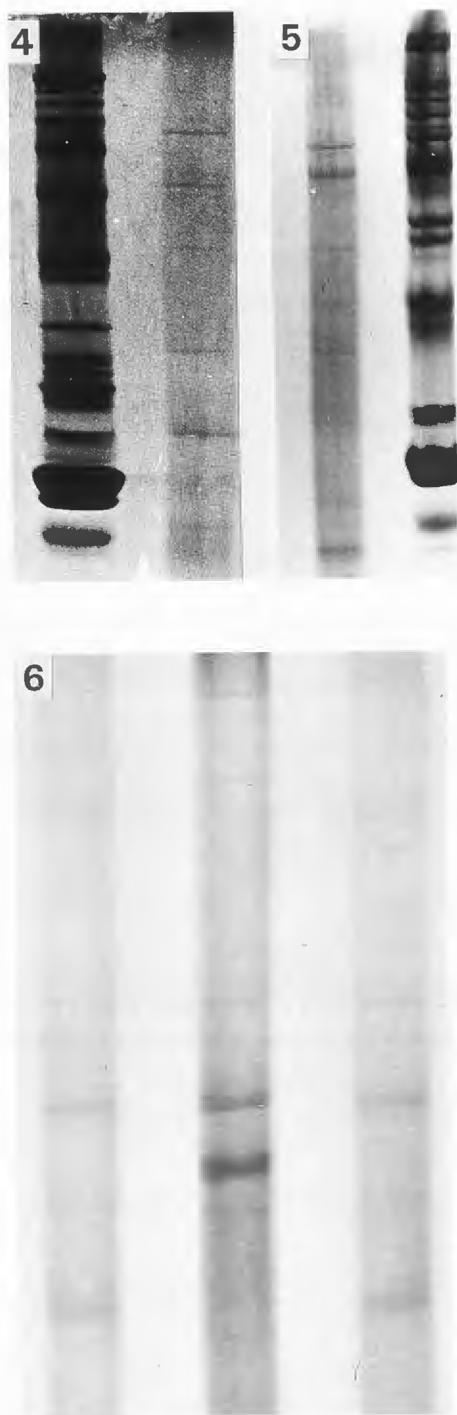



**Figure 4**. Gradient gel electrophoresis in Tris-borate EDTA buffer at pH 8.3. Left lane pooled sera, renal stone extract on right.

**Figure 5**. Left lane bladder stone extract, pooled sera on right.

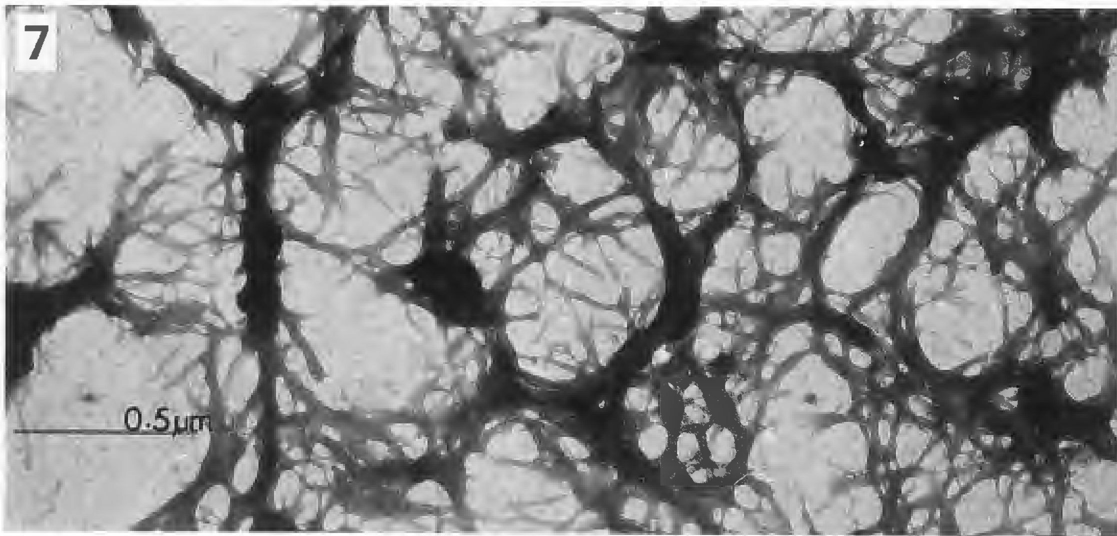
**Figure 6**. Prostate stone extract in center lane flanked by core and shell extracts of a renal urate-calcium oxalate stone.

entire matrix of a calcium oxalate stone had been solubilized [17]. We were unable to achieve this result with urate-calcium oxalate stones after weeks of extraction with dilute followed by more concentrated EDTA solutions and had to proceed to electrodialysis to achieve further extraction which even then remained incomplete.

Two other results of EDTA extraction have been largely ignored. Studies of the structure of the matrix have been carried out on EDTA extracted stones frequently after prolonged extraction and high concentration of EDTA, while a mild form of the treatment clearly altered the matrix of the stones we studied, a result anticipated by Stacholy and Goldberg [20]. One may also question the biological validity of studies of the function of the matrix thus depleted. Clearly, the characterization and function of the EDTA extracted protein(s) need to be pursued, as well as the other proteins selectively incorporated into stones. However, the ultimate role of these proteins may be realized only by their incorporation into a complex unit of biomineralization, the matrix. The organization of these components into a structure which likely serves as the framework for the stony architecture and its similarity with the matrix of the intima of rabbit aorta prepared by freeze fracture [7] suggest the biological importance of the structure and the possibility of the sharing of components, as suggested in this study.

The stone matrix which we have studied by electron microscopy also displays a varied architecture and an uneven distribution of its components which may explain the presence of the rings observed on the cut surface of the core of the urate-calcium oxalate stones [2]. This and the other structural features noted are the more relevant since the matrix has been exposed to a minimum of mechanical and chemical disturbances before examination thus allowing further studies of this and other stones similarly prepared.

We also have attempted to avoid denaturation of proteins in the extraction process with the use of electrodialysis, a technique which in recent years has served to elute proteins from gels after electrophoresis [1]. It is a versatile technique which can extract proteins regardless of the inorganic phase composition of the stones. It is also a mild technique: the mobility and antigenicity of the protein extracted from stone and the protein isolated from urine appear to be identical, evidence that electrodialysis did not denature the matrix protein. Electrodialysis has also confirmed the selective incorporation of proteins into the matrix of urinary tract stones, the diversity of the protein composition of the matrix and has uncovered the sharing of proteins with cholesterol gall stone extracts. The characterization and study of the origin and function of these shared proteins are of particular interest and should complement the study of the intact matrix.

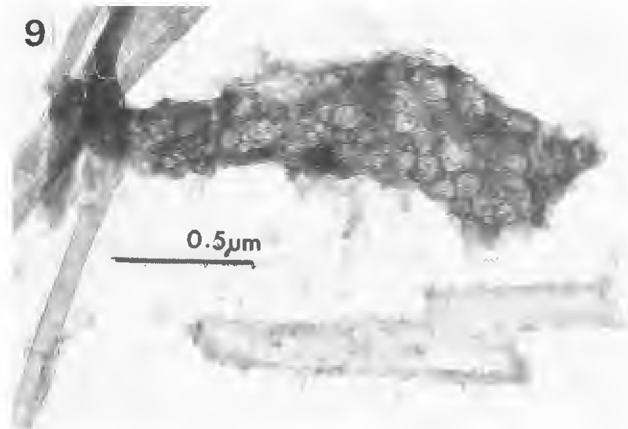


**Figures 7, 8, 9**. Electron micrographs of sections of core matrix of urate-calcium oxalate renal stone.

**Figure 7**. Section of matrix negatively stained with uranyl acetate shows coarse fibers sprinkled with dark deposits forming walls interconnected with more slender fibrils. Bar = 0.5 $\mu$m.

**Figure 8**. Section of stone core after demineralization and negative staining with uranyl acetate shows more slender fibrils and altered organization. Bar = 0.5 $\mu$m.

**Figure 9**. Section of stone core showing coarse crystals dissociated from the matrix network. Bar = 0.5 $\mu$m.



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## Discussion with Reviewers

**M. Akbarieh**: The authors report that the matrix as a complex unit of biomineralization may explain the presence of the rings observed on the cut surface of the core of the urate-calcium oxalate stones. If this structural feature is a result of a re-modulation of the stone by a subsequent phase transformation of the initial mineralization, how do the authors rationalize?
**Authors**: These stones are morphologically heterogeneous as demonstrated by the presence of rings on the cut surface and the uneven distribution of crystals revealed by electron microscopy. They also are chemically heterogeneous with calcium oxalates and urates as major components. Our statement reflects these observations and we propose the following explanations. The core of these stones is formed in successive waves beginning with the deposition of calcium oxalates unto the walls of the ultrastructural units or their constituents followed by encirclement with urate crystals. These events are non-random but the result of salt protein interactions. The organic structure or its components may react differently with different salts thus accounting for the rings or may in turn, depending on local conditions, inhibit or stimulate nucleation. This possible duality of function has been featured again recently (Nancollas *et al.*, ref. 24).

**M. Akbarieh**: The statement "proteins selectively incorporated into stones" is in contradiction with the possibilities concerning the presence of the organic matrix between crystals in urinary stones is its accidental trapping during stone formation. How do the authors explain?
**Authors**: The incorporation of proteins into these stones is clearly selective as several candidates including albumin are under or not represented. It is also likely that entrapment does occur in the course of stone

formation accounting for an unknown percentage of the total protein content of the stones. However, we suggest that the proteins which form the walls of the ultrastructures through charge, configuration and geometrical arrangement trap the calcium oxalate salts from solution and thus play a central role in stone formation. We wish in this context to call attention to two interesting reports published 60 years apart, the first by Huggins [23] demonstrating the osteogenic ability of urinary tract epithelium, the other [25] finding high levels of osteogenic protein (OP-l) mRNA in mouse kidney and bladder. Could it be that proteins involved in bone formation also perform in stone formation and tissue calcification? The tools are at hand to answer this question.

**Y. Nakagawa**: Which method was employed to determine the composition of the urinary calculi?
**Authors**: X-ray powder diffraction and infrared analyses were employed.

**Y. Nakagawa:** How high was the uric acid content and how was the uric acid distributed in the stone?
**Authors:** We do not know the content of uric acid. Please also see above answers to Dr. Akbarieh for proposed distribution.

**Y. Nakagawa:** What role does the urate calcium oxalate stones play in the accumulation of proteins?
**Authors:** We have no revealed knowledge of the role of urates "in the accumulation of proteins". However, we applaud the late conversion of Ryall *et al.* [27] to the salting-out of calcium oxalate and wonder if the concept could be expanded to the salting-out of proteins in the environment provided by some renal tubules. Salting-out, as is well known, is both an old concept and an old technique used successfully for decades in the preparation of protein fractions from plasma.

**Y. Nakagawa:** In your opinion, are the fibrillae crystallization seeds or merely incidental deposits during stone growth?
**Authors:** The deposits removed by EDTA are clearly associated with the walls of the ultrastructural units suggesting that configuration and geometry may determine the location of these deposits. The role of the structures and their components may be elucidated by the study of proteins removed by EDTA and the proteins left behind, and by the use of the chelator BAPTA [1,2bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid] which reportedly is highly specific for $Ca^{2+}$ with little non specific effect thus possibly preserving the structure for further study [28, 29]. The similarity of some ultrastructural features of aorta (rabbit) and stone matrix and the protein sharing (human) are intriguing as we know all too well the potential for calcification of our arteries. As stated in the Introduction, this work was intended to provide materials and guidance for further studies which should include components and assembled matrix.

**Y. Nakagawa:** What do you know of the role played by glycosaminoglycans in the matrices of calcium oxalate stones?
**Authors:** The inhibition of calcium oxalate crystallization by glycosaminoglycans seems well established as it has been reported by eight different groups since 1973 [22]. The significance of these models and the extrapolation of these findings are fraught with uncertainties and difficulties: the glycosaminoglycans, except hyaluronic acid, are generally covalently bound to a protein to form proteoglycans, which in turn are incorporated into the matrix, GAGs are selectively incorporated (heparan sulfate, hyaluronic acid) into the matrix, the milieu in the renal tubules is undoubtedly different from *in vitro* conditions, sialic acid is present in most stones investigated. It is also of interest that the GAGs tested in the crystallization models did not include heparan sulfate, one of the GAGs definitely detected in the stones analyzed by Roberts and Resnick [26]. The selective incorporation of GAGs into the matrix and the demonstrated inhibition of crystallization by the series tested in the models suggest duality of results depending on the environment: inhibition if in solution, stimulation of stone formation if part of the matrix, binding salts selectively.

**Y. Nakagawa**: The authors state that matrix protein is extracted in non-denatured state by electrodialysis. This argument has no strong support. The buffer solution used was 0.03 M sodium citrate, pH 6. Thus I imagine that Ca might be removed from protein as Ca-citrate complex. In the strict sense, if Ca is removed from calcium binding proteins, usually conformation change will occur. Please comment.
**Authors**: We agree with Dr. Nakagawa that calcium might be removed from the protein(s) extracted from stones by electrodialysis and, if so, we would expect conformation change to occur. However, we do not consider such change equivalent with denaturation, for, if it were, we can assume that our life span would be greatly shortened.

**E.L. Prien**: You have apparently studied some of the 17 urate-calcium oxalate stones retrieved from one patient. You also have studied one or several (the number is unclear) single stones recovered from 21 individuals. Please clarify and comment on the composition of these stones.
**Authors**: All the 17 stones obtained from the same individual were selectively studied as it is obviously not possible to apply all the techniques used in this study to the same specimen. The other 21 stones were recovered from different individuals and levels of the urinary tract. Several of these stones were analysed using the techniques mentioned in the paper; their inorganic compositions were different. We were interested in the protein composition of various stones and Figures 4, 5

and 6 demonstrate the variations between stone proteins extracted by electrodialysis.

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