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J. P. Binette
State University of New York at Buffalo

M. B. Binette
State University of New York at Buffalo

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SEQUENCING OF PROTEINS EXTRACTED FROM STONES

J.P. Binette* and M.B. Binette

Veterans Administration Medical Center and School of Medicine and
Biomedical Sciences, State University of New York at Buffalo

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Abstract

Proteins from urinary tract and gallbladder stones were extracted and characterized to determine the composition of the matrix and possibly unravel the role of the organic phase in stone formation. Proteins from crushed stones were extracted by electrodialysis and concentrated in the Amicon centricon cartridge or by lyophilization after dialysis against distilled water. Aliquots were first analyzed by isoelectric focusing in gel and if suitable subjected to two-dimensional (2D) electrophoresis. The most promising spots were harvested and the N-terminal amino acids sequenced, thus providing maximum information with minimum expenditure of material. The 2D separations and amino acid sequences of several protein extracts demonstrated similarities and differences in composition and achieved the identification or demonstration of previously and recently detected polypeptides.

Key Words: Stone, protein, isoelectric focusing, two-dimensional electrophoresis, amino acid, sequence.

*Address for Correspondence:
J.P. Binette
Veterans Administration Medical Center, 151
3495 Bailey Avenue,
Buffalo, NY 14215
Telephone number: (716) 862-3312
or: (716) 834-9200, Ext.: 2378
FAX number: (716) 862-3415

Introduction

The role of the organic phase or matrix in the formation of stones remains speculative and a challenge to our understanding of stone disease. Two related lines of inquiry need to succeed to meet the challenge and resolve the issue: the investigation of the composition of the matrix, with which this report is concerned, and the *in vitro* study of the properties of the isolated components as inhibitors and/or promoters of crystal aggregation culminating in the investigation of the role of the matrix itself in the process as the chemical reactions of the individual components may be altered in significant ways when incorporated into a matrix structure endowed with distinct properties and part of the complex milieu of the renal tubule.

The first step itself, the composition of the matrix, has proven difficult and few proteins isolated from kidney stones have been characterized: albumin [5], Tamm-Horsfall protein [6], uropontin (osteopontin) [8], abbreviated α-1 antitrypsin [16], a prothrombin-like crystal matrix protein [13], and a new glycoprotein from human urine [1]. Nephrocalcin [9], an otherwise well studied protein, remains incompletely characterized. This report enlarges the field as we present our early harvest of stone proteins.

Materials and Methods

Urinary and gallbladder stones obtained at post mortem or at surgery were stored at 4°C under the protection of sodium azide until the proteins were extracted. A total of about 80 stones was used in this study. The extraction, after pulverization of the stones, was carried out by electrodialysis in the ISCO (model 1750, Lincoln, NE) apparatus originally devised to extract proteins from gels [2]. The solution was 0.05 M sodium citrate at pH 6.0, the current was maintained between 6.0-7.0 mA and the wattage is set at 3.0 W. Sodium azide was added in trace amounts to this solution and the proteins were harvested daily from the anode cup with a Pasteur pipette and the electrodialysis continued until the

readings at 280 nm in the spectrophotometer were negligible. The extracts were pooled and usually dialyzed in the cold room against large volumes of reverse osmosis distilled water and lyophilized or concentrated in the Amicon (Beverly, MA) centricon utilizing a refrigerated centrifuge at 5,000 rpm. Some gallbladder and a number of kidney stone extracts were concentrated by polyethylene glycol (PEG) [3], applied to a column at 4°C (Ultrogel AcA34 or AcA44-IBF; IBF Biotechnics, Villeneuve-la-Garenne, France) employing Tris-glycine elution buffer at pH 8.2. The eluents were separated by their optical densities at 280 nm, concentrated by electrodialysis, dialyzed and lyophilized or concentrated after dialysis against distilled water by the Amicon centricon method.

BioRad's amplified alkaline phosphatase immunoblot assay kit was used to detect the presence or absence of α-1 acid glycoprotein [3] (Bio-Rad, Chemical Div., Richmond, CA). α-1 (anti-human) acid glycoprotein was obtained from Sigma (St. Louis, MO).

Aliquots were first checked by isoelectric focusing in agarose gels (pH 3-10 and 4-7 from FMC Bioproducts, Rockland, ME) using the Hoefer cooling platen (Hoefer Scientific Instrument, San Fernando, CA). They were also subjected to gradient (4/30) vertical and sodium dodecyl sulfate (SDS) 8-18 excel gel-horizontal (Pharmacia LKB, Piscataway, NJ) electrophoresis and if suitable were analyzed by two-dimensional (2D) electrophoresis according to the method of O'Farrell [10]. The gels were then transblotted onto PVDF (polyvinylidene disulfide membranes-immobilin-Millipore) (Millipore Corp., Bedford, MA) overnight at 4°C in 12.5 mM Tris, pH 8.8, 86 mM glycine and 10% methanol at 200 mA and approximately 100 volts per gel. The PVDF membranes were then stained with 0.01% Coomassie blue in 50% methanol and rinsed 4 minutes in ultrapure distilled water. Pin holes serve to mark the placement of the gel on the PVDF membrane.

The marked spots were excised and sequenced on an Applied Biosystems 470A gas phase sequencer / 120 PTH analyzer for NH_2-terminal sequence; 20 cycles were run on each (Applied Biosystems Inc., Foster City, CA).

Results

Isoelectric focusing (IEF) on agarose gels revealed the presence of protein bands from pI 3.0-8.5 in kidney, gallbladder and bladder stone extracts. Figure 1 shows IEF electrophoresis of a gallstone extract. Three gallbladder stones (not shown) had bands just beneath the cytochrome standard (pI 10). Four kidney, four gallbladder, and two bladder stone extracts were tested for reactivity to α-1 acid glycoprotein. All showed a positive reaction and each had a negative control.

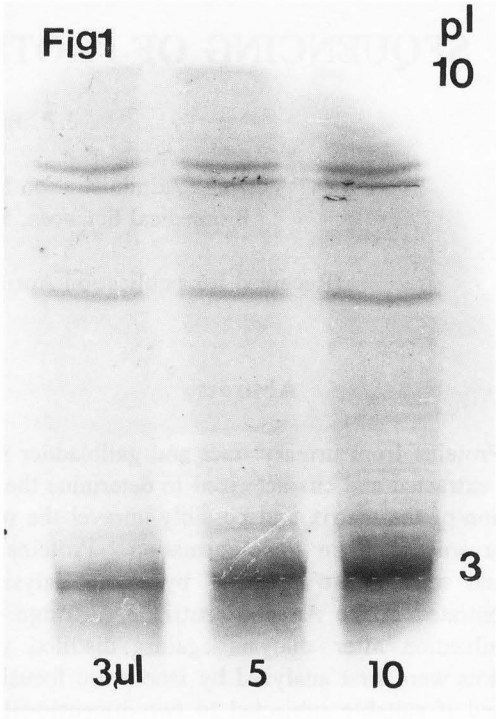

Figure 1. Isoelectric focusing (IEF pH 3-10 on FMC Bioproducts agarose plates) of extracts from a pigmented gallbladder stone. Collections from electrodialysis were concentrated, dialyzed against reverse osmosis distilled water and passed through an AcA34 column in the cold room, concentrated by Amicon centricon after dialysis and electrophoresed for 90 minutes on the Hoeffer cooling platen. From 3-10 μl were applied to each lane and stained with Coomassie blue.

A gallbladder stone extract (Fig. 2) with many bands on IEF and SDS gel also displayed a considerable number of polypeptides on 2D electrophoresis with spots ranging from less than 14 kD to over 94 kD. Unfortunately, it did not transfer well for sequencing. One bladder and three kidney stone extracts revealed spots in the same area on 2D (Fig. 3a). All four specimens had the spots migrating to the same area sequenced after transblotting and the results were identical. The sequence for this protein (Spot 3) follows: A V V G G G A T L P E K L Y G. The approximate molecular weight (MW) was 40,000 and the pI was 5.4. This sequence is not in the data base (Protein Identification Research, Protein Sequence Data Bank, National Biomedical Research Foundation).

Spot 4 from the same three kidney stone extracts gave the following sequence: A E Y V L P D L A Y D Y G A L E X H I. The molecular weight was approximately 22,000 and the pI was 4.8. There is no exact match for this protein but it appears to be a superoxide

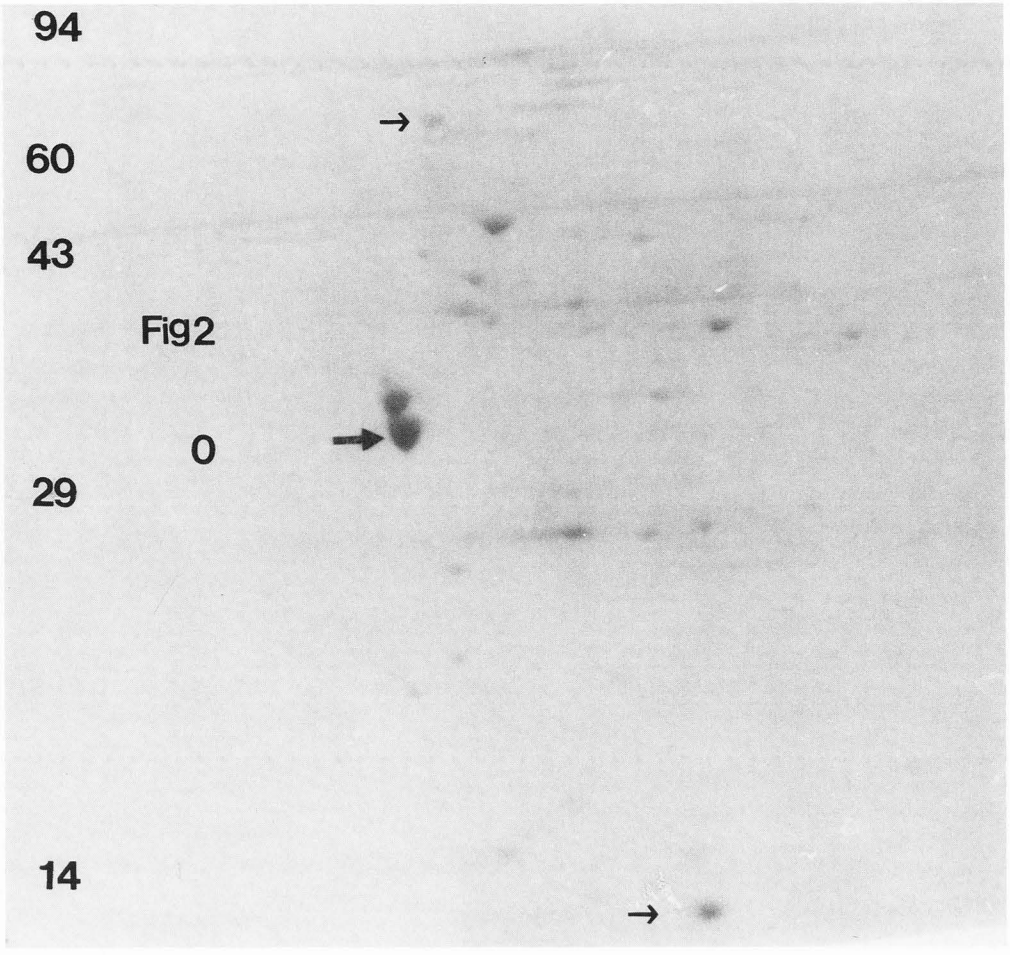

Figure 2. Two-dimensional (2D) electrophoresis of gallbladder stone (same specimen as Fig. 1). The IEF internal standard of 1 μg of tropomyosin, MW 33,000 and pI 5.2 is indicated by the lower left arrow. Protein standards were added to the agarose which sealed the tube gel to the slab gel. These markers are represented by horizontal lines from 220 to 14 kD. The upper left arrow indicates an 82 kD protein.

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dismutase. The composition: 80% calcium oxalate monohydrate, 10% uric acid, 3% ammonium acid urate, 3% cryptocrystalline salt of uric acid, and 4% protein.

One urate-calcium oxalate stone (Fig. 3b) from a series which had been extensively investigated provided three protein sequences. The sequence for Spot 2 is: N D Q E Q S K G F V E D. There is no exact match in the data base; MW = 41,500, and pI is 5.5.

The sequence for Spot 4 of the same stone is: K D Y E L L N V S Y D (P) T (R) E L (Y) Q. This is a sulfate binding protein with a MW around 34,506 and pI of 5.0.

Spot 6 A, B, C from the same stone extract had the following sequence: A F E L P P L P Y A (H) D A L Q P (H) I S. The MW is approximately 21,516 and the pI 4.7. This is a superoxide dismutase. This particular stone extract did not have detectable levels of the protein identified as Spot 3 in Fig. 3 above but other extracts of the same series of stones obtained from one individual clearly did as identified by migration and sequence.

Figure 4 shows a 2D electrophoresis of a urate-calcium oxalate stone extract. The arrows at 3a and 3b indicate proteins which have been sequenced but are not found in a protein data base. Most of the specimens tested so far have spots on 2D with molecular weights clustered around 12-14, 25-43, 70-80 kD with pI ranging from 3 to 8.5 [7].

Discussion

This report is viewed as the opening chapter in the endeavor to map completely and identify accurately the

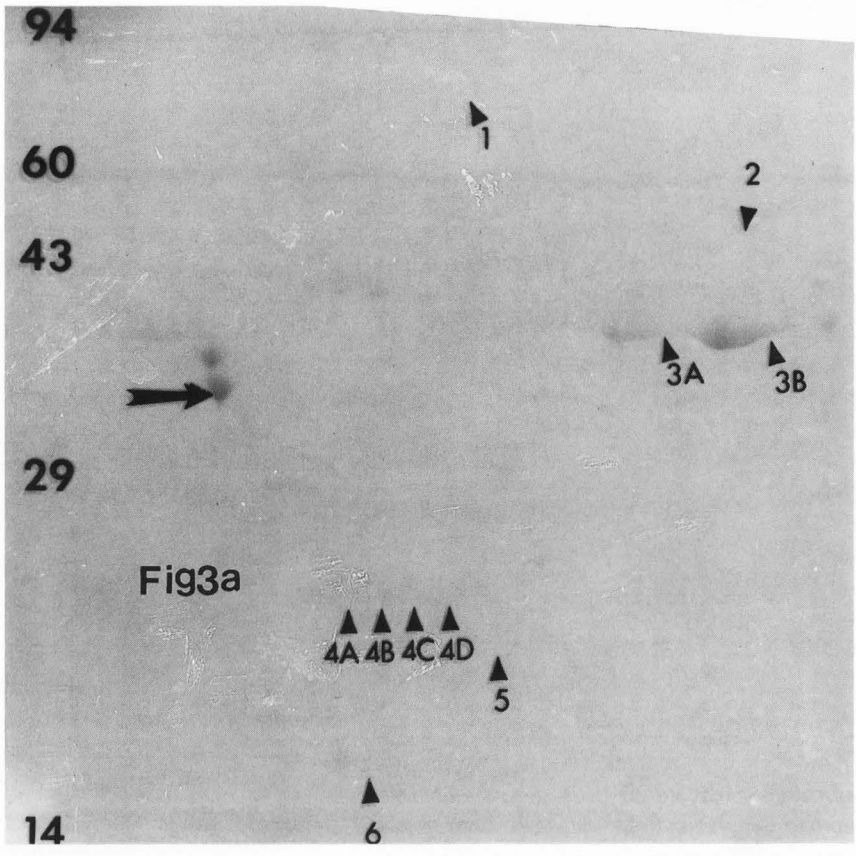

Figure 3a. 2D electrophoresis of a kidney stone. The sequence A V V G G G A T L P E K L Y G has not been located in a protein data base after a careful computer search. It has an approximate MW of 40,000. No exact match has been found for the protein in spot 4 region of 22,000 MW. The internal standard of MW 33,000 is indicated by an arrow.

proteins incorporated into the matrix of stones. Few such proteins have been characterized, an acknowledgement of the difficulties involved in their extraction prompting the substitution of crystalluria spontaneous and enhanced for the study of crystal bound proteins. This compromise has resulted in the isolation and characterization of a prothrombin-like protein and is expected to remain productive but is limited by the escape of unbound components of matrix and the exclusion of the possible role of the matrix structure in stone formation.

The methodology employed in the present work expands the recovery of matrix proteins, some present in small amounts thus challenging even the most economical techniques of purification and characterization. However, it is important that the effort succeed. If we accept that the incorporation of proteins into stone matrix is selective, which the evidence gathered so far suggests, it follows that such selected components should have roles as molecules and possibly as part of structures in the formation of stones.

The resolution of the debate on the active or passive role of matrix proteins in this process requires their isolation, characterization and the study of their individual and conglomerate functions. The isolation of stone proteins of known function may be specially helpful in the elucidation of the elusive mechanism of their formation. The identification of shared proteins selectively incorporated into stones would enhance the possibility of an active role. The possibility that protein(s) present in stones may or may not be detectable in the urine of stone formers or in normal urine, having been removed from tissue, can now be investigated in a refined manner. Even the presence of proteins without *in vitro* inhibition/promotion of crystal aggregation should be considered relevant to the process of stone formation as their location and interactions with other proteins and their environment may affect structure and function in the dynamic setting of the renal tubule. The possibility of protein alteration in structure and function has been raised in the case of nephrocalcin, deficient in gamma carboxyglutamic acid [9], and more recently by the sequencing of α-1 antitrypsin [16] from stones and the prothrombin-like protein isolated from crystals [13]. However, the role of the environment as more significant than the amino acid sequence in determining protein

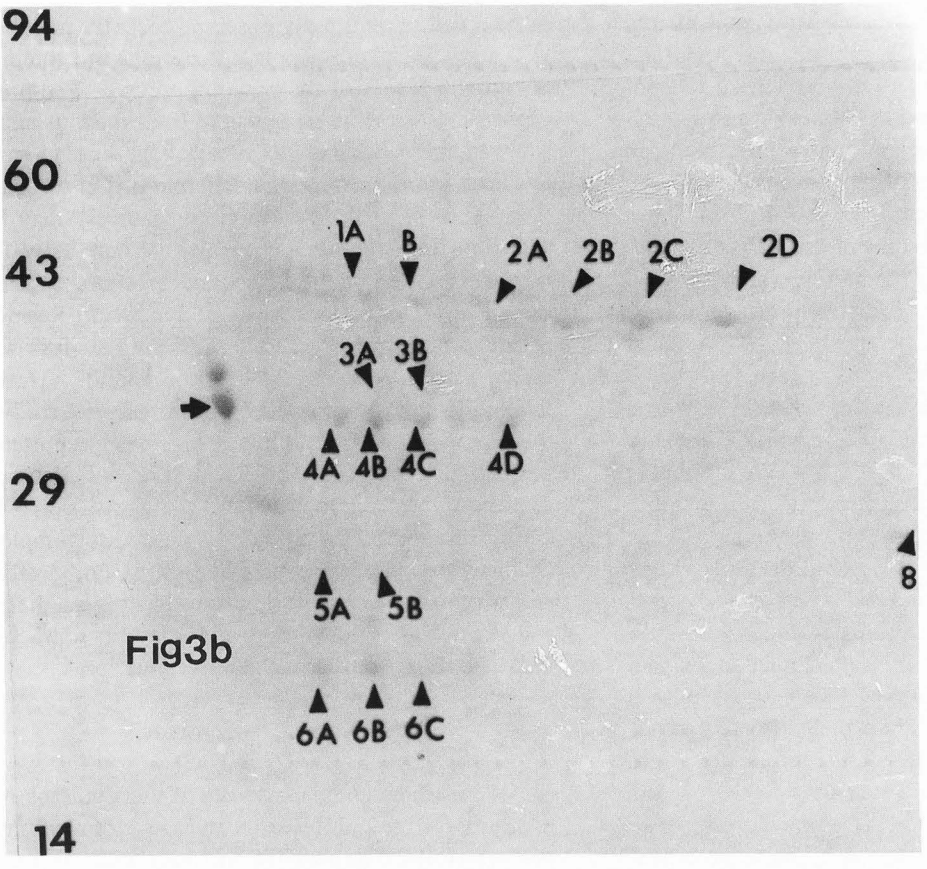

Figure 3b. 2D electrophoresis of a urate-calcium oxalate stone. Polypeptides are indicated by arrows and isoforms by letters. Three areas marked 2 A,B,C; 4 A,B,C,D, and 6 A,B,C have been sequenced. An arrow indicates the tropomyosin standard at pI 5.2 and MW 33,000.

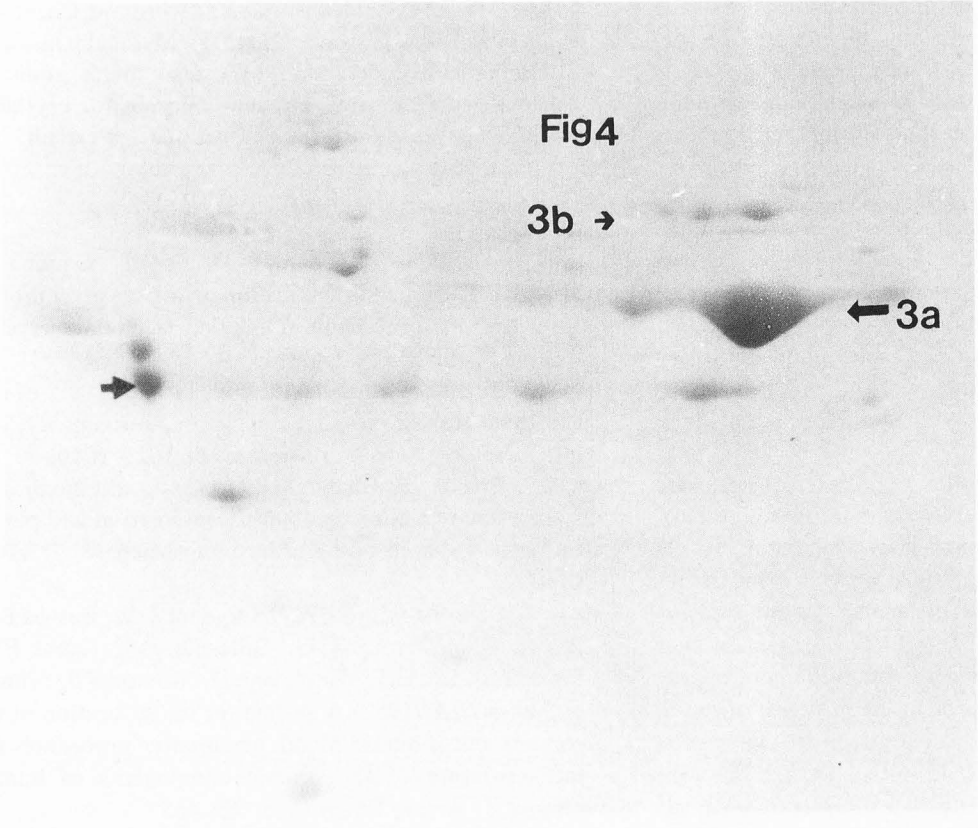

Figure 4. 2D electrophoresis of a urate-calcium oxalate stone extract. Arrows at 3a and 3b indicate proteins which have been sequenced but are not found in a protein data base. Two other stones from different individuals revealed the same sequences. A bladder stone extract (composition 2% calcium oxalate monohydrate, 95% uric acid and 3% protein revealed the same area on 2D but was insufficient for sequence. All specimens were obtained at different times and were processed weeks apart. The heavy arrow at 33,000 is the tropomyosin standard.

structure (α-helical or β-strand) has been documented in recent work [17]. The pH of the solution, one important variable in the environment and a well known component of a scheme of fractionation [4], has been reported recently to be determinant along with concentration in the production of a predominant random coil from β-AP 25-35 at pH 5.5 and a β-structure at pH 7.4 [14]. These states and transitions, should some of the matrix proteins be susceptible to the large pH variations which occur in urine, could also explain their inhibitor/promoter role(s) in stone formation.

The techniques employed in the present work were selected to yield the maximum information with the small amounts of material available and look especially for proteins of known function(s) such as enzymes [11] and shared proteins selectively incorporated into stones suggesting a role in their formation. Proteins of each category have been found in stones and the search continues following the pioneering 2D study of Jones and Resnick [7] on proteins extracted from stones with 10% acetic acid. Findings shared by both studies include an 80kD protein present in all kidney stone extracts as well as several low molecular weight proteins. Much variability is observed in the intermediate area of the gel in both studies. However, the intensity of the low kD spots in the Jones-Resnick study [7] is much greater than in ours. One explanation proposed by these investigators is the degradation of the larger proteins producing small species. Limited hydrolysis by enzymes present in stones could have produced such a result.

The degree of concordance between the two studies and the reproducibility of the 2D electrophoretic patterns of stone proteins extracted at different times argue for the validity of the findings. Of the protein sequences reported thus far only two osteopontin (uropontin) and the prothrombin-like peptide have a known role in calcium binding and presumably in stone formation. The presence of enzymes and membrane associated proteins and the absence or very low levels of more abundant urinary proteins again document the selectivity of protein incorporation and suggest a role in stone formation which remains to be determined.

Proteins shared by stones of different origin and composition may be particularly relevant in both biliary and kidney stone formation. They include an 84 kD protein reported earlier, a 43 kD protein identified in this work and several low molecular weight proteins with respective pI of 8.5, 4.5 and 3.0-3.5. Should their amino acid composition favor β-structure under appropriate conditions of pH and/or in the presence of membrane phospholipids [15] as recently reported, the resultant decrease in solubility could initiate the see saw cascade process of stone formation in a supersaturated salt solution. The process could be further complicated by protein fluxes reportedly observed recently in gallstones [12] and which could explain variations in protein composition in stones of the same individual. The presence of enzymes in some stones may be fortuitous and their role remains ill-defined as none of the known enzymes has been found in stones of different origin or composition. Enzymes, in specific circumstances, may be responsible for truncated proteins such as the α-1 antitrypsin reported, or the presence of small species observed on 2D electrophoresis of Jones and Resnick [7] or the processing of proteins resulting in less soluble residues as in the case of β-amyloid peptide. However, the known proteins sequenced in the present study have the same molecular weights as originally reported suggesting that they are whole and three smaller species sequenced but not reported here are not segments of known proteins. Further work is needed to complete the characterization of the stone proteins present in sufficient amounts to be amenable to study by this methodology and the residues remaining after protein extraction of some stones require complete investigation.

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Discussion with Reviewers

Reviewer IV: How do you know that spot 4 in Figure 3b is a sulfate binding protein?
Authors: The amino acid sequence of the protein identified as spot 4 in Figure 3b is identical to the known sequence of a sulfate binding protein which is in the data bank (Protein Identification Research, Protein Sequence Data Bank, National Biomedical Research Foundation). This analysis provides very strong evidence that the two proteins are identical.

Reviewer IV: Why was reactivity to α-1 acid glycoprotein tested?
Authors: The reactivity to α-1 acid glycoprotein was tested because of personal association with the protein when the world was young [Nature (1961) **190**, 630-631; Biochim Biophys Acta (1968) **154**, 234-235; Biochem J (1974) **143**, 253-254)] and the report of Abei *et al.* [Gastroenterology (1993) **104**: 539-548] on the isolation and characterization of a cholesterol crystallization promoter from human bile. A tryptic peptide sequence of 14 amino acids matched the α-1 acid glycoprotein sequence perfectly. Immunoreactivity further confirmed the identity. Could α-1 acid glycoprotein be a prompter of crystallization in urine as well as in bile? The above question may provide a partial answer to the possible role of α-1 acid glycoprotein in stone formation.

Reviewer IV: In the **Abstract** you state that the aim of the paper is to "... possibly unravel the role of the organic phase in stone formation"; this is more than just sequencing!
Authors: A complete and satisfactory answer to the central question of the role of proteins in nephrolithiasis is not available and was not the aim of our paper. The aims and achievements of this ongoing work are more modest as stated in the opening sentences of the **Abstract** and the **Discussion**. Many factors which may impinge on stone formation are mentioned in the **Discussion**, and these extend beyond the role of proteins in the very complex milieu of the renal tubule. We are wary of simple answers to complex problems and our work is only a contribution to the knowledge of the composition of stones. We consider this approach logical and necessary if we are to understand how they are formed. Shared proteins and GAGs are of special interest and, for example, it may be pointed out to the readership that heparan sulfate, reportedly the major GAG associated with stones, shares the same association with amyloid, another difficult to dissolve protein.

Reviewer IV: Were protease inhibitors included during the extraction process? Azide absorbs in the UV range; how do the authors know that azide did not contribute to the absorbance that they attributed to proteins?
Authors: We have used protease inhibitors and we have checked electrodialysis extracts with and without azide and by other protein detection methods.