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URINARY STONE PROTEINS: AN UPDATE

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Abstract

The discovery of an organic component in kidney stones dates back to 1684. More than 150 years elapsed before the incrustation of this organic component, which is now called the matrix, was proposed as the mechanism of stone formation. The composition of the matrix remained largely unknown until the development of electron microscopy and the advances in biochemistry combined in the 1950's to usher in the modern era of renal stone matrix investigation. Composed mainly of selectively incorporated proteins generally characterized by high glutamic and aspartic acid content and the frequent occurrence of γ-carboxyglutamic acid, the matrix displays a variable and complex composition and shares a few proteins in many stones. The embryonic stone may first appear in the renal tubules where it can acquire the blood and cell membrane proteins recently found by analysis of stone protein extracts. The combination of supersaturation, an appropriate environment, the availability of calcium binding proteins which may be abnormal, and the incorporation of proteins extracted from leukocytes and cell wall membranes may induce stone formation.

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

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Introduction

The discovery that the "stone," a long known source of torment for humankind, has an organic component dates back to 1684 when Von Hyde found that the calculus has an organic framework which we now call the matrix [5]. The following century was remarkably silent on this mundane topic until 1780 [26], when scientists began to name and classify the organic components of stones and eventually suggested incrustation of such as the mechanism of stone formation [5].

Another century elapsed before Boyce, combining the advances in electron microscopy and biochemistry, brought the investigation of the organic component (matrix) into the modern era. Other research groups have further expanded knowledge of the field and have progressively unravelled the composition of the matrix, identifying many of its protein components. What follows is an account of this collective effort spanning some 40 years and includes our unpublished work. It is presented in the form of tables accompanied by a textual commentary and key references.

Materials and Methods

Urinary and gallbladder stones obtained at surgery or postmortem were stored at 4°C until the proteins were extracted. Sodium azide was used as a preservative. The stones were pulverized and the **extraction** was performed in the model 1750 ISCO (Lincoln, NE) apparatus, originally devised to **elute** proteins from gels. 0.05 M sodium citrate buffer with a pH of 6.0 was cooled by a refrigerated circulator with the current between 6.0-7.0 mA and the wattage at 3 W. Electrodialysis was continued until the readings at 280 nm were negligible [1, 2]. The extracts were pooled, concentrated and dialyzed against reverse osmosis distilled water, passed through an AcA44 column (IBF Biotechnics, Villeneuve-la-Garenne, France) employing Tris glycine buffer (0.023 M Tris, 0.008 M glycine adjusted to a pH of 8.2 with standardized 1 N HCl). The eluents were separated by their optical densities at 280 nm, concentrated after dialysis and lyophilized. Aliquots were

screened by isoelectric focusing (IEF) (FMC Bioproducts, Rockland, ME) using the Hoefer cooling platen (Hoefer Scientific Instruments, San Fernando, CA) to ensure suitability for two-dimensional (2-D) electrophoresis (method of O'Farrell) [2]. Briefly, IEF was carried out in glass tubes (2.0 mm inner diameter) using 2.0% Resolytes pH 4.8 ampholines. Tropomyosin was the internal standard: lower spot of molecular weight (MW) 33 kD and isoelectric point (pI) 5.2.

After 10 minutes in buffer "0" {10% glycerol, 50 mM dithiothreitol, 2.3% sodium dodecylsulfate (SDS) and 0.0625 M tris; pH 6.8}, the tubes were sealed to the top of stacking gels which were on top of 10% acrylamide slab gels (0.075 mm thick), and SDS slab gel electrophoresis was carried out for 4 hours at 12.5 mA/gel. The following protein standards were used: myosin (220 kD); phosphorylase A (94 kD); catalase (60 kD); actin (43 kD); carbonic anhydrase (29 kD); and lysozyme (14 kD). These standards appear as horizontal lines when stained with Coomassie Brilliant Blue R-250. The electrophoresed gels were 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, approximately 100 volts per gel. Membranes were stained with 0.1% Coomassie blue in 50% methanol, rinsed four times in ultrapure distilled water and spots were marked, excised and sequenced on an Applied Biosystems (Foster City, CA) 470A gas phase sequencer/20PTH analyzer for NH_2-terminal sequence, 20 cycles were run on each.

Results and Discussion

Not surprisingly, the first proteins extracted from stones by Boyce and his associates included albumin and Tamm-Horsfall protein (THP) [33], the two most abundant urinary proteins, as well as other proteins simply classified by their electrophoretic mobility as α-globulins and γ-globulins [4]. Boyce realized that incorporation of proteins into stones is a selective process, an observation repeatedly confirmed [6]. Dialysis against ethylenediaminetetraacetic acid (EDTA) containing solutions had been used to remove most of the crystalline component of the stones, leaving a matrix which proved difficult to solubilize. This prompted the use of immunological methods in Boyce's early work.

The most immunologically abundant protein extracted from stones he called substance A, a protein characterized by a relatively high content of glutamic and aspartic acids, a characteristic shared by other proteins subsequently recovered from stones [3]. Moore and Gowland [19], in a later study, were unable to confirm the immunological oneness of substance A and, like Boyce *et al.* [3], could not detect it in normal urine, only in the urine of stone formers and in some pathological conditions [19]. Matrix substance A, because of its reported large contribution to stone matrix composition, is likely to have been revisited by subsequent investigators. Unfortunately, more than one characterized protein has an isoelectric point around 4.5 and a molecular weight between 30-40 kD [20]. The identity of matrix substance A may therefore remain enigmatic.

Boyce [4] had also reported the absence of hydroxyproline and hydroxylysine on amino acid analysis of matrix proteins and had suggested that the matrix of all urinary calculi is similar irrespective of crystalline composition. This assertion was reexamined by Spector *et al.* [29] who analyzed calcium oxalate, uric acid and apatite-struvite stones and confirmed the important contribution of aspartic and glutamic acids, but reported a distinct protein composition of the matrix of these stones. The following year, Lian *et al.* [17] reported that γ-carboxyglutamic acid (GLA) is present in the proteins of calcium containing stones, thus linking stone matrix proteins with bone and blood clotting, calcium-binding proteins.

The resolution of kidney stone matrix proteins was expanded by Sugimoto *et al.* [31] who used high performance liquid chromatography to separate EDTA extracts of stones, but did not characterize their proteins beyond ranges of molecular weights and concluded that the matrix protein composition is identical in all stones. Fraij [9] reached the same conclusion after separating SDS-solubilized matrix proteins by SDS gel electrophoresis, although the amounts of each major protein component, THP and albumin fluctuated widely. No protein of molecular weight around 90 kD was reported by Sugimoto *et al.* [31] while THP (94 kD) was one of the major proteins identified by Fraij [9]. These discrepancies, likely the result of different methodologies (or possibly geography), continue to the present.

One well-characterized protein, ß-2M (or fragments thereof), is the major constituent of matrix kidney stones recovered from uremic patients [18] and also the precursor protein of the amyloid associated with long-term hemodialysis [10]. The formation of this stone matrix, which displays the ultrastructure and the birefringence of amyloid, is reportedly independent of dialysis, while the amyloid which may be deposited elsewhere in the body, particularly the joints, appears only after years of dialysis and is made of intact ß-2M [11]. Soluble proteins have also been extracted from these stones but not investigated further. One of these soluble proteins is likely amyloid P-component which has been found to accompany all forms of amyloid and reportedly shields it from attack by proteolytic enzymes [35]. Such stones would provide a fine opportunity to examine the relationship of the amyloid fibril and the P-component and test the susceptibility of the matrix stone to proteolytic digestion

before and after removal of the P-component.

Remarkably, two serine proteases along with hemoglobin have been reported in formic acid extracted proteins of calcium-containing stones [25]. This suggests a close encounter between crystals, red cells and neutrophils in the history of stone formation, with the cells leaving their signatures in stone to record the event. This injurious encounter may have occurred in the tubule, in which case, other proteins derived from cells lining the tubules would be expected at the center of the stone early in its formation. Could these proteolytic enzymes remain active and participate in stone formation and could their activity be demonstrated after extraction?

Nephrocalcin, the celebrated and shrouded protein, may be an example of a deficient protein, as extracted from stones and the urine of stone formers, possibly less able to bind calcium because of reduced γ-carboxylation of glutamic acid [23, 24, 34]. The mechanism of the GLA deficiency in nephrocalcin remains unknown but has not been reported to be vitamin-K responsive nor warfarin inducible, although osteocalcin reportedly is [16]. A recent N-terminal amino acid sequence identifies nephrocalcin with a light chain of inter-alphatrypsin inhibitor [34], however, the absence of GLA and the lack of homology with osteocalcin suggest that authentic nephrocalcin remains to be sequenced.

Another stone-extracted protein, an important constituent of calcium oxalate monohydrate stones, uropontin, shares the same amino acid sequence as osteopontin of bone and kidney, is rich in acidic amino acids but lacks γ-carboxyglutamic acid [13]. It inhibits calcium oxalate crystal growth *in vitro*, suggesting a shared *in vivo* role in the complex process of mineralization. Uropontin may be an example of a protein exhibiting different behaviors in different pH environments or following post-translational modifications such as phosphorylation [28]. Elucidation of its precise function remains a challenge.

Tamm-Horsfall protein, another major constituent of kidney stone matrix proteins, isolated from urine in 1950 [33] and subsequently extracted from calcium oxalate stones by Boyce [4], has been studied extensively but its function remains elusive. It appears to be a chameleon protein which may function as a promoter of crystallization when exposed to low pH solutions or in the presence of cations such as calcium, magnesium or sodium [12]. Self-aggregation of THP, which may be increased in stone formers, may also increase viscous binding neutralizing the zeta potential of the electrostatic surface charge. If conditions are reversed to high pH and low concentrations of calcium and sodium, THP is transformed into an inhibitor of calcium oxalate crystal aggregation. The ability of citrate to preferentially bind calcium may thus explain its beneficial role in the prevention of calcium oxalate stone formation [12].

Several proteins were added to this list using 2-D electrophoresis of proteins recovered after the dissolution of crystals formed in urine [22] or after formic acid extraction from stones [14]. Most of these proteins have not been characterized beyond the determination of isoelectric point and molecular weight but two interesting findings have emerged from this work: many low molecular weight proteins have been recovered and important differences in electrophoretic patterns can be observed between proteins obtained from crystals and proteins extracted from stones, suggesting caution in the interpretation of results obtained from *ex vivo* experiments [14, 21, 22]. The possibility of artifacts induced by the stone protein extraction procedure or protein degradation during stone formation or tubular wall injury may explain the large presence of low molecular weight proteins.

The discovery of a truncated α-1 antitrypsin in a 4 M guanidine extract of calcium oxalate stones matches proteases discovered earlier in the same stones with the most abundant protease inhibitor in human serum [36]. Both enzyme(s) and inhibitor(s) may derive from leukocytes that were attracted to these foreign crystal formations and lost some of their baggage in the encounter [8]. The connection of blood and stone has been more precisely documented by the isolation from calcium oxalate crystals and stones of a protein which binds calcium avidly, is a potent inhibitor of calcium oxalate crystallization *in vitro* and shares the N-terminal amino acid sequence of a prothrombin fragment recently identified as F1 [30]. Its presence in crystals and stones enhances its credentials as a player in stone formation. It also raises, more pertinently, the importance of the post-translational carboxylation of specific glutamic acid residues required for the binding of calcium and the biological activity of prothrombin, a vitamin K dependent function, again raising the question of the role of vitamin K and its inhibition by warfarin in the formation of stones.

Blood and stones are again linked by a calprotectin-like protein extracted from calcium oxalate stones and originally recruited from neutrophils and macrophages losing weight from 36.5 kD to 30 kD in the process [37]. The abundance of the protein in stones and its inhibitory potency on calcium oxalate crystal growth again suggest a role in mineralization which remains to be determined.

The complex protein composition of stone matrix has been further expanded by the use of electrodialysis to extract proteins from crushed urinary tract stones and gallstones, followed by 2-D electrophoresis and selective N-terminal amino acid microsequencing [1, 2]. The results, not previously reported, are presented in Table 1 which includes many protein sequences not found in the data bank as well as several proteins originating from

Table 1. Stone sequences. Individual specimens are listed alphabetically with chemical composition, type of stone, pI (isoelectric point), approximate molecular weight (in kD), and N-terminal sequence. All specimens were examined by crystallography, polarized light and electron microscopy.

No	Type & Chemical Composition	pI	App MW (kD)	N-Terminal Sequence	Status‡
A.	kidney stone	5.4	40	AVVGGGATLPEKLYGST	Unknown * A56049
	Urate calcium oxalate	5.1	32	DPGSATDYRTAAVGSDT	Unknown - closest homology thrombospondin - PIR_2 Accession C56046
		4.9	30	XXQGAVEGELFYKKQYNDSV	Root adhesion protein
		4.5	17	Blocked: *Tryptic digest*:	
				(Y)LVLQGVAPG(Q)LXLV	Unknown - PIR_2 Accession B56046
		5.6	42	(T)HSYFNDLAAETD	Unknown - PIR_2 Accession H56046
		5.7	44	TNTLRPNFAETAK	Unknown - PIR_2 Accession G56046
		4.7	21.5	AFELPPLPYAHDALQPHTSK	Closest homology - E56049 Superoxide dismutase
B.	kidney stone UrCaOx	5.4 5.1	40 32	AVVGGGATLPEKLYGST N-terminal DPGSATDYRTYAAVGSDT	Unknown - PIR_2 Accession A56049 Accession C56046
C.	kidney stone	5.4	40	AVVGGGATLPEKLYGST	Unknown - *
		4.75	22	AEYVLPDLAYDYGALEX(H)I	Superoxide dismutase C56049
		4.5	17	Blocked NH terminal *B56046	(Y)LVLQGVAPG (Q)LXLV
		4.7	21.5	AFELPPLPYAHDALQPHTSK	Closest homology Superoxide dismutase
		4.8	23	AFELPPLPYAHDALQPHTSK	Superoxide dismutase
		4.8	29	(Q)GQGAVEGELFYKKQYNDSV	Root adhesion protein
		5.0	31	XXXXDLTIAKYDADLFAA	Unknown - PIR_2 Accession E56046
D.	kidney stone Urate calcium oxalate	5.9	40	AVVGGGATLPEKLYG(S)T	Unknown - PIR_2 Accession A56049 - *
		5.1	32	DPGSATDYRTYAAVGSDT	Unknown - PIR_2 Accession C56046
			47.7	GTVTTDGADIVVKTKG	Porin-P; [1] exception residue is at V

D[1].	kidney stone Urate calcium oxalate	5.1	32	DPGSATDYRTYAAVGSDT	Unknown - closest C56046
E.	kidney stone Urate calcium oxalate	6.0	41.5	NDQEQSKGFVED(S)X	Unknown - PIR_2 Accession E56049
		5.2	32.5	KDYELLNVSYD(P)T(R)ELY (Q)	Closest homology D56049 Sulfate binding protein
		4.7	21.5	AFELPPLPYAHDALQPHIS	Superoxide dismutase
F.	Urate calcium oxalate kidney stone	< 4.5	14	TQSLHYSSPRETLTDTIMAA	Unknown - Tyrosine related - PIR_2 Accession F56046
				MLQSQLSQ(T)D-*tryptic digest*	Unknown - PIR_2 Accession D56046
G.	Urate calcium oxalate	5.9	40	AVVGGGATLPEKLYG(S)T	Unknown - *
H.	Urate calcium oxalate	5.9	40	AVVGGGATLPEKLYG(S)T	Unknown - *
I*.	115 mg stone (AcA34)	5.4	40	AVVGGGATLPEKLYS(S)T	Unknown
	kidney stone		27	HTDLSGKVFVFPRESVTDHV	Serum amyloid P-component (SAP)
	UrCaOx		27	HTDLSGKVFVFPRESVTDHV	Serum amyloid p-component (SAP)
				(R)TPEVTXVVVDV	Ig gamma-x+C
				RTPEVTXVVVDV	Ig gamma chain
J.	kidney stone (UrCaOx)	4.8	< 18	ASGNVKFTGEIVQSTXKV	Unknown
K.	kidney stone	5.65	43	MIINHNIAALNTYNRLSAN(N)	Similar to flagellin in B-subtilis
		5.5	33.5	AEIYNKDGNKLDLYGKVDAR	
		4.65	21	MFSIPEQFSSATKTNLEAQF	Unknown - PIR_2 Accession A56046
		< 4.5	14	MLQSQLSQTPRLALADTVI(D)	Unknown - PIR_2 Accession D56046
L.	CaOx, monohydrate = 18% $MgNH_2PO_4$ = 1%; CaOx	5.4	33	AEIYNKDGNKLDLYGKIDG	Outer membrane protein
	dihydrate = 64%; $CaPO_4$ (carbonate) = 6%; $CaPO_4$ (hydroxyl form) = 7%; Protein & bl = 15%	5.5	33.5	AEVYNKDGNKLDLYGKVTA	Outer membrane protein similar to Porin P-differs at 18-19
	kidney stone	5.5 < 4.5	33.5	AEIYNKDGNKLDFYGKV MLQSQLSQTPRLALADTV(I)D SQLSQTPRLALADTV(I)	2:1 lower = upper minus 3 NH terminal residues D56046
		< 4.5	14	TQSLHYSSPRETLTDTIMAA	Unknown - F56046

M.	Urate - CaOx kidney stone	< 4.5		MLSLATNAALSAQS	Unknown (immediate early protein) - PIR_2 Accession I56046
				MFSIPEQFSSATKTNLEAQF	Unknown - PIR_2 Accession A56046
				MLSLHTNAALSAQS	Unknown - PIR_2 Accession I56046
				MLSLHTNAAAL(S)AQS	Unknown - PIR_2 Accession I56046
				(D)VNGGGATLPQ(P)LYQ	Unknown
N.	Calcium Phosphate 20% (carbonate form)		67	XAHKSEVAHRFKDLGE	Serum albumin
	Calcium Phosphate 65% (hydroxyl form)	5.6	43	MTPFMTEDFLLDTEFARRLY	Unknown - PIR_2 Accession A56045
	Protein 15% kidney stone	5.1	32	AEVYNKDGNKLDLYGKVDGI	Outer membrane protein
		5.2	32.5	AEIYNKDGNKVDLYGKAVGL	Outer membrane
			†	All slightly different	Accession D56046
O.	CaOX = 80%; Uric acid = 10% (mono); NH_3 Acid Urate = 3%; Protein = 4% kidney stone	< 4.5	15	(Tryptic Digest): MLQSQLSQ(T)P-NH terminal fragment from stone P#78	
P.	CaOX = 18%; CaOX = 64%; (mono) (dihydrate)			XDVTGAGASFP	
	$MgNH_4PO_4 \cdot 6H_2O$ = 1%; $Ca_{10}PO_4$ = 6%	5.2	33.5	AEIYNKDGNKLDLYGKIDGL	Outer membrane protein
	$Ca_{10}(PO_4)_6$ = 7%; Protein = 4%		53.3	AVINTNNLLSLTTQINNLNK	
	kidney stone	< 4.5	14	TQSLHYSSPRETLTDTIMAA	PIR_2 Accession F56046
		< 4.5	15	MLQSQLSQTPRLALADTVID	PIR_2 Accession D56046
Q.	CaO_X = 2% (mono); Uric acid = 95%; Protein = 3%	6.6	47.7	XXVT(T)DGADIVVKTK	Porin-P
				DVNGGGATLPQPLYQ	Unknown same as M#49 Accession A56045
		5.4	40	AVVGGGATLPEKLYGST	Protein 3 - PIR_2 A56049
		4.8	29	(Q)GQGAVEGELFYKKQYNDSV	Root adhesion protein B50H100 Same as 6H61#8H69

		< 4.5	15	MLQSQLSQTPRLALADTVID	(K5#46; #45 x-59; e#78; P6 - PIR2 Accession D56046
R.	CaOx (mono) = 83%; $CaPO_4$ (carbonate form) = 3%	5.5	33.5	AE(M)YNKDGNKVDIYX KVD MLXELEXAL	
	$CaPO_4$ (hydroxyl) = 12%	4.9	30	AEVYNKDGNKDVYGKVD MLXELE(K)AL	
	Protein = 2%	4.7	22	MLTELEKALNSIIDVYHKYS	Calcium binding
	kidney stone	< 4.0	10.5	MLXELEKALNXIID	
				ML(T)ELEKALNXIIDVYHKY	Calcium binding
S.	Bladder stone $MgNH_4PO_4 \cdot 6H_2O$ (struvite) = 50%	5.5	33.5	AEMYNKDGNKVDIYXKVD ML(T)ELEKALNS	
	$Ca_{10}PO_4(3OH)6OH_2O$ = 38% (carbonate apatite)	4.9	30	(1) AEVYNKDGNK(L)DVYGKVD (2) MLTELEKALNSIIXVY	Ratio of (1) to (2)-3:2
	$NH_4H \cdot C5H_2O_3N_4 \cdot H_2O$ = 7%; Protein = 5%	4.75	22	MLTELEKALNSIIDVYHKYS	Calcium binding
		< 4.0	10.5	MLTELEKALNSIIDVYHKYS	
		5		(1)ML(T)ELEKALN(S)IIDV	Calcium binding
				(2)XKMXQXERNIE	MRP-14
		6		MLXELEKALN(S)IIDVY	
T.	Bladder stone	5.4	40	AVVGGGATLPEKLYGST	Unknown *
			43.5	XXVT(T)DGADIVVKTK	Porin - P
	CaOX(monohydrate) = 2%	< 4.5	15	MLQSQLSQTPRLALADTVID	Unknown D56046
	Uric acid = 95%	4.8	29	(Q)GQGAVEGELFKKQYNDS	Root adhesion
	Protein = 3%			DVNGGGATLPEKKYGST	Unknown
U.	Gallbladder stone	5.4	40	AVVGGGATLPEKLYGST	*
	Cholesterol = 93%; Calcium bilirubinate = 4%; Mixed bile pigments = 3%; (Fraction II from AcA44 column)				

I*: contained serum amyloid p-component (SAP) and was obtained from a patient with Alzheimer's disease.

†: Blocked.

‡: The Accession numbers, asterisk and PIR2 indicate NH-terminal sequences of new protein fragments which we have entered in the databank (Protein Information Resource, National Biomedical Research Foundation, Washington, DC).

cell membranes and a few from bacterial cells. These findings are consistent with the presence of bacterial DNA in cholesterol gallstones [32] and the selectivity and diversity of matrix proteins. They also provide some evidence for the crystal injury of tubular epithelial cells early in stone formation [15].

A different matrix protein profile recently obtained by SDS gel electrophoresis of the pooled EDTA extracts of five types of kidney stones features albumin as the major component which is proposed as the protein that binds most other proteins to form the matrix of all stones [7]. This is a startling proposal which has the following limitations: (1) The authors, in order to gather enough material to study, pooled the stone extracts, thus excluding the possibility of detecting individual variations in composition; (2) SDS gel electrophoresis may have failed to detect differences in protein composition which could have been revealed by 2-D electrophoresis. This technique would probably have resolved the large protein component migrating as 67 kD on SDS gel electrophoresis; (3) No mention is made of the residue after extraction; (4) Proteins considered by other investigators as major components of matrix are not reported.

We can summarize the present state of knowledge of stone matrix proteins and "look into the seeds of time and say which grain will grow and which will not" [27] as follows: (**1**) The incorporation of proteins into the matrix is selective: not all urinary proteins are present nor are they democratically represented. (**2**) Considerable evidence sustains the diversity of protein composition of stones of different types. (**3**) Stones, blood and tears appear to have special affinity. (**4**) Some proteins are shared by many stones but none is shared by all individual stones. (**5**) The presence of cell wall proteins in stones is consistent with tubular wall injury as crystals navigate the narrows of renal tubules or sink into the walls [15]. (**6**) The protein volume expansion induced by calcium binding may further impede the navigation of incipient stones. (**7**) The role of vitamin K in γ-carboxyglutamic acid formation and calcium binding, and its inhibition by warfarin lead to interesting theoretical and therapeutic considerations. (**8**) The expanded development of antisera may serve to visualize the distribution of specific proteins on the cut surface of the stone and to localize them in the renal tubule. (**9**) Local conditions, such as, urine pH, specific ion concentration, degree of hydration, and flow velocity may be important factors in stone formation, affecting the solubility and binding affinity of some proteins present in stones. (**10**) Some stone proteins may be intrinsically different through amino acid substitution, abnormal GLA formation, carboxylation or phosphorylation.

Thus, the confluence of supersaturation with one or more favorable local conditions including eclectic protein participation, cell wall injury and phagocytosis may result in the formation of a stone.

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Discussion with Reviewers

P.K. Grover: Though I agree that identification of matrix proteins is essential to define their role in stone genesis, can the authors discuss or speculate as to why these proteins are there?

Authors: Before the question concerning the role of proteins (matrix) in stone formation can be answered,

proteins in stones should be extracted and characterized.

The method of extraction has been reported in previous publications and it results in extensive recovery, so only a small portion of organic material remains that can be measured as protein. These extracts are concentrated by lyophilization or centricon centrifugation prior to isoelectric focusing and, if adequate, 2-D electrophoresis. This method, besides its resolving power, allows microsequencing of N-terminal amino acids or digestion of the polypeptide if the N-terminal is blocked. Proteins are finely characterized including isoelectric point and approximate molecular weight in a process which opens the possibilities of cloning and antisera production.

The future of this approach appears brighter with the development of electrospray and laser desorption mass spectrometry combined with computer generated access to massive data and 2-D reproducible electrophoresis [39]. Such advances will be necessary to characterize the proteome; study of urinary and stone proteins could be beneficiaries. However, this achievement will not provide knowledge of single protein function in urine, stones or cells but will expand the challenge to explain the role of the organic phase in stone formation.

Difficulties include incomplete knowledge of the interactions between proteins and Ca [40, 44], the alterations in protein conformation and function brought about by variable environments, and the organization of proteins into the matrix. Experiments have been designed to dissect these problems and investigate them in simplified systems with interesting results but incomplete explanations. Increased complexity of experimental conditions, such as reported by Lieske *et al.* [42, 43] and by Hammes *et al.* [41], whereby characterized chemical entities may be tested in cell cultures, should expand the knowledge of the process of stone formation. The recent development of a genetic model of urolithiasis in the rat may link the foreseen accomplishments in protein isolation and characterization with the genetics of the rat model, providing the means to study the proteins and gene(s) involved and their role in stone formation [38].

B. Hess: How do the authors explain why proteins originating from cell membranes exclusively are present in stones containing struvite or carbonate apatite, i.e., in stones formed by infection with urea-splitting bacteria?

Authors: The most important urea splitting organisms are proteus, micrococcus urea, and aerobacter. Some strains of pseudomonas, klebsiella, providencia, serratia and staphylococcus are less frequent offenders. Certain strains of escherichia can form traces of ammonia.

We found one sequence with partial homology with Porin P in a stone which contained 1% struvite. The same sequence was in a stone with no struvite, only calcium phosphate (in carbonate and in hydroxyl form). We think it likely that struvite stones acquire membrane proteins through intimate cell contacts and are reminded that stones, including struvite stones, are not pure.

K. Kohri: The authors identified many kinds of proteins as stone matrix substances. However, the effect of these proteins on urinary stone formation is not known. I think that these proteins are mostly derived from miscellaneous substances, such bacterial structure materials, substances adhered to stone surface, and urinary excretion components. The authors have to describe the characters and the actions of the identified matrix proteins.

Also, we do not know what proteins are important for stone formation, because the matrix proteins are different in the same stone mineral components.

Authors: We have determined the N-terminal sequences of a number of stones, not just ones given in Table 1. We have found MRP-14, calmodulin, serum amyloid P-component, albumin, cal-protectin-like protein and several with homology to thrombospondin, a widely distributed protein. Upon submission to the data bank, the fourteen new sequences had no homologies with known proteins. Their accession numbers are listed in Table 1.

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