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Invited review

Glycosaminoglycans, proteins, and stone formation: adult themes and child's play

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Abstract. The relative infrequency of renal stones in children is probably the main reason for the paucity of literature devoted to the study of urolithiasis in pediatric patients. Nonetheless, when pediatricians do address the issue, the contents of their papers reflect those prevalent in the adult literature; with one notable exception. Papers dealing with the potential role of urinary macromolecules in pediatric stone disease are very scarce indeed; to my knowledge, only four have been published in the English literature in the last 15 years. One of these is to be found in this issue and, like the remaining three, it compares the urinary excretion of glycosaminoglycans in healthy children and those with stones. This article briefly reviews the history of the association of urinary macromolecules, particularly glycosaminoglycans and proteins, with calcium oxalate urolithiasis, and discusses in more detail the published experimental evidence for their fulfilling a determinant role in stone formation.

Key words: Glycosaminoglycans – Proteins – Stone formation

Visiting the sins of the fathers

The literature on human kidney stones is probably much like that devoted to any other human pathology, abounding in clinical controversy, experimental contradiction, and scientific polemic. However, on one point there seems to be no argument: in comparison to their seniors, kidney stones seldom occur in children. This rarity undoubtedly accounts, at least in part, for the scarcity of papers related to pediatric stone disease. A cursory electronic scan of the urolithiasis literature between 1991 and 1995 reveals that of a total of 553 papers, only 46 were specifically concerned with children – marginally fewer than those dealing with urinary stones in a remarkable assortment of animals, ranging from hens, dalmations, goats, sheep, horses, monkeys, and cats, through to, of all things, the green iguana!

Nonetheless, the leitmotivs contained in papers about stones in childhood tend to mirror the recurring themes that continue to appear, with almost predictable regularity, in the adult literature. This habit seems to have persisted to the present time. Thus, recent pediatric stone papers comprise, in addition to the inevitable case reports, papers describing the epidemiology of stone disease [e.g., 1–3], findings of retrospective case note reviews [e.g., 4–6], or results of the performance of metabolic studies in juvenile stone formers [e.g., 7–10] or normal controls [11], in which were determined the urinary excretion of what are popularly referred to as promoters (calcium, oxalate, uric acid) and inhibitors (citrate, pyrophosphate, magnesium) of stone formation. Of the metabolic factors presumed to be important in stone formation, hypercalciuria has commanded more than its share of attention [e.g., 8, 12, 13] – as it has always done in adults, although probably with questionable justification [14]. Furthermore, the current predominance of studies evaluating extracorporeal shock wave lithotripsy (ESWL), now so obvious in the adult stone literature, is also becoming distressingly evident in pediatric papers [e.g., 15, 16]; distressing, not because such essential studies are being performed, but because the widespread use and success of ESWL have created the unfortunate illusion that the stone problem has been solved, and we can all go home and forget about basic metabolic research [17].

It might seem then that children are, at least as far as the urolithiasis literature is concerned, just little adults. But are they? Unfortunately, we do not know, because there is one glaring discrepancy between the adult and pediatric stone literature: virtually all the published basic research on kidney stone formation has been performed in adults. This is hardly surprising, since children with stones are unusual – unlike their parents, who form stones with convenient frequency, can consent to participation in research, and, most attractive of all, excrete gallons of urine just begging to be studied. Moreover, where basic metabolic information has been sought, it has been concerned primarily with the inorganic constituents of urine – particularly calcium. Consequently, during the last 15 years only a handful of studies have addressed the possible role of urinary macro-

molecules in the formation of stones in children, and these have been concerned with the excretion of *total* urinary glycosaminoglycans (GAGs) in stone-forming children and healthy controls [18–20]. Contained in this issue is another comparative study by Harangi et al. [21], which differs in one important respect from its predecessors: these authors compared the excretion of *individual* GAGs in their pediatric subjects. This distinction is crucial, because, as will become evident in the ensuing discussion, recent research in adults has demonstrated that it is no longer possible to draw conclusions about the possible influence of macromolecules in stone formation simply by studying them as a single, combined whole. Human urine fairly bristles with an enormous range of macromolecules, each of which will bring to bear its own individual influences in the formation of stones, and it is these that form the basis of this review.

Kidney stones: more than meet the eye

All kidney stones contain macromolecules. These constitute what has come to be known as the *organic matrix*, by analogy with their counterparts in salubrious forms of biomineralization, where they fulfil a range of functions ranging from provision of a structural framework to controlling the initiation and cessation of crystal deposition [22]. The organic matrix is interlaced throughout the entire stone structure, occupying far more space than would be suggested by its contribution of only 2.5% to the total weight [23]. For many years, our knowledge of matrix composition was limited largely to a series of papers published by Boyce et al. in the 1950s and 1960s. Boyce [24] reported that the organic component of the EDTA-soluble portion of matrix consisted of 64% protein, 9.6% nonamino sugars, 5% hexosamine as glucosamine, 10% bound water, and the remainder, "inorganic ash." We now know that GAGs, previously more commonly known as mucopolysaccharides, comprise a significant proportion of stone matrix [25]. Rich in saccharides, they probably account for the sugars detected by Boyce. Stones also contain lipids [26], but because their contribution to matrix is minor in comparison with those of proteins and GAGs, inevitably, it is the study of these latter two classes of macromolecules that has dominated research on stone matrix.

Although published some 30 years ago, the quantitative composition of matrix published by Boyce continues to be cited in the stone literature, principally because the investigation of stone matrix is fraught with such immense difficulties that, until relatively recently, few workers possessed the fortitude, the inclination, or the technical means to identify its components, much less attempt to elucidate their possible role in stone pathogenesis. Matrix is obstinately difficult to dissolve: three-quarters of the material released by demineralization of stones by EDTA treatment remains as an undissolved precipitate [27], and this hampered past attempts to identify its component macromolecules. The interpretation of findings was also complicated by the recognition that alterations to the molecular structure of the matrix macromolecules could occur during their initial deposition in the stone structure, as well as during their storage and isolation [28]. These considerations are further compounded by the derivation of matrix components from two different sources – normal macromolecules present in urine, which become embedded inside stone crystals when they first precipitate in the renal collecting system, and others not usually found in urine, but which are released as a result of injury to the urinary tract caused by those crystals or the developing stone, or both [24, 29] – which are chickens? Which are eggs?

Although these difficulties, for many years, severely impeded the molecular characterization of individual matrix components and the investigation of their possible functions in stone pathogenesis, they have, fortunately, now been largely eliminated by the study of mineral *crystals* freshly precipitated from urine [29–31]. Such crystals, which are the precursors of stones, contain only those macromolecules normally present in urine, and their study eliminates any confounding involvement of macromolecules released as a result of any injurious effects of the stone itself. Progress has also been accelerated by the development and widespread availability of modern physical, biochemical, and immunological techniques, which have enabled the positive identification of a number of specific macromolecular matrix components. This represents an enormous advance in the study of matrix macromolecules, for only by knowing their individual identities will it be possible to deduce their functions, if at all, in stone pathogenesis.

Molecules in search of a function

As adults, we all face the daily threat of kidney stones, for the simple reason that our urine is supersaturated with calcium oxalate (CaOx) under everyday conditions [32]. It is no surprise then that our urines contain crystals of CaOx, the major component of most renal calculi, from time to time. But we don't all form stones. In fact, approximately 90% of us will never succumb to the disease. This immunity is generally attributed to a lack in our urine of substances that promote the nucleation, growth, or aggregation of CaOx crystals, or alternatively, an abundance of molecules that inhibit these processes. The urines of stone formers, however, are supposedly rich in promoters, or deficient in inhibitors, thus explaining their tendency to excrete CaOx crystals in greater quantities and clustered into larger aggregates than those in the urine of healthy control subjects [33, 34]. However, while the roles of urinary calcium, oxalate, and uric acid as promoters of CaOx crystallization, and citrate and magnesium as inhibitors, are widely acknowledged, the functions of urinary macromolecules have, for the reasons outlined above, remained somewhat of a mystery. The simple fact that they are *there* suggests that they must fulfil some function in stone formation. And it is imperative that we identify that function, since it is possible that urinary macromolecules may perhaps direct the course of stone pathogenesis as modulators of crystallization: they might induce CaOx crystal nucleation and promote the subsequent growth and/or aggregation of those crystals; actively bind to the surfaces of preformed crystals and retard (but not completely prevent) further solute deposition or crystal aggregation; or bind to the

crystal surfaces yet have no influence on further crystallization events. In fact, they probably do all of these, depending upon ambient conditions [35], and ultrastructural evidence indicates clearly that macromolecules are involved in both the crystal nucleation and enlargement phases of stone pathogenesis [36].

However, the mere presence of macromolecules in stone matrix tells us nothing about the nature of that involvement, because paradoxically, irrespective of whether urinary macromolecules act as passive adsorbants, promoters, or inhibitors, *they will still be present in the final stone*. Despite years of research designed to unravel the role of macromolecules in stone pathogenesis, the area continues to excite debate and generate confusion: we still do not know, with any degree of certainty, why macromolecules are in stones, how they came to be there, what effects their inclusion into the structure may have had, and whether their presence reflects cause or effect of the stone. What we *do* know, however, is that generalization is impossible. Each urinary macromolecule will have its own specific effects, and to discover what they are, it is first necessary to determine which ones are actually present in stones and then to study those effects in detail.

Glycosaminoglycans

Experimental findings

A possible role for GAGs, or mucopolysaccharides as they were then known, in CaOx stone formation became apparent almost 30 years ago when a group of chemists showed that heparin, chondroitin sulfate (ChS), and hyaluronic acid (HA) affected the precipitation of CaOx [37]. It was not long before the significance of the findings was realized by workers in the stone field, and GAGs soon came to be regarded as potential inhibitors of stone formation, if not naturally, then at least as possible therapeutic agents. Moreover, their study proved remarkably easy: unlike proteins, there are few GAGs, and a number of these are freely available from commercial sources. A veritable rash of studies soon followed the revelations of Crawford et al [37]. Most of these have consisted of testing the effects of a pure GAG, usually ChS or heparin, on various aspects of CaOx crystallization in inorganic milieux. A list of the findings of these studies, which is by no means complete, is shown in Table 1.

Table 1. Effects of glycosaminoglycans (GAGs) on calcium oxalate (CaOx) crystallization in inorganic reaction media

GAG	Effect	Reference
ChS	Inhibits "growth and aggregation"	Robertson et al. 1973 [38]
	Inhibits nucleation	Pak et al. 1979 [39]
	Inhibits growth, aggregation	Ryall et al. 1981 [40]
	Inhibits growth	Fellström et al. 1986 [41]
	Inhibits growth, agglomeration, mass deposition	Robertson, Scurr, 1986 [42]
	Promotes nucleation	Robertson and Scurr 1986 [42]
	Inhibits agglomeration	Scurr and Robertson 1986 [43]
	Inhibits nucleation; promotes growth rate, suspension density	Kohri et al. 1989 [44]
Heparin	Inhibits "growth and aggregation"	Robertson et al. 1973 [38]
	Inhibits nucleation	Pak et al. 1979 [39]
	Inhibits growth, aggregation	Ryall et al. 1981 [40]
	Inhibits growth	Ryall et al. 1981 [40]
	Inhibits growth, agglomeration, mass deposition	Robertson and Scurr 1986 [42]
	Inhibits agglomeration	Scurr and Robertson 1986 [43]
	Inhibits nucleation, mass deposition	Kohri et al. 1989 [44]
SPP	Inhibits growth, agglomeration	Norman et al. 1984 [45]
	Inhibits growth	Martin et al. 1984 [46]
	Inhibits growth, promotes agglomeration	Grases et al. 1989 [47]
	Inhibits growth	Osswald et al. 1989 [48]
	Inhibits growth	Suzuki et al. 1989 [49]
	Inhibits growth, agglomeration	Cao et al. 1992 [50]
G871/872	Inhibits growth, agglomeration	Cao et al. 1992 [50]
Urinary GAGs	Inhibit "growth and aggregation"	Bowyer et al. 1979 [51]
HS	Inhibits "growth and aggregation" (not measured directly; inferred	Yamaguchi et al. 1993 [52]

ChS, Chondroitin sulfate; SPP, sodium pentosan polysulfate; HS, heparan sulfate

In addition to ChS and heparin, several synthetic GAGs [sodium pentosan polysulfate (SPP), G871, G872] have been tested to assess their potential usefulness as treatments for stone prevention. With only two exceptions, all the GAGs tested inhibited various aspects of CaOx crystallization. Those exceptions included the demonstration by Robertson and Scurr [42] that ChS promotes CaOx crystal nucleation in an inorganic, artificial urine, and a report by Sallis and Lumley [53] that a GAG fraction isolated from human urine did not affect crystal growth. On balance, therefore, the body of evidence reported in the stone literature demonstrates that GAGs are capable of retarding CaOx crystallization – at least in inorganic media. But can the results be used to draw conclusions about the role of GAGs in stone formation?

Apparently not. The studies listed in Table 1 have been largely responsible for the widely held belief that GAGs are responsible in vivo for contributing to the prevention of calculi in healthy people; but this belief is, in reality, nothing more than misplaced trust in data interpreted beyond the limits of experimental design. First, heparin has been used as the basis for proposing a role for GAGs in stone formation, yet heparin is not present in human urine [54, 55]. Second, to suppose that the action of a GAG, or of any inhibitor in an inorganic reaction medium, can be extrapolated to conclusions about its possible effects under

Table 2. Effects of GAGs on CaOx crystallization in urine and in vivo

GAG	Effect	Reference
ChS	No effect on CaOx deposition in rat kidneys	Osswald et al. 1989 [48]
	No effect on metastable limit, mass deposition, aggregation	Ryall et al. 1991 [57]
	Promotes large calculus formation in rats	Michelacci et al. 1992 [58]
	Promotes nucleation (only from stone formers)	Shum and Gohel 1993 [59]
HS	Enhances nucleation, inhibits growth	Shum and Gohel 1993 [59]
	No effect on metastable limit, mass deposition; inhibits aggregation	Suzuki and Ryall 1996 [60]
SPP	Inhibits growth	Suzuki et al. 1989 [49]
	Inhibits deposition in rats	Suzuki et al. 1989 [49]
	Inhibits deposition in rats	Osswald et al. 1989 [48]
	Inhibits deposition in rats	Miyazawa et al. 1989 [61]

physiological conditions is, indeed, a monumental leap of faith. Literally countless studies (some of them my own) measuring the inhibitory effects of many substances on CaOx crystallization in artifical, inorganic solutions have rewarded us with a great deal of information about the basic inhibitory mechanisms of a variety of natural and synthetic substances. However, although indispensable to the progess of research on the role of inhibitors in stone pathogenesis, their findings simply cannot be presumed to reflect how those inhibitors will act under physiological conditions. Yet the results of such studies continue to be cited as evidence that agents capable of inhibiting CaOx crystallization in a watery medium fulfil some critical role in the urinary soup, and thereby, in stone formation, despite the fact that caution about the practice has been advocated for many years [56] – and caution is clearly warranted.

Table 2 presents the results of studies designed to test the effects of ChS, SPP, and heparan sulfate (HS) on CaOx crystallization in urine, or in animal models in vivo. It is immediately apparent that the inhibitory effects of ChS, so obvious in inorganic solutions, are no longer evident. In fact, the work of Michelacci et al. [58] would suggest that a high urinary concentration of ChS actually encourages stone formation! Both ChS (although only that derived from stone formers) and HS have been reported to encourage the nucleation of CaOx crystals in urine [59], and it may be argued that this could be an advantage, since promotion of nucleation causes precipitation of smaller, more numerous crystals that can be harmlessly flushed from the urinary tract. However, those effects were observed after freezing the urine specimens to induce crystal precipitation, a process that will inevitably alter the solubility of component macromolecules and may therefore affect their activity. At 37 °C [57] ChS has no effect on the amount of oxalate required to induce CaOx crystallization in urine, or on the mass deposition or degree of aggregation of the crystals. However, this work was carried out in ultrafiltered urine which lacked its normal macromolecular complement, and although the results suggest that ChS will have no effect on CaOx crystallization in urine in vivo, we cannot rule out the possibility, however slight, that other urinary macromolecules could potentiate any small effects that ChS might have.

Of greater interest is the observation that HS enhances CaOx crystal nucleation and inhibits growth in frozen urine [59], but more particularly, potently inhibits aggregation in undiluted, ultrafiltered urine at 37 °C [60]. Crystal aggregation is commonly recognized as a critical step in stone formation, since it is the only mechanism by which free crystalline particles can attain a size sufficient to occlude a renal tubule in the time taken for urine to travel through the nephron. Although the synthetic GAG, SPP, is obviously not naturally present in urine, its consistent ability to reduce CaOx crystal growth or deposition, both in vivo and in vitro, certainly suggests that it could, nonetheless, find application in the treatment of stones in recalcitrant stone formers.

I have purposely delayed discussion of studies carried out to determine which GAGs are actually present in kidney stones, because to have revealed the results before reviewing the findings of the inhibitory experiments listed in Tables 1 and 2, would probably have left readers wondering why most of them were ever undertaken at all. The truth is that many of them predate the information about GAGs in stones; but then again, many of them do not.

GAGs in stones and crystals

Although the early work of Boyce [24] suggested that stones contained GAGs, their presence has been confirmed only in the last 10 years [25, 52, 62]. In fact, it can be calculated that GAGs may account for up to 20% of matrix macromolecules, having been shown to comprise between 0.19% and 0.58% of the entire stone weight [25], and this certainly suggests that they may influence the course of stone formation. HS and, to a lesser extent, HA account for all the GAGs in CaOx stones [25, 52, 62]. ChS cannot be detected in CaOx stones, although small quantities of it are reportedly present in magnesium ammonium phosphate and apatite stones [25]. Perhaps the most puzzling feature of these findings, in view of its high concentration in urine, is the complete absence of ChS from stones. ChS is easily the most abundant of the GAGs in normal human urine: these comprise approximately 60% ChS, 15%–20% HS, 11% low-sulfated ChS, and 4%–10% HA [54, 55]. Other GAGs, namely keratan sulfate and dermatan sulfate, are generally regarded as being present in trace quantities. Harangi et al. [21] reported their relative contributions to the GAG fraction of children's urine as 7.3% and 3.3%, respectively. The mismatch in GAG pattern between stones and urine suggests that the incorporation of GAGs into stones is selective, with HS and HA being included in the structure at the expense of the more plentiful ChS. This selectivity is also evident in studies of GAG inclusion into CaOx crystals precipitated from urine: such crystals, like stones, contain only HS [52, 63]. In fact ChS can be detected in CaOx urinary crystals only in the complete absence of HS [63], indicating that the two probably compete for the same

Table 3. Excretion of GAGs in stone formers (SF) and normal subjects (N)

Finding	Comment	Reference
SF < N		Roberston et al. 1978 [64]
SF = N		Samuell 1981 [65]
SF = N		Caudarella et al. 1983 [66]
SF = N		Ryall and Marshall [67]
SF = N	children	Baggio et al. 1983 [18]
SF = N		Ryall et al. 1984 [68]
SF = N	male > female	Hesse et al. 1986 [69]
SF < N		Baggio et al. 1987 [70]
SF = N	SF > N in type I AH	Hwang et al. 1988 [71]
SF < N	recurrent SF only	Nikkilä 1989 [72]
SF = N	unselected SF	Nikkilä 1989 [72]
SF < N	recurrent SF	Nikkilä 1989 [72]
SF = N	children	Lama et al. 1990 [20]
SF < N	children > adults	Michelacci et al. 1989 [19]
SF < N		Nesse et al. 1992 [73]
SF = N		Akinci et al. 1992 [7]
SF = N	children: differences in some individuals GAGs	Harangi et al. 1996 [21]

AH, Absorptive hypercalciuria

binding sites on the CaOx crystal surface. The most important ramification of these findings is that, with the benefit of hindsight, many of the studies examining the effects on CaOx crystallization of several GAGs, particularly ChS and heparin, now seem illogical. So too does the measurement of total urinary GAG excretion as a diagnostic marker of stone formation.

Urinary GAG excretion

The flurry of experimental activity following the discovery of Crawford et al. [37], as well as the revelation that several GAGs could inhibit various aspects of CaOx crystallization, prompted people to test whether differences in the excretion of urinary GAGs could account for the occurrence of stone disease. As with most urinary parameters proposed as diagnostic markers for urolithiasis, the results are disappointingly inconclusive. Table 3 lists a large number of studies, again not exhaustive, in which the urinary excretion of GAGs in stone formers was compared with that in healthy controls. Although a number of them report a deficiency in the urine of stone patients, an even greater number find no such distinction. Several studies on GAG excretion in children are presented in Table 3 [18–20], including that of Harangi et al. [21] in this volume. With only one exception [19], no differences between juvenile stone formers and healthy children could be discerned, although Harangi et al. [21] found that patients with renal hypercalciuria excreted significantly less keratan sulfate than did the normal children or stone formers with absorptive hypercalciuria. However, their output of dermatan sulfate was considerably greater than that of the other patients and healthy controls, so that, overall, the total GAG excretion was the same in all groups. The physiological significance of this difference in pattern of GAG excretion can only be guessed at, but in view of the known selectivity of GAG incorporation into CaOx crystals and stones, and the differences between the inhibitory effects of ChS and HS, the findings are sufficiently interesting to tempt us to discover more about urinary GAGs and their potential effects in stone formation. There is some evidence that keratan sulfate may be present in stones of various mineral types [62], although its inhibitory effect on CaOx crystallization has never, to my knowledge, been measured; neither has that of dermatan sulfate.

It is therefore obvious that a reduced excretion of total urinary GAGs is not a consistent feature of kidney stone disease – either in adults or in children, which is hardly surprising when we consider the fact that ChS, which accounts for more than half of the GAGs in urine, is not in stones or crystals, and does not inhibit CaOx crystallization in urine. On the basis of available information, there may be some merit in assessing the urinary output of HS as a factor contributing to stone formation, but there is little, if any, evidence to support a function for ChS in the genesis of the disease. The routine measurement of *total* GAG excretion in the investigation of stone formers is therefore indeed "irrational" [7] and should be discontinued.

Proteins

Although it has been known for many years that proteins constitute approximately 60% of the EDTA-soluble organic matrix of stones [24], most proteins now known to be present in stones defied identification until only recently. Human serum albumin and α- and β-globulins were specifically detected by Boyce et al. [27] in 1962. Boyce and Garvey [23] also reported the presence of Tamm-Horsfall glycoprotein (THG), which they called "uromucoid," but the finding was called into doubt by the absence from matrix of sialic acid, which forms the side chains of THG [74]. This apparent inconsistency was, for a number of years, neatly circumvented by hypothesizing that the incorporation of THG into matrix was accompanied by desialylation, a notion that was rapidly despatched when later studies by Melick et al. [75] demonstrated the unequivocal presence of THG in stones. The presence of THG in stones of various composition led these authors to surmise that THG must be passively absorbed into the structure, a speculation which, as we shall see, is supported by recent experimental evidence. The remaining proteins found in stone matrix have all been identified within the last 10 years – a welcome consequence of technical advances in molecular biology and protein chemistry, and the widespread commercial availability of specific antibodies.

The number of individual proteins detected in stones increases each year, and those whose presence has been unequivocally demonstrated are listed in Table 4. However, as discussed earlier, the mere presence of a protein in stone matrix does not automatically bestow upon it a defined function in the stone's formation, and at the present time little can be said about the possible roles of most of the proteins listed in Table 4. However, recent years have seen rapid progress in the study of several stone proteins, and the remainder of this section will be devoted to a discussion of what is currently known about them.

Table 4. Proteins detected in CaOx kidney stones

Protein	Reference
Human serum albumin	Boyce et al. 1962 [27]
α and γ-globulins	Boyce et al. 1962 [27]
Tamm-Horsfall glycoprotein	Melick et al. 1980 [75]
Nephrocalcin	Nakagawa et al. 1987 [76]
α-1-Microglobulin	Morse and Resnick 1988 [30]
Hemoglobin	Petersen et al. 1989 [77]
Neutrophil elastase	Petersen et al. 1989 [77]
Transferrin	Fraij 1989 [78]
Uropontin (osteopontin	Shiraga et al. 1992 [79]
α-1-Antitrypsin	Umekawa et al. 1993 [80]
CD59 protein (protectin)	Binette and Binette 1993 [81]
Superoxide dismutase	Binette and Binette 1994 [82]
β_2-Microglobulin	Dussol et al. 1995 [83]
α_1-Acid glycoprotein	Dussol et al. 1995 [83]
Apolipoprotein A1	Dussol et al. 1995 [83]
Retinol-binding protein	Dussol et al. 1995 [83]
Renal lithostathine	Dussol et al. 1995 [83]
Urinary prothrombin fragment 1	Stapleton et al. 1996 [84]
Inter-α-trypsin inhibitor	Unpublished findings from the author's laboratory

Tamm-Horsfall glycoprotein

THG has been the subject of intense investigation for many years and it would be impossible to review all that has been published about it: so much is known about THG that it has been the subject of at least two recent reviews [85, 86]. The protein has long been a puzzle. Although it is the most abundant protein in human urine and the predominant component of renal casts, its precise function remains unknown. It is found in the kidneys of all placental invertebrates [87], where it is localized to the luminal aspect of the epithelial cells of the distal convoluted tubules [88] and distributed throughout the epithelial cells of the thick ascending limb of the loops of Henlé [89]. THG is excreted in large quantities, 20–200 mg each day [90], so it is somewhat surprising that it is present in stone matrix in only trace amounts and is completely absent from CaOx crystals precipitated from whole human urine [29]. These observations suggest that it is incapable of binding irreversibly to CaOx crystals.

THG has a monomeric molecular mass of approximately 80 kiloDaltons (kDa), but exists in urine as polymeric monsters with molecular masses of several millions, which are easily visible to the naked eye. This curious property of the molecule is undoubtedly at least partly responsible for the frustrating ability of THG to exhibit a range of effects on CaOx crystallization, depending upon ambient conditions and methodology. In synthetic inorganic media, THG has been reported to inhibit deposition of CaOx [41, 43, 91, 92]; but in urine it is capable of promoting both CaOx [93, 94] and calcium phosphate [95] precipitation. Despite this, it is a potent inhibitor of CaOx crystal aggregation in undiluted, ultrafiltered urine [57, 96]. Furthermore, its effect on crystal aggregation apparently results from steric hindrance [57], rather than binding of the protein to the crystal surface, which is the most commonly accepted explanation for an inhibitor's effect.

The intriguing and contradictory properties of THG have contributed greatly to the general confusion surrounding its function in stone formation, and this is reflected in the fact that the excretion of THG does not seem to differ between stone formers and healthy subjects [65, 97, 98]. One notable exception to this is the study of Baggio et al. [18] which, in addition to urinary GAG excretion, compared that of THG in pediatric stone patients and healthy children. They found that the daily excretion of THG was raised in the stone-forming group; however, there was no difference in urinary concentration.

Years of study of THG have therefore yielded less bounty than we might have hoped. We know that the protein can act as both a promoter and an inhibitor of CaOx crystallization processes in experimental systems, but what it does in vivo remains a mystery. Of one thing though we can be reasonably confident – it is certainly not the only urinary protein likely to be involved.

Nephrocalcin

Nephrocalcin (NC) enjoys the distinction of being, after THG, the most extensively studied urinary protein in the stone literature. The first report of the protein came from Nakagawa et al. in 1978 [99], who first described it as an unidentified acidic glycopeptide, and then for a time as a glycoprotein inhibitor of CaOx crystal growth [100–105]. Its present name, NC, was conferred 9 years after its initial discovery [76], by analogy with the bone protein osteocalcin. Like THG, NC has features which do not endear it to scientific investigators. Its molecular weight varies widely, reportedly as a result of polymerization, with the molecular weights of the monomer, dimer, trimer, and tetramer being 14–15, 23–30, 45–48 and 60–68 kDa, respectively [76, 100, 104, 106]. Histochemical studies have mapped the location of NC to the epithelium of the proximal tubules and thick ascending limb of the loops of Henlé, in both human and murine kidneys [107]. Originally isolated from human urine, the protein has been isolated from a number of sources, including rat kidney and urine, the culture medium of human kidney cell lines [100, 108], kidney stones [76], and the urine of patients with renal stones [76, 103, 104] and renal cell carcinoma [109].

NC has been reported to occur in urine at concentrations ranging from 5 mg/l [101] to 16 mg/l [110], and to contain between two and three residues of γ-carboxyglutamic acid (Gla) per molecule [91, 100, 101, 103–105], deficiencies in which have been cited as the reason for the protein's reduced inhibitory activity in stone formers [111], and an explanation for the occurrence of stones [103] in those individuals. However, despite many years of investigation and claims that NC accounts for approximately 90% of the total inhibitory activity of urine [100–102], a figure which was recently modified to 16% [112], it must be remembered that the protein's effects have never been assessed in urine. Furthermore, its molecular structure is still unknown; NC has never been sequenced. However, recent evidence suggests that it may possibly be a fragment of the light chain, bikunin, of inter-α-trypsin inhibitor (ITI) [113],

a protein which, as will shortly be seen, has also been implicated in CaOx stone formation. Therefore, although much evidence points to a possible involvement of NC in stone urolithiasis, as it is undoubtedly a potent inhibitor of CaOx crystal growth under inorganic conditions, it is becoming increasingly apparent that this potency is shared with several other urinary proteins, one of which is osteopontin (OP).

Osteopontin (Uropontin)

OP has long been known to be associated with bone mineralization, in which it is assumed to anchor osteoblasts to bone [114]. It is a member of a superfamily of proteins rich in aspartic acid that have been shown to exhibit stereospecific binding to the surfaces of crystals [115], a property which probably accounts for its reported effects on CaOx crystal growth. A possible involvement of OP in stone formation became apparent when Shiraga et al. [79] isolated from human urine a protein which they had previously shown to exhibit maximal inhibition of CaOx crystal growth in an inorganic crystallization system, and which they called uropontin (UP). N-Terminal amino acid sequence analysis [79] demonstrated complete identity of UP with the N-termini of both OP [116] and lactopontin. Moreover, total amino acid analysis, molecular weight estimations, and identical nucleotide sequences of cDNAs encoding OP from human kidney [79] and bone [117] indicate that UP is not a distinct protein, but rather a urinary form of OP. Although acknowledging these facts, Hoyer has advocated retention of the name uropontin to reflect its urinary source and presumed role in the urinary tract, a practice with the potential to cause confusion. Worcester et al. [118], who isolated the protein from cultured mouse kidney cortical cells, preferred the name osteopontin, as did Kohri et al. [119], who cloned and sequenced the cDNA encoding the same urinary protein. These authors also demonstrated, using in situ hybridization, that OP mRNA in the kidneys of rats in which stone formation had been induced by lithogenic drugs increased in proportion to the dose and duration of drug treatment [120]. Although prima facie, these findings could be interpreted as reflecting a cause and effect relationship between OP and CaOx stone formation, the possibility cannot be discounted that the effects resulted from cellular injury caused by action of the CaOx crystals themselves.

OP is widespread in humans, being found on the luminal surfaces of specific epithelial cells in the gastrointestinal tract, gallbladder, pancreas, urinary and reproductive tracts, lung, breast, and salivary and sweat glands [121]. Although this ubiquitous distribution would tend to militate against its fulfilling a specific function in stone formation, the protein's potent effect on CaOx crystallization might, nonetheless, be a useful adjunct in the body's defense against urolithiasis – an example, perhaps, of the right result for the wrong reason. OP is certainly found in CaOx stones and in normal adult urine at a concentration of around 6×10^{-8} mol/l [122], which, assuming a molecular weight of 50 kDa, converts to a value of approximately 3 mg/l. Nevertheless, current evidence connecting OP to stone formation is largely circumstantial. The protein's inhibitory effect on CaOx crystallization in urine has never been examined; nor has its influence on aggregation been determined – in any medium. Therefore, like all the other macromolecules discussed in this review, unequivocal demonstration of a role for OP in the pathogenesis of stones depends upon the generation of further information.

Urinary prothrombin fragment 1

A possible link between urolithiasis and urinary prothrombin fragment 1 (UPTF1) was first suspected when this protein was found to be the principal proteinaceous component of the organic matrix of CaOx crystals freshly precipitated from whole human urine [29]. A glycoprotein with a molecular mass of approximately 31 kDa, UPTF1 was first thought to be a previously undescribed protein, and was initially called crystal matrix protein. However, subsequent N-terminal amino sequence analysis demonstrated identity with human prothrombin [123], and a later study showed conclusively that the protein is the F1 activation peptide of prothrombin [124], thereby establishing, unambiguously, the existence of a link between human blood clotting and urolithiasis. To avoid confusion, the protein is no longer called crystal matrix protein, but UPTF1, to distinguish it from its counterpart in serum and to indicate its urinary origin. The chromatographic properties of the serum form of F1 differ from those of the urinary form [124], which suggests that UPTF1 may be modified in the kidney, or manufactured there, rather than in the liver, where prothrombin is thought to be exclusively synthesized [125].

UPTF1 possesses all the properties that would reasonably be expected of a protein fulfilling a function in stone formation. It is present in kidney stones [84], having been detected in nine of ten stones containing calcium, and in all composed principally of CaOx. Of most significance is that it was absent from two struvite calculi, indicating that its presence in stones is not a consequence of bleeding induced by the stone itself, but the result of selective and avid binding of the protein to the crystalline surface. The protein is found in specifically circumscribed regions of the human nephron, where it was shown, immunohistochemically, to be located in the thick ascending limb of the loops of Henlé and the distal convoluted tubules, including the maculae densae of a subset of nephrons [126]. Limited data also revealed [126] that UPTF1 is present in the kidneys of stones formers in greater quantities than in healthy controls, a finding which suggests that its levels may rise in response to lithogenic conditions. However, of greatest importance is that UPTF1 is a potent inhibitor of both CaOx crystal growth and aggregation in undiluted, ultrafiltered human urine [127], a property that can almost certainly be attributed to its Gla domain. This region of the molecule, close to the N-terminus, contains ten Gla residues – a legacy from its parent prothrombin, whose function in blood coagulation depends absolutely on the integrity of its Gla domain.

There is good evidence, therefore, that UPTF1 may fulfil some directive role in stone formation, but further evidence is required before it will be possible to state with

certainty that variations in the amount of the protein excreted in the urine, or alterations in its molecular structure, predispose some individuals to urolithiasis.

Inter-α-trypsin inhibitor

A possible connection between stones and ITI was first suggested in 1990, when Sørensen et al. [128] isolated from urine a glycoprotein that inhibited the growth of CaOx in an inorganic medium. Although they described it as "unidentified," amino acid sequencing showed that it was related to ITI, a complex Kunitz-type plasma proteinase inhibitor comprising three chains encoded by three different genes on three different chromosomes [129]. ITI has no known function – despite its presence in plasma at a concentration of around 500 mg/l. Three years after the publication of Sørensen's paper, Atmani et al. [130] isolated what appeared to be a similar protein. It had a molecular mass of 35 kDa and also inhibited CaOx crystal growth under similar conditions. Analysis showed that it was a glycoprotein, containing 8.5% carbohydrate, and that it was rich in uronic acid, which suggested that it might be covalently bound to a GAG. They named the protein uronic acid-rich protein, and later that same year demonstrated by Edman degradation that it shared homology with ITI [131]. They also showed that the protein's activity was unaffected by chondroitinase, hyaluronidase, or glycanase treatment, but disappeared after proteinase treatment and that the protein derived from the urine of stone formers had a reduced inhibitory effect on CaOx crystallization in comparison with the protein isolated from healthy subjects [132].

Since that time, the protein has also been shown to be present in the urine of rats [133], in which it exhibits similar characteristics to its human cousin. Like the human protein, the one isolated from rat urine also cross-reacts with an antibody to ITI, which, combined with its N-terminal identity to ITI, suggests that it belongs to the ITI superfamily. These data have been independently corroborated in my own laboratory, where we have isolated from human urine a 35-kDa protein with an N-terminal primary amino acid sequence identical to bikunin, the light chain of ITI, which is covalently bound to the two heavier chains by a ChS moiety [113]. It is likely that the protein isolated by Sørensen et al. [128], the one described by Atmani et al. [130–133], and the 35-kDa protein isolated in my laboratory [113], are one and the same – probably bikunin. They all have similar molecular weights and immunoreact with antibodies to ITI. Most importantly, they all inhibit CaOx crystal deposition in an inorganic crystallization system, which, like the other proteins singled out for attention in this review, seems sufficient justification for adding ITI to the list of urinary proteins with the potential to influence the course of CaOx stone disease. Unfortunately, however, the protein's effects have not yet been tested in urine or in an animal model.

It is therefore obvious that further studies are required in order to ascertain whether bikunin would retain its inhibitory activity under physiological conditions. Results of such investigations may prove even more interesting, in view of the suspicion that NC may also be a relative of ITI: recent findings have suggested its identity to HI-14 [113], a fragment of bikunin known to be present in human urine [134].

Lessons from our juniors?

In reviewing the more recent literature linking urinary macromolecules and CaOx stone formation I have been obliged merely to skim the surface; many more papers have been published than have been mentioned here. Nonetheless, their addition would have done little to clarify the matter. Years of research in adults have identified a confusing array of proteins and GAGs that *may* play some role in the pathogenesis of stones, yet have not to this day demonstrated with absolute certainty that the initiation or progress of the disease is significantly influenced by any one of them. However, it is imperative that the work continues, for amongst all the confusion and doubt it is manifestly obvious that the occurrence of stones relies as much on the enlargement and retention of crystals as it does on their initial precipitation; and these almost certainly are affected by urine's organic constitutents [36].

But where are the children in all this? Although evidence indicates that they exhibit the same urinary inorganic derangements as their seniors, to my knowledge, with the exception of the paper by Harangi et al. [21] in this issue, no study has ever examined in detail individual organic components of children's urines and attempted to relate them to the occurrence of stones; and yet, avoiding stone formation might prove to be simply a matter of child's play and urinary macromolecules the toys. The study of children's urinary macromolecules may therefore be regarded as a tempting, locked chest containing a rich booty: perhaps the time has now come for adults to see if their sons and daughters hold the key.

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