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Analysis of the soluble organic matrix of five morphologically different kidney stones

Evidence for a specific role of albumin in the constitution of the stone protein matrix

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Abstract Our aims were to analyze the protein composition of the organic matrix of urinary stones and to investigate the role of albumin in its constitution. Five different morphological types of stones were studied. Proteins extracted from the stone were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with antibodies to 13 urinary proteins. Nine of the 13 proteins were found in all types of stone: human serum albumin (HSA), α_1-acid glycoprotein (α_1-GP), α_1-microglobulin (α_1-M), immunoglobulins (Igs), apolipoprotein A1 (apo-A1), transferrin (Tr), α_1-antitrypsin (α_1-T), retinol-binding protein (RBP) and renal lithostathine (RL). The β_2-microglobulin (β_2-M) was present only in calcium oxalate and uric acid stones. In contrast, ceruloplasmin, haptoglobin and Tamm-Horsfall protein (THP) were detected in none of them. Because HSA appeared as the major protein component in all stones, we wondered whether it might play a specific role in the constitution of the stone matrix. Association of HSA with urinary proteins that were present in stones was demonstrated by showing that proteins present in the matrix comigrated with HSA on gel filtration, whereas proteins that were absent did not. Moreover, HSA induced the binding of stone matrix proteins to an albumin-specific affinity column. Finally, we evidenced HSA binding to calcium oxalate monohydrate (COM) crystals in a solution similar to urine. It was concluded that (1) only a subset of urinary proteins is present in stone matrix, (2) the same proteins are found in all types of stones, (3) HSA shows significant affinity for several proteins of the matrix, but not for proteins absent from stones and, (4) HSA also displays significant affinity for COM crystals.

Key words Urinary stones · Organic matrix · Albumin
Tamm-Horsfall protein · Renal lithostathine

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Human renal stones are composed of a crystalline phase which, in 80% of cases, is either calcium oxalate (CaOx) or calcium phosphate, and a supporting structure, the "matrix". This matrix, which constitutes on average 1–3% of the stone weight, consists mainly of a mixture of proteins, carbohydrates, glycosaminoglycans, lipids and organic ash [2]. Our knowledge of the components of the matrix is incomplete. Boyce showed that only 25% of lyophilized matrix is readily soluble [4]. Some proteins of the matrix have been characterized in different types of stones, e. g., human serum albumin (HSA) [12], Tamm-Horsfall protein (THP) [12-13] and transferrin (Tr) [12]. Keutel and King isolated matrix substance A but the composition of this glycoprotein is currently not completely known [16]. Nephrocalcin, an inhibitor of CaOx crystal growth, has been evidenced only in the matrix of calcium oxalate monohydrate (COM) stones [22]. However, there is controversy about the presence of THP and HSA since they are not always detected in crystal extract [11]. Indeed no exhaustive study of the protein composition of the matrix has ever been conducted.

Some authors believe that stone matrix formation is the result of the selective incorporation of specific urinary macromolecules into crystals and that this matrix serves as a substrate upon which heterogeneous nucleation of urinary crystals occurs [3, 32]. This is supported by Leal and Finlayson, who have shown that in urine every CaOx crystal can be covered on up to 75% of its surface by a protein layer which can bind other crystals to form an aggregate [20]. Others believe that stone matrix formation is the result of nonspecific adsorption of urinary proteins onto urinary crystals and that it does not act as a nucleating agent [21, 31].

In an effort to gain insight into the mechanism of urinary lithogenesis, we investigated the role of the kidney stone matrix in the formation of kidney stones. Using an immunological approach, we first characterized the protein matrix of five of the most common classes of renal stones: COM (whewellite), calcium oxalate dihydrate (weddellite), anhydrous uric acid and uric acid dihydrate, a mixture of carbapatite and struvite, and cystine. Since the protein composition was highly similar in all these and mainly constituted of HSA in all types of stone, we investigated the possible relationship between these proteins and the crystal- and protein- binding properties of human albumin.

Materials and methods

Materials

Human renal stones. Human renal stones were obtained from the Laboratoire Cristal (Saint-Cloud, France) and were subjected to a three-step analytical procedure: careful morphological examination involving stone dissection, infrared spectroscopy and microchemical analysis followed by classification according to Daudon et al. [9].

Chemicals. Reagent-grade chemicals were used without further purification. All solutions were made up with deionized, distilled water. Ethylenediaminetetraacetic acid (EDTA) was from Boehringer (Mannheim, Germany), and phenylmethylsulfonylfluoride (PMSF) and morpholinoethanesulfonic acid (MES) from Sigma (St. Louis, USA). Di-isopropyl fluorophosphate (DFP) and thiodiglycol were purchased from Aldrich (Milwaukee, USA). Sodium dodecyl sulfate (SDS) and tris-(hydroxymethyl)aminomethane (TRIS) were purchased from Pharmacia (Uppsala, Sweden). Human serum albumin was from Institut Mérieux (Lyon, France).

Antibodies. All antibodies or antisera were obtained from Immunotech (Marseille, France) except for rabbit antisera against human plasma α_1-acid glycoprotein (α_1-GP), HSA and α_1-microglobulin (α_1-M), which were purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands). Rabbit antiserum against human pancreatic lithostathine [26] was prepared as previously described [10]. Western blot experiments were performed at a serum dilution of 1/2000 except for HSA (1/10 000).

Methods

Extraction of proteins from stones. Four grams of each morphological class of urinary stone was washed in 0.15 *M* NaCl with gentle stirring for 48 h. The stones were then ground in a mortar. Calcium was extracted by suspending the powder in 140 ml 0.05 *M* EDTA, pH 8, 1 m*M* PMSF, 1 m*M* DFP and 1% thiodiglycol and by stirring for 4 days at 4°C. The suspension was centrifuged for 30 min at 10 000 *g* at 4°C and the supernatant kept at –20°C. The same extraction was repeated on the pellet 10 times. Supernatants were pooled, filtered on 0.8-µm cutoff membranes and concentrated 20 times (to about 70 ml) by ultrafiltration with an artificial kidney (Nephross Andante H.F., The Nederlands) fitted with a cuprophane fiber (cutoff 1500 Da, Organon Technica, The Netherlands) and then with an Amicon apparatus (YM5, cutoff 3500 Da, Grace, Witten, Germany). After several washes, the final volume, about 5 ml, was stored at –20°C. It was not possible to estimate the amount of protein in the stone because the supernatants contained numerous pigments which interfered with colorimetric and UV protein assays [5].

Purification of urinary proteins. Proteins were extracted from urine of healthy subjects as already described [30] and their concentration adjusted to 5 mg/ml. HSA was separated from the other urinary proteins by using a Sephacryl S-200 (Pharmacia) column (144×2 cm). Elution buffer was 0.02 *M* TRIS, 0.15 *M* NaCl, 0.02% NaN3, pH 8, at a flow rate of 0.18 ml/min. Four fractions were collected (data not shown). As a control in the affinity chromatography experiments (see below), we used the fractions without albumin.

Crude urine preparation. Fresh urine (500 µl) obtained from healthy subjects was lyophilized. The residue was suspended in 100 µl distilled water and centrifuged at 1000 *g*. The supernatant was kept at –20°C.

Preparation of COM crystals. COM crystals were prepared by mixing volume to volume 0.01 *M* $Na_2C_2O_4$ with 0.01 *M* $CaCl_2$ at 25°C for 2 h. After the seeds appeared, they were allowed to settle and the supernatant was discarded. They were washed twice with methanol and dried at 65°C for 30 min. The crystals were then stored in this form at 25°C until use.

High performance liquid chromatography (HPLC). Chromatography was carried out with a GOLD delivery system (Beckman Instruments, San Ramon, Calif., USA) consisting of a module 126 programmable solvent and a module 166 programmable detector coupled to a Gilson fraction collector. Size-exclusion chromatography was performed on a TSK-G 2000 SW column (TosoH, Japan). About 20 mg urinary proteins was then dialyzed against 0.02 *M* TRIS, pH 6, containing either 0.15 *M* or 1 *M* NaCl during 48 h. HPLC was performed with the same buffers at a flowrate of 0.5 ml/min. The elution of the proteins was monitored by UV absorbance at 280 nm. We pooled different fractions and analyzed the protein pattern by immunochemical methods.

Affinity chromatography. We prepared a 2-ml column of Affi-Gel blue (Bio-Rad, Richmond, USA) with specifity for albumin in a syringe and used according to the recommendations of the supplier. Briefly, the column was washed with 0.02 *M* phosphate buffer at pH 7.1 (buffer P). The samples were equilibrated in buffer P before being applied to the column and incubated for 20 min. Flow-through was recovered in buffer P and the proteins bound to the column were eluted by mixing 1 ml Affi-Gel blue with SDS sample buffer (3% SDS, 0.065 *M* TRIS, pH 6.8, 5% β-mercaptoethanol, 10% glycerol). The mixture was then boiled 5 min and centrifuged at 1000 *g*. The supernatant containing the eluted proteins was recovered. With this procedure, the elution of proteins was complete. The fractions were then submitted to SDS-PAGE followed by Coomassie blue or silver nitrate coloration, or the proteins were transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, USA) for immunodetection.

Human serum albumin adsorption onto COM crystals. The affinity of HSA for COM crystals was evidenced by immunodetection after incubation of the protein with COM crystals. Five sets of incubation conditions were used: 0.002 *M* MES pH 5.7 (buffer A), 0.005 *M* $CaCl_2$ in buffer A (buffer B), 0.01 *M* $CaCl_2$ in buffer A (buffer C), 0.02 *M* phosphate buffer pH 7.1 (buffer D), 0.002 *M* $CaCl_2$, 0.133 *M* NaCl, 0.05 *M* Na acetate and 0.05 *M* Na barbiturate, pH 5.7 (buffer E). One milligram of COM crystals was added to 200 µl of each buffer in the presence of 8 mg HSA. The mixture was gently shaken at 37°C for 2 h, then centrifuged at low speed (1000 *g*) for 2 min and the supernatant stored (supernatant 1). The pellet was resuspended in 200 µl H_2O, shaken vigorously for 2 h and centrifuged as before. This procedure was repeated 3 times, yielding supernatants 2, 3 and 4. Supernatants 1–4 were dialyzed overnight against distilled water. The pellet was resuspended in 40 µl SDS-PAGE sample buffer and centrifuged (supernatant 5). The fractions were then loaded on SDS-PAGE and stained by Coomassie blue. We also tested the putative adsorption to COM crystals of the three other proteins: α_1-GP, β_2-microglobulin (β_2-M) and immunoglobulins (Igs).

Electrophoretic techniques. Proteins were separated by electrophoresis in 15% polyacrylamide gels in the presence of SDS (SDS-

PAGE). The gels were then stained either with a commercial Coomassie brilliant blue preparation (R-250, Bio-Rad) or with silver nitrate according to Blum et al. [1]. Apparent molecular weight (*Mr*) of proteins was determined using a low and high molecular weight calibration kit (LMW and HMW, respectively, Pharmacia) and comparison with standard proteins. Proteins were transferred onto PVDF membranes according to Burnette [6]. Proteins were immunodetected with peroxidase-conjugated $(Fab')_2$ fragment of anti-rabbit or anti-sheep IgGs (Immunotech, Marseille, France) followed by revelation with 0.05% diaminobenzidine tetrahydrochloride and 0.01% H_2O_2.

Results

Mineral constituents of the five morphological types of urinary stones (Table 1)

Type Ia consists of 95% whewellite (CaOx monohydrate) and type IIa of 80% weddellite (CaOx dihydrate) with small amounts of carbapatite and whewellite. Type IIIb is made up of 96% anhydrous or dihydrate uric acid with small proportions of whewellite. Type IVb is a mixture of carbapatite and struvite (95%) and whewellite (5%). Type Va is made up of pure cystine.

Protein composition of the organic matrix of urinary stones: comparison with that of normal urine and urinary proteins

Migration patterns on SDS-PAGE. Figure 1 shows typical electrophoretic patterns of crude urine preparation (lane U), urinary proteins (lane P) and proteins extracted from kidney stone matrix of the five types of stone (lanes Ia, IIa, IIIb, IVb and Va). A 67-kDa protein appears to be by far as the major constituent in all types of stones.

Western blot experiments of kidney stone matrix proteins (Figs. 2–4). As shown in Fig. 2, immunodetection of β_2-M was possible in crude urine, in urinary proteins and in three types of stones (lanes U, P, Ia, IIa and IIIb). α_1-Microglobulin, α_1-GP, α_1-T and Tr were detected in urine, in urinary proteins and in all kidney stones. Figure 3 shows five proteins evidenced in all calculi: HSA, Igs, renal lithostathine (RL), apolipoprotein A1 (apo-A1) and retinol-binding protein (RBP). RBP and apo-A1 could not be detected in crude urine preparation (lane U) because of their minute concentration. The 67-kDa protein is HSA, which

Table 1 Percentage of mineral constituents of the five types of kidney stone

	Ia	IIa	IIIb	IVb	Va
Whewellite	95	10	4	5	0
Weddellite	0	80	0	0	0
Uric acid	0	0	96	0	0
Carbapatite/struvite	5	10	0	95	0
Cystine	0	0	0	0	100

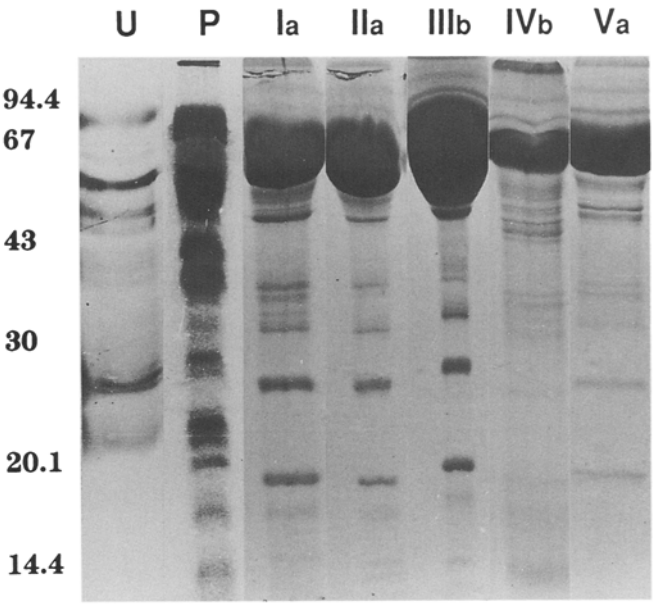

Fig. 1 SDS-PAGE analysis of the proteins extracted from the kidney stone matrix. Crude urine (500 µl, lane U), proteins purified from concentrated urine (100 µg, lane P) and proteins extracted from stones (10 µl, lanes Ia-Va) were submitted to SDS-PAGE and stained with silver nitrate (lane U) or Coomassie blue (lanes P and Ia-Va). Molecular weight standard values are indicated *on the left*. Sizes are given in kilodaltons

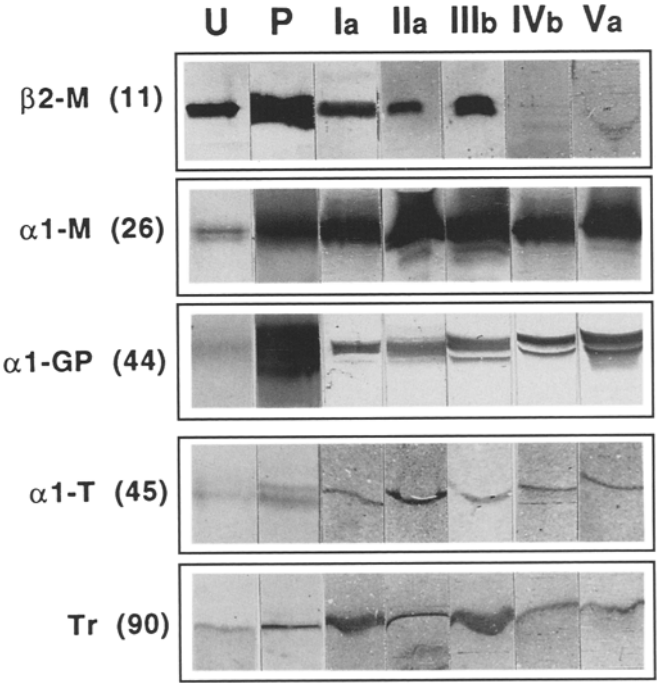

Fig. 2 Immunodetection of proteins in urine and kidney stones. Crude urine (500 µl, lane U), proteins purified from concentrated urine (100 µg, lane P) and proteins extracted from stones (10 µl, lanes Ia-Va) were submitted to SDS-PAGE, transferred to nylon membranes and incubated with various antibodies against β_2-M, α_1-M, α_1-GP, α_1-T and Tr, respectively. Apparent molecular weights are given in *brackets*

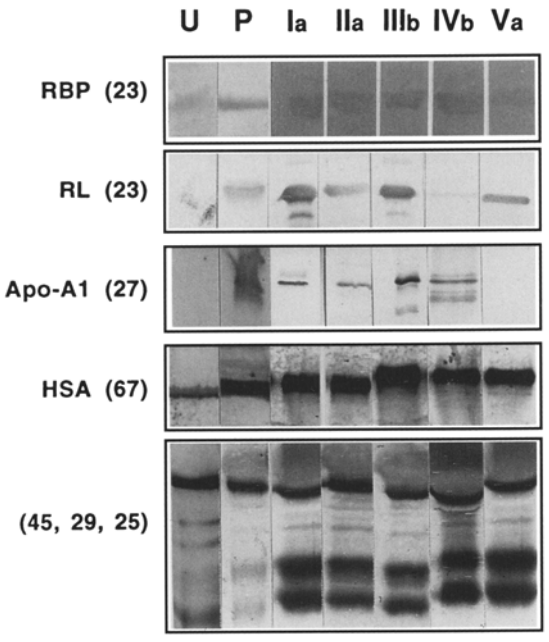

Fig. 3 Immunodetection of proteins in urine and kidney stones. Crude urine (500 µl, lane U), proteins purified from concentrated urine (100 µg, lane P) and proteins extracted from stones (10 µl, lanes Ia-Va) were submitted to SDS-PAGE, transferred to nylon membranes and incubated with various antibodies against RBP, RL, Apo-A1, HSA and Ig A, G, M, respectively. Apparent molecular weights are given in *brackets*

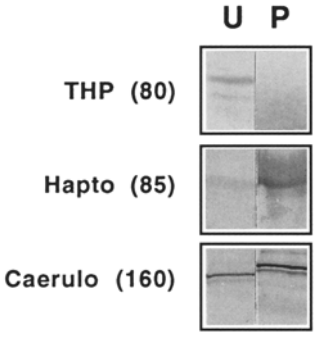

Fig. 4 Immunodetection of proteins in urine. Crude urine (500 µl, lane U) and proteins purified from concentrated urine (100 µg, lane P) were submitted to SDS-PAGE, transferred to nylon membranes and incubated with antibodies against THP, haptoglobin and ceruloplasmin respectively. Apparent molecular weights are given in *brackets*

appears as the most abundant protein in all types of stones. Immunoglobulins were present in all samples, producing three bands representing heavy and light chains. Tamm-Horsfall protein, haptoglobin and ceruloplasmin could not be evidenced in any of the five types of stone, although they are normally present in crude urine and in urinary proteins (Fig. 4). We detected THP in urine, but not in the protein prepared from urine. Such a loss has already been described by others [11, 12], who have shown that centrifugation steps induced THP aggregation into a pellet. We investigated whether this could not explain the absence of THP from the protein extracted from stones, by analyzing the EDTA extract of the five stones without centrifugation. Even with this simplified procedure, THP could not be detected by immunoblotting (data not shown) whereas in urine samples from healthy subjects THP was detected with the same antibody used for kidney stone matrix.

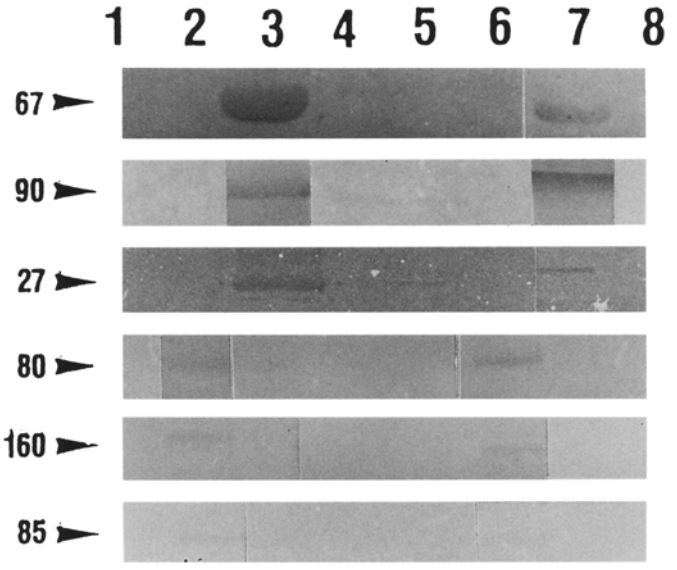

Fig. 5 Immunodetection of HPLC fractions. Fractions 1–8 were submitted to SDS-PAGE, transferred to nylon membranes and incubated with antibodies against HSA, Tr, α_1-M, THP, ceruloplasmin. Apparent molecular weights are indicated *on the left*

Involvement of HSA in the constitution of the kidney stone matrix

The possible occurrence of specific interactions between HSA and some urinary proteins was investigated by gel filtration and affinity chromatography.

Gel filtration. Urinary proteins were subjected to chromatography on a TSK-G 2000 SW column using an isocratic gradient of either 0.15 *M* (fractions 1–4) or 1 *M* NaCl (fractions 5–8). The fractions were collected at the same time at both ionic strengths (elution profile not shown). They were then submitted to SDS-PAGE and transferred to PVDF membranes. Immunoblotting with specific antibodies showed that HSA, Tr and α_1-M comigrated (fractions 3 and 7) in spite of their difference in M. In contrast, THP, ceruloplasmin and haptoglobin were found in other fractions (fractions 2 and 5) even though haptoglobin whose M is very similar to that of HSA, should have comigrated with HSA (Fig. 5). This result demonstrates that proteins present in kidney stone matrix comigrate with HSA and, conversely, urinary proteins not present in kidney stone matrix migrate independently. Hence these proteins can be classified as: (1) proteins with affinity for HSA which are found in the matrix and (2) proteins with no affinity for HSA which are not found in the matrix.

Affinity chromatography. Figure 6A shows the electrophoretic pattern of HSA, urinary proteins and proteins extracted from stones after fractionation on an affinity column for HSA (Affigel blue-gel). As expected, HSA was com-

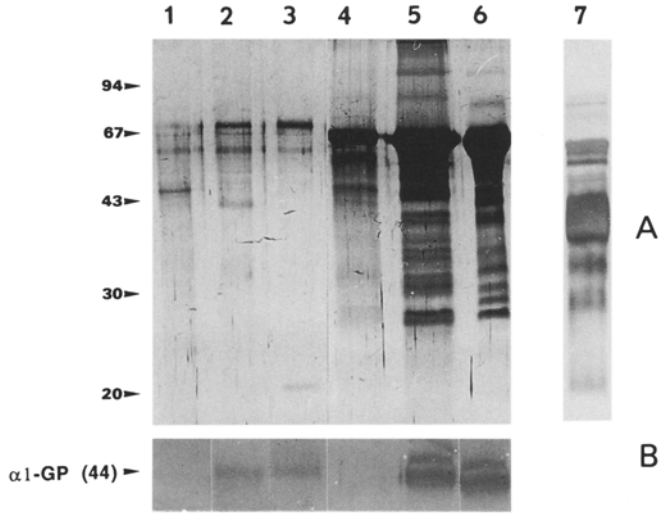

Fig. 6A, B Affinity chromatography of proteins extracted from stones. **A** HSA (*lanes 1, 4*), proteins purified from concentrated urine (*lanes 2, 5*), proteins purified from concentrated urine without HSA (*lane 7*) and proteins extracted from kidney stone matrix (*lanes 3, 6*) were loaded onto an Affigel-blue gel column. The flow-throughs (*lanes 1–3, 7*) and eluted proteins (*lanes 4–6*) were then subjected to SDS-PAGE and stained with silver nitrate. Molecular weight standard values are given *on the left*. Sizes are given in kilodaltons. **B** Immunodetection of α_1-GP: after SDS-PAGE, the proteins were transferred to nylon membranes and incubated with antibodies against α_1-GP (*apparent molecular weight in brackets*)

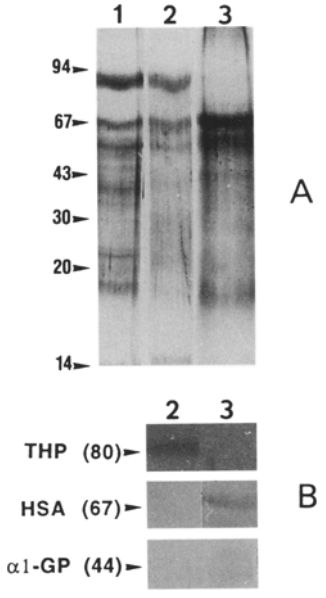

Fig. 7A, B Affinity chromatography of crude urine. Crude urine samples (500 µl) were loaded onto an Affigel-blue gel column. Flow-through (*lane 2*) and eluted proteins (*lane 3*) were then submitted to SDS-PAGE, stained with silver nitrate (**A**) or transferred to nylon membranes (**B**) and incubated with antibodies against THP, HSA and α_1-GP. Apparent molecular weights are given in *brackets*. As a control, 500 µl crude urine was run and treated in parallel (*lane 1*). Molecular weight standard values are given *on the left*. Sizes are given in kilodaltons

pletely bound to the column (lane 4), but most of the urinary proteins and all proteins extracted from stones were also retained (lanes 5 and 6, respectively). Using specific antibodies against α_1-GP in immunoblot experiments, we confirmed that this protein, normally expected in the flow-through (Fig. 6B, lanes 2, 3), was mostly found in the bound fraction, together with HSA (lanes 5, 6). Conversely, urinary proteins depleted from albumin did not bind to the column (lane 7). The possible interaction of THP with HSA could not be analyzed using purified urinary proteins as the starting material, because of the technical problem with centrifugation discussed above. The binding experiment was therefore conducted with crude urine samples. The results clearly demonstrated that THP, which is not found in kidney stone matrix, flowed through the column while HSA and α_1-GP were retained (Fig. 7A, B). Further evidence of a strong interaction between HSA and α_1-GP was obtained by showing that α_1-G alone did not bind to the column (Fig. 8A, lane 1). Furthermore, when an equimolar mixture of HSA and α_1-GP was incubated for 1 h at room temperature and then loaded onto the column, we observed a partial fixation of α_1-GP (lanes 3, 4), which was confirmed by Western blot analysis (Fig. 8B, lanes 3, 4).

Human serum albumin adsorption onto COM crystals. Adsorption of HSA onto COM crystals was tested by incubating the protein with crystals and looking for the presence of HSA in successive rinses of the crystals. Five different incubation buffers were tested. The results are presented in Fig. 9. At pH 5.7 without $CaCl_2$, albumin was still present in the third incubation supernatant, suggesting weak interaction (lane A). Binding was much stronger with 5 m*M* $CaCl_2$ (lane B), a significant amount of protein being retained in the final pellet (lane 5). The binding was totally abolished when $CaCl_2$ was raised to 10 m*M* (lane C). With the same buffer as used in affinity chromatography experiments, we observed no significant change in the behavior of HSA, although the pH was different (D). In contrast, using a buffer with pH, ionic strength and $CaCl_2$ concentration similar to those of urine [33], the adsorption of HSA was complete (E). No adsorption to COM crystals could be observed, whatever the incubation buffer used, with α_1-GP, β_2-M and Igs.

Discussion

We have demonstrated that only a subset of urinary proteins are present in stone matrix, the same proteins being found in all types of stones. All these proteins are bound to albumin, the most abundant species of the protein mixture. On the other hand, albumin shows strong affinity for COM crystals. Hence, urinary proteins that bind to albumin are found in COM-containing stones because of albumin adsorption onto COM crystals.

Studies by Boyce and King showing that urinary stones were composed of crystal aggregates held together by an organic matrix were an important step in the understanding of urolithogenesis [2, 19]. On a weight to weight basis, they found that the ratio of protein matrix to crystals was usually 1:50 (i.e., 2%) but with considerable variations of up to 300% [8]. Our analysis by infrared spectroscopy confirmed that proteins were always present, their amount varying with the type of stone (not shown). The relationship of these proteins to lithogenesis is still a mat-

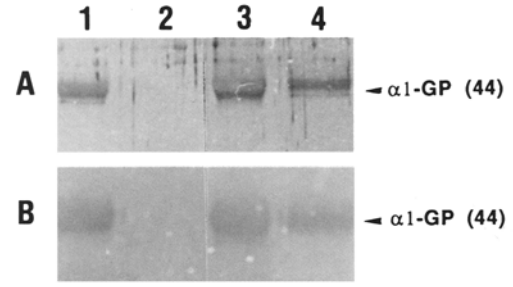

Fig. 8A, B Affinity chromatography of a mixture of HSA and α_1-GP. One hundred micrograms of α_1-GP (*lanes 1, 2*) or a pre-equilibrated equimolar mixture of α_1-GP (50 μg) and HSA (78 μg) (*lanes 3, 4*) was loaded onto an Affigel-blue gel column. The flow-throughs (*lanes 1, 3*) and the bound fractions (*2, 4*) were submitted to SDS-PAGE, stained with silver nitrate (**A**) or transferred to nylon membranes (**B**) and incubated with antibody against α_1-GP (*apparent molecular weight in brackets*)

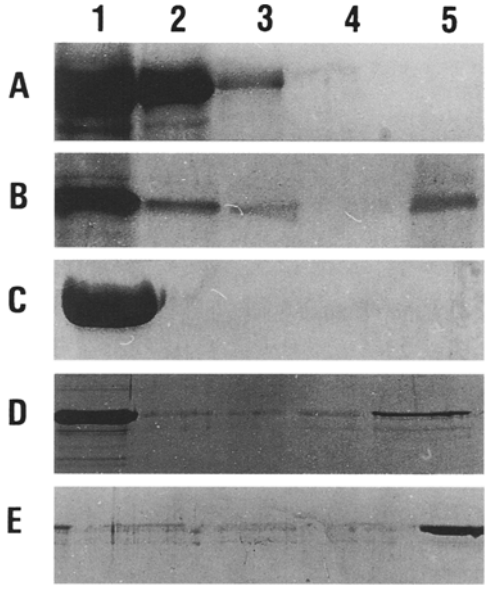

Fig. 9A–E Adsorption of HSA onto COM crystals. Eight milligrams of HSA was incubated with COM crystals in various buffers as described in "Materials and methods": **A** 0.002 *M* MES pH 5.7, **B** 0.005 *M* $CaCl_2$ in buffer A, **C** 0.01 *M* $CaCl_2$ in buffer A, **D** 0.02 *M* phosphate buffer pH 7.1, **E** 0.002 *M* $CaCl_2$, 0.133 *M* NaCl, 0.05 *M* Na acetate, 0.05 *M* Na barbiturate, pH 5.7. *Lanes 1–4* correspond to supernatants of successive incubations of albumin with the crystals. *Lane 5* corresponds to protein extraction from the last pellet with SDS buffer. Gels were stained with Coomassie blue

ter of debate. This work aimed at further characterizing the protein composition of stones in the hope of obtaining additional information regarding their possible role in stone formation.

Our analysis was conducted on 12 of the most abundant proteins present in urine [24] and on RL, an inhibitor of calcium carbonate ($CaCO_3$) crystal growth, recently identified in urine [29]. Of these 13 proteins, 9 were present in all types of stone: HSA, α_1-GP, α_1-M, Igs, apo-A1, Tr, α_1-T, RBP and RL. The β_2-M was present only in CaOx and uric acid stones. Our findings are in agreement with those of Sugimoto et al. [28], who showed that the stone matrix is the same regardless of mineral composition. Similarly, immunological results obtained by Fraij [12] demonstrated that the same three proteins are present in kidney stone matrix of CaOx, calcium phosphate and uric acid stones.

Three urinary proteins were not detected in the matrix: THP, ceruloplasmin and haptoglobin. There has been a long controversy about the presence or absence of THP in the matrix of stones. It has been shown to be present in the core of renal stones by Grant et al. [13], Keutel et al. [17] and Fraij [12]. Conversely Doyle et al. were unable to detect THP in CaOx crystals [11]. Recent work by Worcester et al. found that THP does not adsorb to COM crystals [33]. Hess et al. have shown that THP adsorbs onto COM crystals, thereby inhibiting their aggregation if pH is above 7 and the ionic strength weak [15]. If pH is below 7 and the ionic strength high, THP is auto-aggregated and loses its inhibiting properties [27]. The conflicting results in the literature about the presence or absence of THP in kidney stones may be explained by the different physicochemical conditions found in urines.

By studying a larger number of proteins we were able to demonstrate that not all urinary proteins were present in the matrix of stones, and that, of the 13 proteins analyzed in this study, the same subset of 9 proteins was found in all 5 types of stones. This interesting finding suggests that all nine proteins are involved in interactions with components common to all stones. For three of them, Igs, apo-A1 and RL, a specific binding to the mineral component of stones can be considered. Immunoglobulins and apo-A1 might be bound to the lipid fraction of stones, since their strong pronucleating and inhibiting activity, respectively, towards cholesterol crystal formation is well documented [14, 18]. The presence of RL in stones might be associated with $CaCO_3$. RL is functionally similar to pancreatic lithostathine [10], which is the major protein component of pancreatic stones and which inhibits the growth of $CaCO_3$ crystals [29]. Supersaturation in $CaCO_3$ was recently reported in the thin segment of Henle's loop, and small $CaCO_3$ crystals bearing trapped RL molecules might have provided suitable nuclei for the formation of crystals found in urinary stones [7].

The seven other proteins do not appear to play a role in stone core formation. However, HSA, which is known to stick to COM crystals without influencing their growth [33] and also to exhibit strong interactions with many proteins, may somehow be implicated [25]. We demonstrated that proteins of the matrix were indeed strongly bound to albumin and that, in an ionic environment similar to urine, HSA was completely adsorbed onto COM crystals. Albumin binding to uric acid was also reported [23]. Altogether these results suggest that proteins are selectively incorporated into the matrix by their binding to HSA, rather than because of their direct binding to the crystals. We are now trying to determine whether HSA (1) could act as a template for nucleation growth or aggregation of crystals and (2) could be one of the driving forces of the nucleation process.

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