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# Immunohistochemical distribution and quantification of crystal matrix protein

ALAN M.F. STAPLETON, ANTHONY E. SEYMOUR, JAMES S. BRENNAN, IAN R. DOYLE, VILLIS R. MARSHALL, and ROSEMARY L. RYALL

*Departments of Surgery and Histopathology, Flinders Medical Centre, South Australia 5042, Australia*

**Immunohistochemical distribution and quantification of crystal matrix protein.** The aim of this study was to determine the immunohistochemical distribution and quantification of crystal matrix protein (CMP). CMP, a 31 kDa glycoprotein, is the principal macromolecule found in calcium oxalate crystals generated in human urine, and is a potent inhibitor of crystal aggregation. A polyclonal rabbit anti-human CMP antibody was used to examine renal tissue by immunohistochemical techniques and light microscopy ($N = 45$). Twenty-five other human organs were similarly assessed. Quantification was performed using a visual analogue scale. CMP was visible as cytoplasmic staining in the epithelial cells of the TALH and the distal convoluted tubule including the macula densa in a subgroup of nephrons. CMP was not identified elsewhere in the urinary tract or in the extrarenal organs examined. Despite a trend indicating that the kidneys of normal men had more CMP than those of normal women, the difference failed to reach significance ($P = 0.11$). There was, however, more CMP in the stone formers group compared with either normal men ($P < 0.01$) or normal women ($P < 0.01$). This protein may be an important determinant of calcium oxalate kidney stone disease.

Throughout all kidney stones there exists a latticework of organic material commonly referred to as the matrix [1]. Its importance in the pathogenesis of stone disease must not be overlooked, for it may hold the key that will allow us to manipulate the natural history of this condition in the future. More than 70% of urinary calculi contain calcium oxalate (CaOx) as their principal component [2], and in these stones the matrix constitutes approximately 2.5% of the stone's dry weight and is distributed throughout the entire structure [3–5].

Chemical analysis of stone matrix has proved difficult because of its poor solubility [6], and the fact that its dissolution and extraction prior to analysis necessitates the use of aggressive procedures, such as acid hydrolysis, which may themselves alter its chemical structure. Furthermore, stones take varying periods to grow before becoming clinically apparent, and are often stored for years prior to analysis [7]. As a result, consequent chemical cross-linking and degeneration of proteins may alter the final composition of matrix so that it bears little resemblance to that prevailing when the core of the stone was initially formed. If this were not enough to undermine the validity of analytical findings, the matter is further complicated by the fact that organic material in the stone's crystalline structure may derive from two distinct sources, namely constituents normally present in urine and those liberated variably from the epithelial lining or the interstitium of the urinary tract as a consequence of trauma induced by the enlarging stone [8].

Thus, recent attention has turned to the extraction and analysis of organic matrix from the precursors of stones, CaOx crystals, freshly precipitated from whole urine [8, 9]. Since these crystals are grown in the absence of substances that could be termed opportunistic contaminants of the growing stone's matrix, their organic matrix should be representative of normal urinary macromolecules associated with CaOx crystal formation in stone pathogenesis.

In an extension of the study by Morse and Resnick [9] we have analyzed the protein content of CaOx crystals harvested in this manner and have isolated a urinary protein not previously described. This has been named crystal matrix protein (CMP), a 31 kDa glycoprotein [8]. Unpublished studies from our laboratory have shown that it is a potent inhibitor of calcium oxalate crystal aggregation, and is present in CaOx crystals in quantities far in excess of those expected from its concentration in urine. This lends support to the findings of Morse and Resnick [9] that the incorporation of urinary proteins into CaOx crystals is a highly selective phenomenon.

The anatomical origin of CMP is unknown. Doyle, Ryall and Marshall [8] did not detect CMP in human serum, indicating that it may be produced and secreted within the urinary tract. Since this protein may be important in the control of stone pathogenesis, and perhaps even have a role in renal physiology and homeostasis, the aims of this study were: (1) to delineate the distribution of CMP within the human urinary tract; (2) to investigate its presence in other human tissues; and, (3) to assess histologically the quantity of CMP in men, women, and CaOx stone formers.

## Methods

### *Preparation of CMP antibody*

Ten milliliters were bled from each of three New Zealand white rabbits. Pre-immune serum was prepared and stored at −70°C, prior to being treated in a similar way to the immune serum as detailed below.

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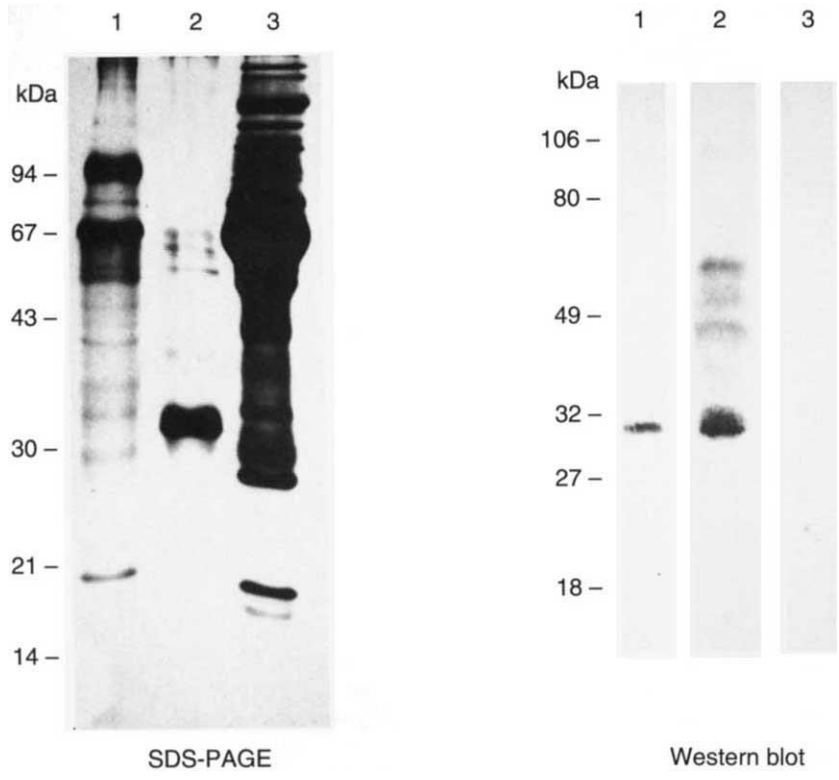


**Fig. 1.** *Nine to 18% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of human urine (centrifuged 5000 rpm for 5 min and passed through a 0.2 μm filter) (lane 1), crystal matrix extract (lane 2), and human serum (lane 3), all stained with silver.* Alongside is the associated Western blot of the same samples (lanes 1 to 3) using anti-human crystal matrix protein antibody (1:1000), detected with anti-rabbit antibody conjugated with peroxidase (1:1000) and stained with diaminobenzidine in the presence of 0.2% $H_2O_2$. Despite the many proteins in urine only a few are incorporated in CaOx crystals, the most prominent being at 31 kDa, called crystal matrix protein (CMP). On the Western blot, CMP is detected in urine and in the crystal matrix extract, and is absent from serum.

CMP was isolated from urine collected over a 48 hour period from four healthy volunteers, aged between 19 and 42 years, and processed as described by Doyle et al [8]. Briefly, crystallization was induced in the pooled urine by the addition of an oxalate load. The crystals were isolated, washed briefly in water and 0.1 M/liter sodium hydroxide and demineralised in 0.25 M/liter EDTA at pH 8.0. The extract was electrodialyzed over seven days and then passed through a 0.22 μm Millipore filter, before being lyophilized. Since this crystal matrix extract consisted principally of CMP [8], as shown by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [10], the extract was used without further purification.

Three hundred micrograms of the crystal extract were dissolved in 3 ml of water and emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA). Each of three New Zealand white rabbits was injected subcutaneously at 20 sites along the back with 0.1 ml aliquots of the CME adjuvant. At days 14 and 77 the immunizations were boosted with the same inoculum except that Freund's incomplete adjuvant was substituted for Freund's complete adjuvant (Difco Laboratories). The rabbits were exsanguinated on day 87, yielding 65 ml of blood.

The antiserum was preabsorbed once with human serum immunoabsorbent as described by Avrameus and Ternynck [11] to reduce nonspecific staining. The specificity of this antiserum was assessed by its use as a primary antibody in the immunochemical staining of Western blots [12] of proteins [including Tamm-Horsfall glycoprotein (THG)] separated by 9 to 18% gradient SDS-PAGE (Mini-Protean II apparatus, Bio-Rad Laboratories, Richmond, California, USA) from human urine, crystal matrix extract, and human serum. The urine sample was prepared by centrifugation at 5000 rpm (J-21B Beckman®, USA) for five minutes, then passed through a 0.2 μm filter (Millipore Corporation, Bedford, Massachusetts, USA) and lyophylized. Venous blood was drawn from a normal volunteer into a plain tube and allowed to congeal at 22°C for two hours then centrifuged at 2000 rpm for five minutes (Labofuge M, Heraeus-Christ GmbH, Germany), and the serum harvested. Proteins of interest were transferred to nitrocellulose (Amersham International, Buckinghamshire HP79LL, UK) using the Mini-Protean II apparatus (Bio-Rad Laboratories) and CMP was detected with anti-rabbit antibody conjugated with peroxidase (Bio-Rad Laboratories) (1:1000) and stained with diaminobenzidine (Sigma Chemical Company, St Louis, Missouri, USA) in the presence of 0.2% $H_2O_2$ (Western blots) (Fig. 1).

### *Anti-epithelial membrane antigen antibody*

Goat anti-human epithelial membrane antigen (EMA) monoclonal antibody was obtained from DAKOPATTS (DK-2600 Glostrup, Denmark). It was diluted 1:1000 in normal horse serum (DAKOPATTS) prior to use.

### *Tissue*

Fresh human kidney was obtained from 10 nephrectomy specimens removed because of renal cell carcinoma (7) or transitional cell carcinoma of the renal pelvis (3). There were seven males and three females, median age 74, range 57 to 86 years. Macroscopically normal tissue was sampled away from the site of known pathology and subsequently shown by light microscopy to be within normal morphological limits. Each specimen was processed as follows:

(1) Tissue blocks not more than 3 mm thick were embedded in cryomoulds containing OCT Compound (Miles Laboratories), quenched in an isopentane bath cooled in liquid nitrogen, and stored at −70°C.
(2) Tissue blocks approximately 5 mm thick were placed in Zamboni's solution [13] for 16 to 24 hours, washed three times in dimethylsulphoxide, washed three times in phosphate buffered saline (PBS) at pH 7.0, transferred to PBS containing 30% sucrose and 0.2% sodium azide, and stored at 4°C. Tissue was embedded in OCT Compound and frozen at −20°C prior to sectioning.
(3) Tissue blocks, not more than 5 mm thick, were fixed in 10% buffered formalin (pH 7.4) for 12 to 24 hours and embedded routinely in paraffin wax.
(4) Blocks similar to those in (3) above were fixed in 2.5% glutaraldehyde for 2 to 24 hours and then embedded in paraffin wax.

Archival renal tissue (formalin-fixed paraffin-embedded) was obtained from the routine tissue blocks of kidney specimens from 35 individuals, stored in the Histopathology Department. Nephrectomy had been performed in patients for parenchymal carcinoma (19), transitional cell carcinoma (9), infection (3), trauma (1), renal arterio-venous malformation (1), renal oncocytoma (1), and partial nephrectomy for renal calculi (1), between 1986 and 1992 at the Flinders Medical Centre (FMC). The median age of these patients was 63, range 16 to 86 years, and 24 of them were men. Included in these groups were five patients known to have had one or more CaOx renal stones who had their renal surgery because of renal cell carcinoma (1), renal leiomyoma (1), partial nephrectomy for renal calculi (1), renal abscess (1), transitional cell carcinoma (1). Four were males and one was female.

Adult human tissue other than kidney was either gathered from storage (formalin-fixed paraffin-embedded) or obtained fresh from surgical resection within the Department of Histopathology at FMC.

To assess the influence of the time tissues spend in fixative on the quantification of subsequent positive staining, adjacent tissue blocks from a single kidney were fixed in 10% formalin for two hours 48 minutes, 4, 6, 8, 12, 18, 24, 36, and 48 hours, prior to standardized processing that occurred with all specimens (viz Hypercenter® Tissue Processor, Shandon Scientific, Cheshire, England).

### *Immunohistochemistry*

Five micrometer frozen sections were cut using a cryotome (Tissue-Tek II microtome 4451, Miles Laboratories, Illinois, USA), placed on chrome-alum gelatin coated slides, air dried and washed in Tris (Analar, BDH Chemicals, Vic 3137, Australia) buffered saline, pH 7.6 (TBS) prior to immuno-staining.

Five micrometer paraffin sections were cut using a standard microtome (Anglia Scientific, Type 300 Cambridge, UK), placed on acid cleaned glass slides, deparaffinized in xylene and passed through graded alcohol before placing in TBS prior to immuno-staining.

To evaluate the effect of protein digestion on immunostaining, a series of formalin-fixed paraffin-embedded sections was placed on acid cleaned glass slides and incubated in a solution of 0.1% trypsin in 0.1% $CaCl_2$, (pH 7.8) at 37°C for 20 minutes, before being washed in TBS prior to immuno-staining.

Endogenous peroxidase activity was blocked by incubating sections for 10 minutes in a solution of 0.1% $H_2O_2$ in 100% ethanol.

Each primary antibody (Ab) was diluted in the normal serum of the species used to generate the secondary Ab, and the sections incubated in a humid chamber overnight at 4°C. All subsequent incubations were performed at room temperature. The anti-CMP Ab was routinely diluted 1:400, and the anti-EMA Ab 1:1000.

For single staining, the avidin-biotin immunoperoxidase technique was used as previously described by Hsu, Raine and Fanger [14] using a Vectastain® ABC Kit (Vector Laboratories, Burlingame, California, USA). 3:3-diaminobenzidine (DAB; Sigma Chemical Co, St Louis, Missouri, USA) was the chromogen ($1.7 \times 10^{-3}$ M/liter) used in the presence of 0.024% hydrogen peroxide to give a brown insoluble precipitate which was stopped with water after three minutes. Sections were counterstained in Mayer's hematoxylin, acid differentiated, blued in lithium carbonate, dehydrated, and mounted in PIX. Several sections were treated with periodic acid Schiff (PAS) reagent prior to counterstaining.

Sections were double labeled using a combination of the immunoperoxidase described above with an alkaline phosphatase method. The immunoperoxidase/DAB technique was used first since it was observed that this relatively insoluble product was less likely to diffuse during subsequent incubations than the water soluble alkaline phosphatase/fast red combination. So, after immunolabeling as detailed above, the tissue was incubated with the second primary Ab overnight. After incubation with the second biotinylated linking Ab directed against the second primary Ab, 0.5% streptavidin alkaline phosphatase (Biogenex Laboratories, San Ramon, California, USA) was applied for 30 minutes. Naphthol AS-TR phosphate (Sigma Chemical Co.) was dissolved in dimethylformamide and added to Gomari's buffer (pH 9.8) containing 0.03% levamisol. The chromogen, Fast Red TR Salt (F1500, Sigma Chemical Co.), was added to give a characteristic red reaction which was stopped after 10 minutes by adding excess water. Sections stained by this method were subsequently air dried and mounted in Locktite® (Locktite, UK Cat No. 35768, Ireland) which was polymerized under ultraviolet light for 15 minutes. Between each of the incubation steps sections were washed three times in TBS for five minutes.

Light microscopy was performed using a Leitz Wetzler Diaplan microscope and photography using a Wild MPS 45 camera system with Kodak 35 mm 50ASA professional color film.

### *Controls*

All sections were run in parallel with a negative control of pre-immune rabbit serum (diluted 1:400) for the anti-CMP antibody, and TBS for the anti-EMA Ab. Each batch was accompanied by at least one renal section that had been found consistently to be positive for CMP.

The anti-CMP Ab was assessed by an affinity-absorption test, whereby aliquots of CMP, purified by gel filtration and noted to display a single band on SDS-PAGE, were incubated with the Ab for two hours at 37°C, and overnight at 4°C. Maximum

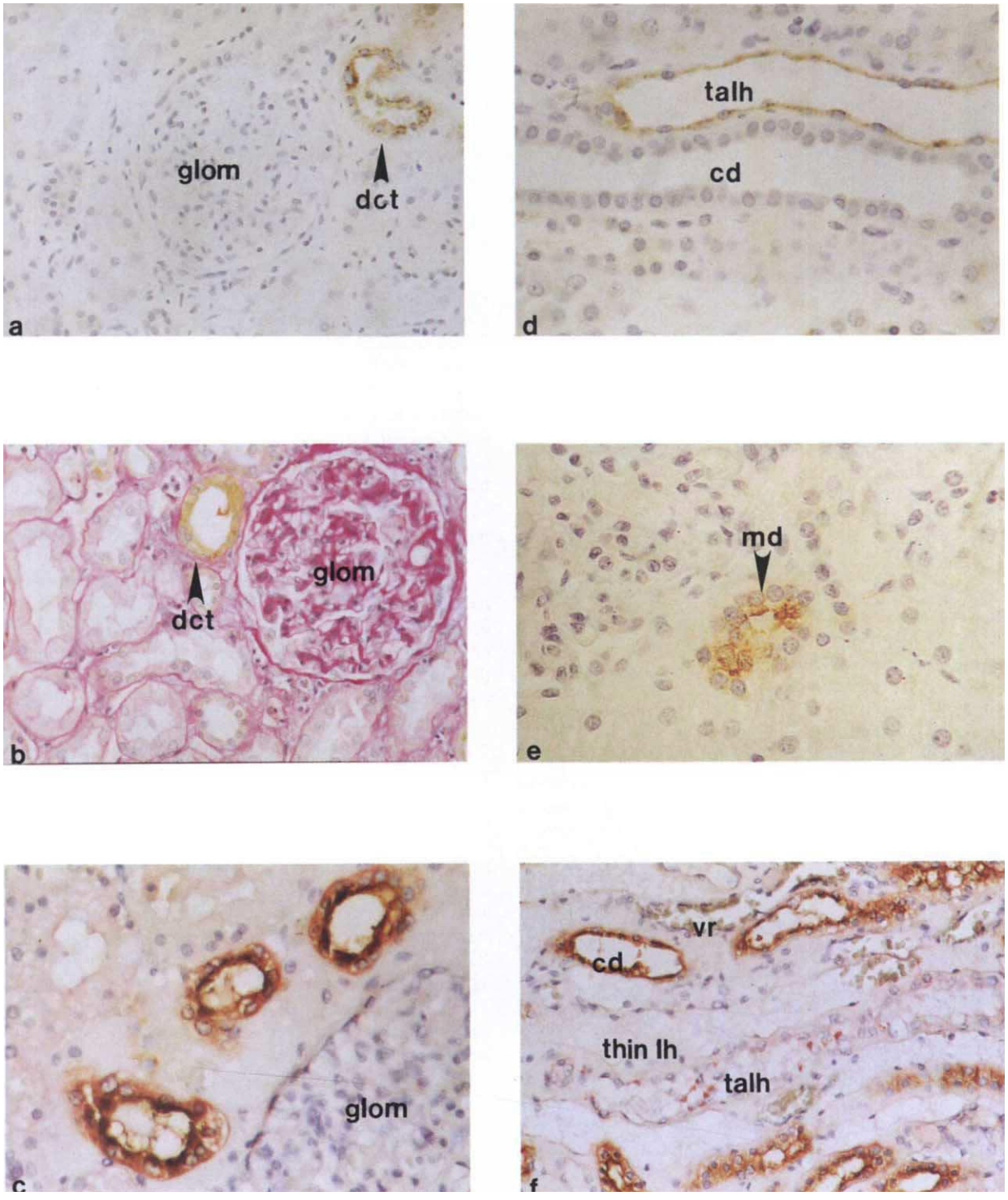


**Fig. 2.** *Photomicrographs* **(a), (b), (d)** *and* **(e)** *show sections of human kidney labeled immunohistochemically with anti-human crystal matrix protein antibody detected with diaminobenzidine to give the brown color.* The blue nuclear counterstain is hematoxylin; (a) cortex, intensity of staining 3+, magnification 560×, (b) cortex counterstained with periodic-acid-Schiff reagent to highlight the carbohydrate moieties, particularly on the brush border of the proximal convoluted tubular epithelia and absent from the distal tubular epithelia, magnification 560×; (d) outer medulla, intensity of staining 2+, magnification 900×, (e) cortex showing the macula densa cytoplasm stained positively for crystal matrix protein, magnification 1125×. Photomicrographs (c) and (f) show sections of human kidney double-labeled immunohistochemically with anti-human crystal matrix protein antibody detected with Fast Red and anti-human epithelial membrane antigen detected with diaminobenzidine shown as brown. The blue nuclear counterstain is haematoxylin; (c) periglomerular tubules of the renal cortex showing crystal matrix protein in the cytoplasm of cells positive for epithelial membrane antigen, a marker of the distal convoluted tubule, magnification 900×, (f) outer medulla showing crystal matrix protein (red) in the cytoplasm of tubular epithelia of the TALH with the brown stain corresponding to epithelial membrane antigen, a marker for the collecting ducts. Red blood cells can be seen in the vasa recta of the medulla, magnification 900×. Abbreviations are: dct, distal convoluted tubule; talh, thick ascending limb of the loop of Henle; cd, collecting duct; glom, glomerulus; vr, vasa recta; md, macula densa; lh, loop of Henle.

concentrations of CMP were estimated to be in excess of the anti-CMP Ab by a factor of 50 to 100×. The samples were then centrifuged at 3000 rpm for 15 minutes (Labofuge M) and the supernatant applied to known positive renal tissue and processed as described above, using the indirect immunoperoxidase method. Included in this batch was a section incubated with an appropriately diluted sample of anti-CMP Ab without added antigen, to act as the positive control.

### *Quantification of CMP*

A separate batch of formalin-fixed paraffin-embedded tissue sections was treated in a standard fashion by single labeling with anti-CMP Ab, keeping uniform the incubation times, washing, development, etc.

Subjective quantification of intracellular CMP was performed independently by two of the authors (AMFS and AES) examining single immunoperoxidase labeled sections using a direct visual analogue system from 0 (absence of staining), through 1+, 2+, 3+, to 4+ (indicating heavy staining). Each final score represents the sum of assessments of the DCT and the TALH. Forty-five kidneys were assessed and the data analyzed using the Wilcoxon rank sum test, to allow comparison between men and women, and between healthy subjects and CaOx stone formers.

## Results

### *Specificity of the anti-CMP antibody*

Immunoabsorption using human serum had no perceptible effect on the titer of the CMP antiserum but the specificity of the antiserum was enhanced. Figure 1 illustrates that despite the many proteins in urine only a few are incorporated in CaOx crystals, the most prominent being at 31 kDa, which has been called CMP [8]. CMP was detected by the antibody in the urine sample and in the crystal matrix extract, and was absent from serum using this technique of Western blotting. There were reactivities in the extract with molecular weights in excess of 31 kDa. Affinity chromatography, using CMP isolated by preparative SDS-PAGE, demonstrated these reactivities principally to be conglomerates of CMP. The antiserum exhibited no cross-reactivity with urinary THG.

### *Controls*

The sections incubated with rabbit pre-immune serum which were used as negative controls in each batch were consistently unstained, unless endogenous peroxidase activity was incompletely quenched. Absorption of the anti-CMP Ab to purified antigen eliminated labeling—as would be expected with an Ab of high specificity.

### *Immunohistochemistry*

At appropriate Ab dilutions background staining was low, and the signal high. Staining for CMP was evident in all but one of the kidney specimens (98%). The typical staining pattern seen in the cortex is shown in Figure 2a, which is an example of indirect immunoperoxidase labeling with DAB, and the nuclei stained blue with hematoxylin. On the basis of morphological criteria, such as the relatively tall and cuboidal cell shape [15], the punctate stain is evident in the distal convoluted tubule (DCT), and absent from the proximal tubular epithelium, glomerulus, and the interstitium. Figure 2b shows a similar peri-

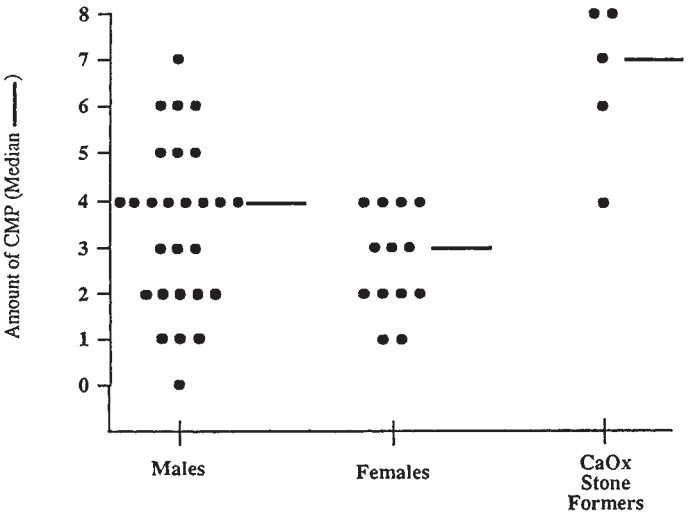


**Fig. 3.** *The intracellular amount of crystal matrix protein (CMP) in the kidneys of normal men, women, and CaOx stone formers.* There is more CMP in stone formers' kidneys compared with those from either normal men or normal women ($P < 0.01$). The difference between normal men and normal women failed to reach significance ($P = 0.11$).

glomerular portion of renal cortex. However, in this section the counterstain includes PAS, which stains carbohydrate moieties pink, and highlights the brush borders of the proximal tubular epithelium which are known to be absent from the distal tubular epithelium [15]. CMP is confirmed to be within the cytoplasm of distal tubular epithelial cells.

These findings were consolidated by the double labeling studies. EMA was noted to be distributed along the apical membranes of the distal convoluted tubule and collecting duct epithelia and absent from the glomeruli, proximal tubules, and the thick and thin limbs of the loops of Henle, as previously reported [16–19]. Figure 2c shows co-localization of CMP (red) and EMA (brown) within periglomerular tubular cells consistent with the DCT.

The distribution of CMP in the TALH is demonstrated in Figure 2d which shows the typical appearance of CMP labeled by the indirect immunoperoxidase method using DAB. On the basis of morphological criteria this brown stain is in a cortical TALH, and the tubule immediately below this is a collecting duct (CD), which is negative for CMP. Double labeling with anti-EMA Ab lends support to these findings. Figure 2f is an example of the double staining technique with the brown DAB label attached to EMA on the collecting duct epithelia, and the red stain precipitated to CMP using the alkaline phosphatase reaction. Clearly, they are in different parts of the nephron; CMP is in the medullary TALH [15] (note the greater cell height here compared to the cortical segment of TALH as shown in Fig. 2d) and the tubule without label above this by comparison is a thin limb of the loop of Henle. Red blood cells are visible in the vasa recta (VR), remaining lightly stained by the peroxidase reaction, despite pretreatment with hydrogen peroxide to reduce endogenous peroxidase activity.

Interestingly, CMP was not detected in all nephrons. In fact staining was heterogeneous amongst the nephron population, such that in any one individual, between 10% and 60% of the nephrons were positive. The proportion of positively stained nephrons was not changed by manipulating the conditions for

**Table 1.** Comparison of macromolecular inhibitors of calcium oxalate crystallization

| | Molecular weight *kDa* | Distribution in the nephron | Contains Gla | Inhibitory activity demonstrated in |
|---|---|---|---|---|
| Tamm-Horsfall glycoprotein | 90 [37, 38] | TALH (MD −ve) early DCT [31, 29] | No [39] | urine [28] |
| Nephrocalcin | 14 [32] | PT, TALH [33] | Yes [40] | inorganicsolutions[40] |
| Uropontin (mice) | 55 [34] | PT, TALH [34] | No [34] | inorganicsolutions[34] |
| Osteopontin (human) | 41.5;75 [44, 45] | DT, CD [36] | No [46] | |
| Crystal matrix protein | 31 [8] | TALH, DCT (MD +ve) | Yes [41] | urine [42] |

immuno-labeling, such as Ab dilution, formalin fixation time, time of development of the end product, etc. No one group, such as cortical or juxtamedullary nephrons, was consistently stained. Material within the lumina distal to the TALH was also noted to be positive for CMP, as one would expect with release of CMP into the urinary space. CMP was consistently demonstrated in the cells of the macula densa in those nephrons that were positively stained elsewhere (Fig. 2e).

The different methods of initial tissue processing did not alter the specific distribution of CMP within the kidney, although background staining was increased in tissue fixed in Zamboni's solution or snap frozen in liquid nitrogen.

Trypsinization of the archival sections did not influence the sites or the intensity of positive staining, indicating that the CMP antigen is not masked by protein cross-linking during fixation or processing of tissue in 10% formalin, 2.5% glutaraldahyde, or paraffin wax embedding.

CMP was not detected despite using varying Ab dilutions, in 27 separate human organs: renal pelvis, ureter, bladder, prostate, testis, epididymis, lung, liver, spleen, pancreas, oesophagus, stomach, jejunum, ileum, colon, skeletal muscle, smooth muscle, heart, skin, thyroid, parathyroid, bone marrow, tonsil, adrenal, uterus, breast, cerebral cortex. It is noteworthy that within the urinary system the kidney was the only tissue to stain positively for CMP.

### *Quantification of CMP in renal tissue*

The intensity of staining of CMP evident after tissue had been fixed in formalin for between two hours 48 minutes, and 48 hours showed no variation. Staining intensity was equal between the TALH and the DCT in 19 cases (42%), the TALH stained more than the DCT in nine cases (20%) and in the remaining 17 cases (38%) the DCT showed greater intensity of staining for CMP than the TALH. The mean scores for each group were: (1) normal males, TALH = 1.7, DCT = 1.8, (2) normal females, TALH = 1.2, DCT = 1.5, and (3) stone formers, TALH = 2.8, DCT = 3.8.

The combined histological quantification data are illustrated in Figure 3. Significantly less CMP was observed in normal women compared with stone formers ($P < 0.01$), and there was less CMP in normal men compared with stone formers ($P < 0.01$). The difference in the amount of CMP detected in kidneys from normal males, as opposed to normal females, failed to reach statistical significance ($P = 0.11$), although the trend suggested the occurrence of less CMP in the latter.

## Discussion

The consistent presence and disproportionate abundance of CMP within the structure of CaOx crystals generated from human urine suggest that the protein may fulfill an active role in CaOx crystallization, and perhaps, therefore, in CaOx stone disease. Using a polyclonal antibody of high specificity we have demonstrated the presence of CMP in the tubular epithelia of the TALH and the DCT of the human kidney. Its precise location in these structures was confirmed by simultaneous staining for EMA, which is known to be confined to the distal convoluted tubules and collecting ducts. CMP was also clearly seen within the cells of the macula densa, but whether it contributes to the function of this complex is not known. The definite location of CMP within the lumen adjacent and distal to these sites suggests that the protein is produced by and secreted from these cells into the urinary collecting system. Furthermore, the protein appears to be specific to the kidney, with none being detected elsewhere in the urinary tract or in other human tissues.

This very limited and precise location, both within the body and the kidney, lends credence to the possibility that it may fulfil some regulatory role in stone pathogenesis. The DCT has a large capacity for calcium absorption, reclaiming as much as 90% of the load presented to it [24], and this transport is active. Transport of calcium also occurs against an electrochemical gradient in the connecting tubule where vitamin D-stimulated calcium-binding protein is present [25]. Some authors have postulated that this portion of the nephron may be the control point for calcium excretion [26]. Given therefore that calcium transport is significantly controlled in this section and that luminal fluid is progressively concentrated dependent upon hydration both here and distally, calcium and oxalate concentrations may be maximal in the collecting ducts. It is noteworthy that histological studies of human kidneys have shown calcium mineral deposits in collecting ducts, but not in the more proximal nephron [27]. Therefore, it would be physiologically advantageous to minimize the risk of crystal precipitation in the collecting ducts by releasing an inhibitor of CaOx crystallization into the luminal fluid immediately upstream in the nephron. Nonetheless, although CMP would appear to fulfill both the anatomical and physicochemical criteria of a critical macromolecular determinant of CaOx stone disease, other urinary proteins can also claim such distinction, and it is salient to compare their properties with those of CMP.

Tamm-Horsfall glycoprotein (THG) is a potent inhibitor of CaOx crystallization in human urine at normal urine osmolality [28] and is localized to the distal tubule of the nephron [29–31]. Using a fully characterized monoclonal antibody, studies of its ultrastructural distribution showed that in the DCT, only the luminal plasmalemma is positive while the basolateral membranes and the cytoplasm are negative. In the epithelial cells of the TALH, on the other hand, THG is distributed throughout

the whole cell plasmalemma [29]. Our light microscopic findings of more centrally placed punctate staining for CMP within the cells of both of these segments demonstrate that the distribution of CMP is different from that of THG. This is supported by our finding that the cells of the macula densa contain CMP and are negative for THG [29]. It is apparent, therefore, that THG and CMP are distinct proteins and, if they are both involved in stone pathogenesis, their roles are unlikely to coincide.

Nephrocalcin (NC) is a 14 kDa glycoprotein which has been reported to inhibit CaOx crystal growth in inorganic solutions, but whose effects in undiluted urine have not been tested [32]. Using a rabbit polyclonal Ab, NC has been shown by Nakagawa and his colleagues to be present in the proximal tubules and the TALH epithelia of both human and mouse kidneys [33]. These authors have therefore proposed that NC protects against CaOx crystallization at two different sites within the nephron. The dissimilar distributions of CMP and NC within the nephron, and the disparity in their molecular weights suggests that they are different proteins and probably fulfill different functions.

More recently, another potentially important urinary protein has been described. Shiraga et al [34] isolated an aspartate-rich protein from urine, which they named uropontin, and showed to be an inhibitor of CaOx crystal growth in inorganic solutions. There is indirect evidence that it is synthesized by cells in the proximal and distal tubules and loops of Henle in mice. Given that there is significant sequence homology between uropontin and osteopontin [34], osteopontin's presence is implied in these areas by the detection of transcripts by Northern analysis using labeled probes encoding osteopontin [35]. Using immunohistochemistry and *in situ* hybridization, Brown et al [36] found osteopontin to be present in the distal tubules and collecting ducts of the human kidney, and also in the transitional epithelium of the renal pelvis and focally in the bladder. Uropontin has a molecular weight of 55 kDa and amino acid analysis reveals an absence of Gla [34], features that clearly distinguish it from CMP. The characteristics of these four known macromolecular inhibitors of calcium oxalate crystallization are summarized in Table 1.

If CMP is indeed a distinct urinary protein which fulfills some regulatory function in stone pathogenesis, we may reasonably expect that its quantity in kidneys from stone formers would differ from that in individuals who have never suffered from urolithiasis. Furthermore, since the incidence of calculi is some three times higher in men than women [43], this difference should be reflected in disparities between the amounts of the protein detected in the kidney tissue of the two sexes. CMP was detected in significantly greater amounts in sections of kidneys from stone formers than in those from individuals unaffected by the disease, and though the difference failed to attain statistical significance, the data also demonstrated a trend for normal males to have greater quantities of CMP than normal females. The observed quantity of CMP therefore mirrors the risk of stone disease, such that an increased amount of CMP correlates with an increased incidence of stone disease. These data appear to conflict with the fact that CMP is a potent inhibitor of CaOx crystal aggregation, where one might expect a greater risk of stone disease to result from a decreased level of urinary inhibitors. A number of explanations can be proposed for this apparent contradiction, including differences in molecular structure (and therefore functional activity), stimulation of the protein's production and secretion under conditions of high urinary supersaturation, and alterations in the transport mechanisms required to deliver the protein into the lumen.

In summary, this study has demonstrated the distribution of a newly described urinary protein, crystal matrix protein, within the TALH and the DCT of the human nephron, and its absence from other areas of the kidney and many other organs. The precise, limited distribution of CMP and its inhibitory effects on CaOx crystal growth and aggregation strongly suggest that it may play a pivotal role in stone matrix deposition, and further studies are clearly warranted to clarify its role in stone pathogenesis.

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*Reprint requests to Rosemary L. Ryall, Ph.D., Department of Surgery, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia.*

## Note added in proof

Since submission of this paper, the authors have shown that CMP is related to human prothrombin. Further analysis indicates that our anti-CMP antibody does stain the cytoplasm of hepatocytes, albeit with quite different characteristics than are observed in the kidney.

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