ANALYSIS OF TAMM HORSFALL PROTEIN
IN A RAT MODEL OF NEPHROLITHIASIS

By

JAYASHREE A. GOKHALE

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Jayashree A. Gokhale
This work is dedicated to my parents Parashuram and Usha Ranade and my husband Atul Gokhale.
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The involvement of Tamm Horsfall protein (THP) in kidney stone formation (nephrolithiasis) was investigated by using an in vivo rat model of nephrolithiasis as well as by examination in in vitro crystallization assays. Immunoperoxidase localization showed that THP associated with the calcium oxalate (CaOx) crystal deposits and appeared in the papillary tubules, where it is normally absent. However, there was no correlation between stone formation and the amount of THP excreted under these conditions. Analysis of THP from both models normal and nephrolithic rats revealed they were biochemically similar. It was observed that although the distribution pattern of THP was altered in these animals, the site of synthesis of THP remained unchanged, as shown by in situ hybridization. Thus from the in vivo analysis it was concluded that THP interacted with crystals and its overall expression remained unaltered.
In vitro crystallization assays performed in rat artificial urine with normal and low citrate revealed that THP purified from normal and nephrolithic rats inhibited crystal aggregation efficiently. These results indicated that under normal and stone forming conditions in vivo, THP, which had similar biochemical composition, interacted with the crystals in a similar fashion. Based on the in vivo and in vitro data it can be speculated that THP-crystal interaction is probably a purely physical phenomenon and it is most likely that THP adheres to crystals which were already retained in the renal tubules.

In vivo localization of a second protein, osteopontin (OPN), which has been shown to inhibit CaOx crystal nucleation in vitro, revealed that OPN also associated with the crystals and appeared abnormally in the papilla, at locations similar to THP. This association was more conspicuous and consistent than that of THP and the crystals. These results have shown for the first time that at least two proteins, THP and OPN, interact simultaneously with CaOx crystals during stone formation, although probably during different stages of crystallization. Taken together, these data emphasize that several factors act concomitantly to influence the stone formation process, and they need to be examined as single entities as well as in combination with each other.
CHAPTER 1
INTRODUCTION

Kidney stone formation or nephrolithiasis, a painful and debilitating malady, affects 1% of the population in industrialized nations, and can persist with serious medical consequences throughout a patient’s lifetime. Although stones can also contain many other components like phosphates and uric acid, the majority of the stones found in man are mainly composed of calcium oxalate (CaOx). As in any other biomineralization, either normal or pathological, the primary phenomenon underlying stone formation is crystallization of the mineral phase. Once the stable crystalline nuclei are formed in the urinary system, the decisive steps are the retention and aggregation of these crystals in the urinary system, leading to the formation of stones.

Due to the complex nature of the urinary environment, this process is under the constant influence of a variety of urinary components. These include ions like calcium and oxalate, small molecules like citrate, and macromolecules like lipids and proteins. For example, presence of high oxalate levels appears to be necessary for stone formation. These various factors interact with each other and with the crystals, and can potentially influence and modulate various stages of crystallization either directly or indirectly. Among these urinary modulators, proteins have received considerable attention for their role in stone formation.
Tamm Horsfall protein (THP), the most abundant protein in the normal urine, has been assessed extensively for its participation in this process. Over the past forty years different laboratories have focused on various properties of THP using different points of view, as well as diverse test systems. For example, one research group may have compared the effect of THP from normal subjects and stone-formers on CaOx in a particular assay system. A second group may have studied it in a different system and a third group may have examined only the biochemical properties of THP, purified from distinctly different sources. Consequently, the conclusions reached regarding its role as a modulator of stone formation are somewhat contradictory. For example, it has been shown to promote, inhibit, as well as have no effect on CaOx crystallization. In summary, its effect on the physical process of stone formation is still controversial and unclear. Therefore, the primary focus of this study was an in-depth analyses of THP under stone forming conditions.

The currently available information on CaOx-THP interaction is derived largely from human THP, and therefore mostly by *in vitro* experimentation. In contrast to *in vitro* assessment, *in vivo* experimentation can provide direct information which is difficult to obtain via *in vitro* methodology. However, at present, there is no known study of THP in an *in vivo* model. Based on this background, in order to understand the precise nature of the THP-crystal interaction, in this study, the analysis of THP was performed under *in vivo* stone-forming conditions. This goal was achieved by using a well defined rat model of nephrolithiasis, which closely mimics stone-forming conditions in humans. This task was made easier by the previous work on other aspects of nephrolithiasis in this system, in the laboratories of Drs. S. R. Khan and R. L. Hackett.
It seems probable that one of the reasons for the contradictory conclusions about THP found in the literature, is the lack of uniformity in assay systems. In this study, such disparity was eliminated by using a combination of \textit{in vivo} as well as \textit{in vitro} methods: THP was analyzed in rats with active deposition of CaOx crystals and in addition, it was also purified, biochemically characterized and tested in \textit{in vitro} CaOx aggregation assays.

The present study primarily addressed two basic questions:

1) Does THP interact with CaOx crystals?

2) What is the nature of this interaction?

This investigation can be defined by the following specific aims:

1. Purification and characterization of THP from normal rats, and its comparison to human THP;

2. Analysis of THP in a semi-acute rat nephrolithiasis model; and

3. Study of THP in a model with chronic hyperoxaluria and analysis by \textit{in vitro} aggregation assays.

Although in aims 2 and 3, two different protocols were used to simulate stone forming conditions, they were both aimed at inducing active crystal deposition in rats. Due to their particular features, it was possible to obtain different types of information from the two models. Based on the literature, it appears that if THP is at all involved with the development of stones, most likely it associates with the aggregation step of the crystallization process. This assumption was the basis for the \textit{in vitro} crystal aggregation assays.

Currently, in addition to THP, there are at least 6 other proteins under scrutiny for their involvement in stone formation. Is it possible that more than one protein is
simultaneously involved with this process? This possibility was explored in aim 3, by examining a second protein, osteopontin, under stone-forming conditions. Within the limits of this study, however, the analysis of osteopontin was limited to its \textit{in vivo} localization only.

This thesis is organized as follows:

(i) Introductory \textit{Chapter 1}

(ii) A brief overview of kidney structure and processes involved in nephrolithiasis is presented in \textit{Chapter 2}. In addition to discussion of the rat and human kidney structure and function, this chapter discusses renal handling of calcium, oxalate and citrate, the three ions in the urine most relevant to this study.

(iii) In \textit{Chapter 3}, a literature review on urinary proteins currently under investigation for involvement in stone formation is presented.

(iv) In \textit{Chapter 4}, isolation and characterization of normal rat THP and its comparison to human THP is described.

(v) \textit{Chapter 5} deals with the semi-acute nephrolithiasis model. The analysis in this model included purification and partial biochemical characterization of THP, scanning microscopic structural profile of crystal deposition, examination of kidneys for crystal deposition, and immunocytochemical staining for THP. Quantitative determination of urinary THP excretion during crystal deposition is also discussed.

(vi) \textit{Chapter 6} describes the experiments performed with the chronic model, which include purification and detailed biochemical analysis of THP, immunocytochemical localization, and \textit{in situ} hybridization for localization of mRNA. In addition, the effect of THP on
crystal aggregation was assessed by spectrophotometry-based assays. Localization of a second protein, osteopontin, is also included in this section.

(vii) Chapter 7 presents a general discussion applicable to the entire study.
CHAPTER 2
NEPHROLITHIASIS AND THE KIDNEY: AN OVERVIEW

The two major objectives of this chapter are as follows: 1) to familiarize the reader with salient features of the structure and function of normal mammalian kidney. Since this study utilizes a rat model of nephrolithiasis which simulates stone formation in humans, the description particularly includes information about rat kidney and its environment. 2) to present a brief account of the physico-chemical events and factors underpinning the process of stone formation relevant to this study.

Anatomy of the Kidney

The extensive information regarding various anatomical, biochemical and functional features of the kidney obtained from rat is largely applicable to the human kidney. Consequently, as it will be discussed in the later chapters, rat provides an ideal in vivo system to study kidney stone formation in humans. Both the primary structure and function of human and rat kidney are very similar, except for the number of papillae. A comparison of some of the features of human and rat kidney is shown in Table 2.1. A complete discussion about rat kidney structure has been published by Bachmann et al. (1988).
Table 2.1

Comparison of physical features of human and rat kidney

<table>
<thead>
<tr>
<th></th>
<th>Size</th>
<th>Weight (gm)</th>
<th>Number of Papillae</th>
<th>Number of Nephrons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>12x6x4 cm</td>
<td>160-175</td>
<td>5-6</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Rat</td>
<td>2x1x0.5 cm</td>
<td>2-3</td>
<td>1</td>
<td>30-35000</td>
</tr>
</tbody>
</table>
In general, the mammalian kidney can be broadly divided into cortex, medulla (inner and outer) and the papilla in which the urine is most concentrated. The nephron (Figure 2.1), the structural and functional unit of the kidney, starts in the cortex with the renal corpuscle or the glomerulus (G). The tubular section of the nephron consists of the proximal convoluted tubule (PT), the loop of Henle, and the distal convoluted tubule (DT). The loop of Henle in turn is made up of the thick and thin descending limbs, the loop, thin and thick ascending limbs (TALs), in that order. As shown in Figure 2.2, based on their anatomical position in the kidney, nephrons can be superficial (closer to the periphery), or embedded deeper into the kidney. In addition, they can have either short or long loops. Due to these differences, although each nephron probably functions in a similar manner, they may appear slightly different in a kidney cross section.

In contrast to the long loops that extend into the papilla, all short loops return within the outer medulla and the transition of thick to thin limb occurs at the turning point. Due to the difference in the loop lengths, the number of loops gradually decrease with only 250 in the last millimeter of the papilla. The thick ascending limb (TAL) passes through the inner and outer medullary region near the proximity of the collecting ducts and enters the medullary cortex. It contacts the glomerulus at the macula densa and ends abruptly with the beginning of the distal convoluted tubule. Consequently, TALs are restricted to the cortex and medulla, and are absent in the papilla.

The last segment of the nephron is the collecting duct (CD). This segment establishes the link between the distal convoluted tubule and the collecting duct system. Anatomically, the structure of the connecting tubules for the superficial and deep
Figure 2.1 Various segments of a nephron. G: glomerulus, PT: proximal tubule, TAL: thick ascending limb, TL: thin loop, DT: distal tubule, CD: collecting duct. (Taken from Dousa, 1978)

Figure 2.2 Diagram of a kidney cross section showing the position of two nephrons with long loop (LP) and short loop (SP). (Taken from Hogg R. J. and Kokko J. P., 1979)
nephrons is different: in contrast to the unbranched tubules of superficial nephrons draining into a single nephron, the branched tubules of the deep nephrons merge to drain into the collecting duct. On an average, in the rat kidney, six nephrons drain into a single collecting duct. Whether all nephrons are functional at all times or only a percentage of them is adequate to carry out the necessary function is not known at this time.

**Ultrastructure and Function**

The mechanism by which urine is processed in the mammalian kidney has been reviewed in detail by Hogg and Kokko (1986). Along the length of the nephron, the cell types show specific changes corresponding to their specialized functions.

**Glomerulus**

The first step in processing the urine is filtration through the glomeruli, situated in the cortical area. However, kidney function occurs successfully in some vertebrates which lack glomeruli (Dousa, 1978). In Figure 2.3, a light micrograph of a cross-section through a normal rat kidney containing the glomeruli and the surrounding tubules is shown. The glomerulus is composed of anastomosing capillaries and consists of three basic cell types: 1) endothelial cells with small nuclei and peripheral cytoplasm line the capillaries, 2) mesangial cells form the interstitial (supporting) portion of the glomerulus and do not communicate directly with the vascular or urinary space. They are associated with an extracellular basement membrane-like matrix containing mucopolysaccharides and glycoproteins, and 3) epithelial cells can be divided into visceral (podocytes) and parietal
Figure 2.3 Light micrograph of a cross section through the cortical area of a rat kidney. G: glomerulus, PT: proximal tubule, CD: collecting duct.

Figure 2.4 Scanning electron micrograph of a glomerulus showing intercalated foot processes (FP) of podocytes forming the filtration slits (FS).
types. The podocytes, as shown in the scanning micrograph in Figure 2.4, are associated with the non-vascular side of the capillary wall and have extended foot processes or pedicles. The ultrastructural details of this area are shown in Figure 2.5. As shown in this figure, the cytoplasmic extensions of these cells are intercalated to form the filtration slits or the fenestrae through which the filtration of the urine takes place.

**Tubular Segments**

Although glomerular filtration is the first step in the formation of the final urine, the indispensable and specialized component of the excretory function of the kidney is the tubular segment. The specific functions are accomplished by diversification of ultrastructure, basic processes involved, and the sequential arrangement of various tubular functions along the length of the nephron (Dousa, 1978; Holley, 1978; Hogg and Kokko, 1986). The two basic mechanisms involved in processing of the urine are re-absorption and secretion. These are conducted via a combination of active as well as passive transport processes. Active transport, which is a localized phenomenon, involves transport of electrolytes and non-polar solutes across the cellular membranes, against either a chemical or electrical gradient. The energy (ATP) required for this transport is provided by the biochemical reactions in the tubular cells. In contrast to active transport, for the passive transport or diffusion, the energy is supplied by the chemical gradient in the same direction as the transport.

The proximal tubule, which is primarily involved in re-absorption of a large volume of fluid, is lined with cells with numerous microvilli (brush border) which
Figure 2.5 Transmission electron micrograph through a glomerulus showing basement membrane (BM), podocyte (P), foot processes (FP), endothelial cells (EC), fenestrae (F) and filtration slits (FS).

Figure 2.6 Transmission electron micrograph showing the deep complex invaginations of the plasma membrane (PM) that enclose the mitochondria (M), similar to that seen in the thick ascending limb.
increase its surface area for maximum re-absorption. These cells are packed with mitochondria, which are necessary for active transport. In this portion, important constituents of glomerular filtrate including glucose, proteins, amino acids, salts, such as phosphate and other components, such as urea, uric acid, etc., are reabsorbed along with water. No evidence of lateral movement of proteins has been found in this segment (Bode et al., 1980).

In the thin loop of Henle, movement of sodium occurs via passive transport. Correspondingly, cells lining this segment are flat and contain very few mitochondria. In addition, this segment is characterized by numerous tight junctions, and membranous labyrinth formed by the interdigitation of the basolateral membrane. The thin loop is one of the sites where osteopontin (OPN), one of the urinary proteins associated with stone formation, has been localized (Kleinman et al., 1995).

Sodium chloride is removed from the glomerular filtrate by the cortical as well as the medullary portion of the thick ascending limb, by active transport, although no significant movement of water takes place in this area. The cortical portion shows thin epithelium and therefore fewer vesicles and mitochondria. In contrast, the medullary region has a thick epithelium packed with mitochondria and a well developed vesicular compartment, as shown in Figure 2.6. In addition to removal of solute, one of the features of the thick ascending limb is complete impermeability to the osmotic flow of water. Tamm Horsfall protein (THP), which is synthesized by these cells, is a specific marker for identification of this segment.
The structure of the distal tubule is somewhat similar to the TAL. High Na-K-ATPase activity takes place in these cells, which are characterized by tall cells, numerous mitochondria and many short microvilli on the luminal membrane.

In conclusion, bulk re-absorption of the glomerular filtrate takes place in the proximal tubule, followed by dilution in the loop of Henle. Next, in the distal nephron, osmotic concentration of the urine takes place through sodium and potassium excretion, and pH is regulated via adjustment of hydrogen and ammonium ions. In addition, re-absorption of water, sodium and urea occurs here.

All of the known urinary proteins discussed in the next chapter are synthesized along the tubular portion of the nephron by different cell populations. The sites of synthesis of these proteins are described in the next chapter. Since all of them primarily are secreted proteins, it is reasonable to assume that they are directly released in the urinary flow, where they have the opportunity to interact with crystal as well as cells along the nephron.

Urine Composition and Renal Handling of its Components

In its final processed form, urine is an extremely complex solution of water, ions, inorganic salts, small molecules such as citrate and phosphate, macromolecules such as proteins and lipids, and various other metabolites. Kidney stone formation, or nephrolithiasis, is a multifactorial phenomenon influenced by all the components mentioned above, some affecting this process more significantly than the others. Nevertheless, a primary requirement for stone formation is supersaturation with respect to
the inorganic salts such as CaOx, which then allows precipitation of the mineral phase and formation of crystals. A comparison of human and rat normal urinary ionic composition is shown in Table 2.2. as seen in the table, calcium in humans is 1.7 fold higher than in rats, oxalate in rats is 3 fold higher than in humans, and citrate in rats is approximately 5 fold higher than in humans.

With respect to the mineral phase, four major abnormalities have been identified in stone-formers, which are usually present in combination: 1) hyperoxaluria (excess oxalate), 2) hypercalciuria (excess calcium) 3) hypocitrauria (reduced citrate) and 4) hyperuricoseuria (excess uric acid). Other ions shown in Table 2.2 also influence stone formation to some extent. For example, magnesium, which is not a focus of this study, can affect the concentration of oxalate significantly. In a study of weanling rats on a magnesium-deficient diet, hyperoxaluria was induced by significantly increasing endogenous production of oxalate in the liver and kidneys (Rattan et al., 1994). However, within the scope of this study, only the significance of the first three ions (oxalate, calcium and citrate) will be discussed below.

Oxalate

It is an established fact that supersaturation with respect to minerals is a primary requirement for stone formation but not sufficient by itself to cause the development of stones (Coe et al., 1979). The source of oxalate in the body can be either endogenous as synthesized by the cells, or exogenous as supplied by the diet. Excess oxalate from the diet can contribute significantly in inducing hyperoxaluria. An absorption kinetic study
Table 2.2

A comparison of ionic composition of human and rat urine

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>Ox</th>
<th>Citrate</th>
<th>NaCl</th>
<th>KCl</th>
<th>MgSO₄</th>
<th>NaH₂PO₄</th>
<th>Na₂SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>5.75</td>
<td>0.318</td>
<td>3.21</td>
<td>105.5</td>
<td>63.7</td>
<td>3.85</td>
<td>32.3</td>
<td>16.95</td>
</tr>
<tr>
<td>Normal</td>
<td>3.39</td>
<td>1.05</td>
<td>16.8</td>
<td>149.0</td>
<td>26.7</td>
<td>21.4</td>
<td>35.8</td>
<td>29.3</td>
</tr>
</tbody>
</table>

* Burn and Finlayson (1980)
**Shevock et al. (1993)
on oxalate suggests that in addition to the small intestine, the stomach may be a critical site for absorption of dietary oxalate (Hautmann, 1993).

Although it is known that oxalate metabolism in humans and rats is similar, mechanisms of oxalate metabolism including transport and excretion are not completely understood. Several separate clinical studies have established that, in general, stone-formers have a higher oxalate level in the plasma and consequently in the urine. For example, in one study, the mean oxalate excretion of normal subjects was 0.28 mM per 24 hours (urine volume=1.5 lit), compared to 0.59 ± 0.03 mM in stone formers (Hatch et al., 1991). In another study, it was demonstrated that in one group of patients the hyperoxaluria could be attributed an increase in dietary oxalate and a decrease in renal clearance. In contrast, in a second group, increased endogenous production of oxalate was identified as the cause of hyperoxaluria (Hatch, 1993).

In contrast to the observations described above, work of Sutton et al. (1994) has shown that although the plasma oxalate levels in normal subjects and stone formers was the same (2.2 ± 0.2 and 1.9 ± 0.1 µm/L), there was significant difference in the urinary oxalate levels (294 ± 26 versus 368 ± 26 µM). This increase also correlated with increase in glycolate (399 ± 32 versus 601 ± 111), a precursor of oxalate (Sutton et al., 1994). In spite of the discrepancy in the results of oxalate levels in plasma in the two studies mentioned above, it is well established that the presence of hyperoxaluria is one of the primary signs for possible development of stones in a subject. The cause of hyperoxaluria in some stone-formers can be partially explained by the observations of Baggio et al. (1994). They found that in addition to a higher trans-membrane oxalate exchange in
stone-formers, the cell membrane band 3 protein phosphorylation was also altered in these patients.

Papillary encrustation and necrosis is a common observation in stone-forming patients and experimental nephrolithic animals, and the papilla plays an important role in experimental CaOx lithiasis (Khan et al. 1982; 1984). In a comprehensive study of distribution of calcium and oxalate in human kidney tissue, it has been shown that the maximum concentration of both calcium and oxalate exists in the papilla (ducts of Bellini) or inner medulla (Hautmann and Osswald, 1980; Hautmann et al., 1980). Such high concentration may be due to increased absorption of oxalate by the papillary epithelial cells as found by Chandhoke and Hruska (1994).

Calcium and oxalate concentrations in the urine affect each other significantly. For example, a cell culture study of normal rat kidney papillary cells showed that incubation with 400 µM of sodium oxalate for 60 minutes induced reduction of ionized calcium in the cells (Yamagawa et al., 1994). The authors speculated that oxalate complexes with calcium, thereby reducing the free calcium concentration in the system. Other additional factors that influence the total oxalate concentrations in the urine include the urine volume and flow rate. In summary, oxalate concentration, transport and metabolism are important factors in the development of stones.

In experimental models, hyperoxaluria can be induced by increasing any of the precursors of oxalate in its metabolic pathway. Ionized oxalate can diffuse freely through biological membranes. Microtubule puncture experiments with rats have shown that under normal physiological conditions oxalate is filtered through the glomerulus, secreted
in the proximal tubule and very little is reabsorbed in the tubule (Greger et al., 1978; Hautman and Osswald, 1978; 1980) and a similar mechanism occurs in man. Since concentration of oxalate in the urine is dependent upon total urinary volume, a lowering of urinary flow rate, and therefore a low volume results in increased oxalate concentration.

Calcium

The normal concentration of calcium in the plasma is 8.9-10.1 mg/ml. After ultrafiltration through the glomerulus, it is reduced to 55%, and finally only 0.5-2% is excreted in the urine (Duarte and Knox, 1978). Prior to this, the proximal tubule is a site of major reabsorption for calcium. For example, under experimental conditions of intravenous loading it has been shown that phosphate overload can lead to deposition of calcium in the tissues.

Hypercalciuria is defined as urinary excretion of more than 0.1 mmol/Ca/Kg/day (4 mg/Kg/24 hrs), and is a common occurrence in stone-formers (Lemann et al., 1991). It can also exist as a familial trait which appears to be transmitted as an autosomal dominant trait (Coe et al., 1979). There is extensive experimental evidence to demonstrate that calcium binds to oxalate, thus reducing the concentration of free oxalate in the system. A study of normal subjects and stone-formers showed that there was a significant correlation between calcium and oxalate excretion, and recurrent stone-formers may have an abnormality of oxalate absorption in relation to calcium absorption (Koide et al., 1985). Calcium also appears to influence the type of oxalate crystals in stone patients.
Conte et al. (1990) observed that in patients with calcium levels in the normal range, 
CaOx monohydrate was more frequent than the dihydrate, which was more common in 
hypercalciuric patients.

Although hyperoxaluria is a crucial factor in the development of renal stones, 
calcium metabolism has a tremendous effect on oxalate kinetics. For example, when rats 
were perfused with saline containing $^{14}$C-labeled oxalate but no calcium, a strong 
unidirectional oxalate flux could be demonstrated (Hautmann and Osswald, 1983). A 
dietary study in 34 healthy adults has shown that when fed with a low calcium diet, 
urinary oxalate excretion increased from $0.31\pm 0.08$ mM per day to $0.45\pm 0.09$ mM per day 
(Zarembski and Hodgkinson, 1969). Similarly, a separate study with a larger sample size 
(n=106) also showed that daily urinary excretion was inversely related to the calcium 
intake, thus indicating that calcium present in the gut binds to oxalate, thus making it 
available for absorption (Lemann et al., 1991).

Citrate

In the plasma, the concentration of citrate in humans and other mammals ranges 
from 0.05-0.3 mmoles/liter (Simpson, 1983). It exists as an alkaline citrate$^{-3}$ (molecular 
weight 189) at the physiological pH in the urinary environment. It is a component of the 
energy-yielding citric acid cycle, which is the main metabolic pathway for oxidation of 
carbohydrates, fats and some amino acids to carbon dioxide and water. As discussed in 
detail by Berg (1990), in humans citrate is freely filtered through the glomerulus and a 
large portion (approximately 75%) is re-absorbed in the proximal tubule, and a similar
processing occurs in rat. The acid-base state of the urine exerts substantial influence on
the excretion of citrate as well as its renal concentration. For example, a major cause of
hypocitriauria in stone patients is acidosis, which reduces urinary citrate via enhancing
renal tubular re-absorption and impairing uptake (Jenkins et al., 1985).

The clinical implication of lower than normal urinary citrate (hypocitriauria) was
established when a comparison of normal subjects and stone-formers showed the stone
formers excreted significantly reduced citrate (Schwille et al., 1982; Kok et al., 1987a;
Minisola et al., 1989). The relationship between hypocitriauria and hypercalciuria was
evident when it was found that patients with hypocitriauria almost always have
hypercalciuria The importance of citrate was further emphasized when treatment of
hypocitriauria resulted in slowing down or even eliminating kidney stone formation (Pak
et al., 1985a; Pak, 1994).

Within the limits of its urinary concentration range, citrate has been shown to
inhibit spontaneous precipitation of calcium oxalate in vitro and to moreover directly
affect nucleation (Nicar et al., 1987). In addition, citrate reduced crystallization indirectly
by complexing oxalate and reducing its saturation. Citrate has also been shown to inhibit
CaOx growth as well as aggregation (Kok, 1991). Thus at least by in vitro experiments it
appears it appears that citrate may modulate crystallization at all three stages and low
urinary citrate promotes stone formation.

As discussed in Chapter 4, in spite of the high oxalate levels that could promote
complexation with calcium and therefore stone formation, rats rarely form stones
naturally. One of the explanations could be that the high level of citrate may at least partially compensate for the high level of oxalate by complexing with calcium.

In addition to the three ions discussed above, urinary pH clearly influences crystallization. In a study of patients with recurrent oxalate stones and hypocitruria, Pak and Fuller (1986) found that treatment with potassium citrate maintained pH at the normal range of 6.5-7.0. It also restored normal citrate excretion and reduced incidence of new stone formation by 89%.

**Process of Crystallization**

As mentioned earlier, the primary phenomenon underlying biological mineralization is the process of crystallization. Biological crystallization can be either normal or pathological. An example of normal crystallization is the formation of bone, which is a programmed, controlled process. In contrast, kidney stone formation is a result of an abnormal occurrence and/or imbalance of one or more components of the urinary environment. None of the biological environments where crystallization occurs are ‘pure solutions’ of minerals, and therefore the outcome of the process is a result of the interaction of various components of that system. Of the various components, proteins are among the most active macromolecules which act as controllers of crystallization (Weiner and Addadi, 1991). Whether crystallization occurs as a normal or abnormal phenomenon, the basic process can be divided into three stages: a) nucleation, 2) growth and 3) aggregation. A brief discussion of these three stages of crystallization is given below.
Figure 2.7 The classical schematic representation of the overall free energy change during nucleation as a function of nucleus size. The overall free energy change (middle curve) is a result of two opposing changes: an increase due to creation of a new interface (top curve) and a decrease due to volume free energy change (bottom curve). The maximum point on the middle curve represents the critical barrier to nucleation or the degree of supersaturation required before nucleation can occur. The nuclear radius at this point is known as the critical radius, because addition of atoms/molecules will decrease the free energy. (Taken from Porter and Easterling, 1981).
Figure 2.8 Comparison of the energy barriers to homogeneous and heterogeneous nucleation as a function of nucleus size ($r$). Note that the energy barrier (equivalent to degree of supersaturation) for heterogeneous nucleation can be several orders of magnitude lower than that for homogeneous nucleation. $DG$ represents the Gibbs free energy change. The subscripts denote heterogeneous (het) and homogeneous (hom), while "*" denotes a critical value of the parameters. (Taken from Porter and Easterling, 1981).
Nucleation

In a system, crystal nuclei can form only after overcoming the energy barrier. Figure 2.7 depicts the general energy requirements for nucleation (Porter and Easterling, 1981). Based on the laws of thermodynamics, any system will try to reach the lowest possible free energy state. One of the primary requirements for crystallization to begin is that the system must be supersaturated with respect to a particular mineral component. Saturation provides the driving force to reach the lowest-energy state. At present, all forms of current treatments for prevention of stone formation (by preventing nucleation) are aimed at reducing saturation levels to some extent (Smith, 1990). Nucleation, which can be seen visually as an increase in turbidity of a clear solution, causes increase in surface area, thus meeting the requirement for a lower energy level. In the system, however, certain 'metastable' areas may exist which, although locally saturated, will not induce nucleation.

Theoretically, in such a system, nucleation can be either homogenous or heterogeneous, the energy requirements for which are shown in Figure 2.8. For homogenous nucleation to occur, the system should be absolutely pure (no contaminants) and the requirement for the level of supersaturation is very high. In a biological system, it is unlikely that these criteria will be satisfied. Therefore, in a biological system, nucleation is almost always heterogeneous. The site for heterogeneous nucleation can be provided by either other existing crystals, degraded cellular components such as cell membranes or even the glass walls of the reaction vessel in the case of an in vitro experimental set-up. Consequently, as shown in Figure 2.8, if there are pre-existing
crystals or other nucleants present in the system, lower supersaturation levels are sufficient to induce nucleation.

In the case of calcium oxalate crystallization, the trihydrate form of the salt will form first, which transforms into the monohydrate form. This is because although kinetically the trihydrate form is favored, thermodynamically the monohydrate form is more stable as shown by the rarity of trihydrate crystals in stones (Heijnen et al., 1985).

Growth

Once stable crystalline nuclei are formed, crystal growth can begin to take place, to lower the energy level even further. Growth occurs by deposition of the solute on the nuclei, and therefore can take place on the same type of nucleus (CaOx on CaOx) or on a different type (calcium oxalate on calcium phosphate nuclei). Therefore, stones with more than one type of crystal are a common occurrence. In addition, when crystals of various sizes are present in a solution, growth of the larger crystals can occur by deposition of smaller crystals on their surface. In this case, which is also known as 'coarsening' of particles, the total crystalline mass in the system remains the same.

In a single crystal, there are specific growth sites or facets, which determine the shape and size of a particular crystal. Crystal growth kinetics have been examined with a variety of matrix components, either as single factors, or as a combined matrix (Nawrot et al., 1976; Wheeler et al., 1981) or in combination (Termine et al., 1981). In a study of calcite crystals, Addadi and Weiner (1985) determined that acidic proteins from mollusks interacted with certain faces of these crystals during their growth. These interacting faces
exhibited a common stereochemical property. As discussed by these authors and shown in Figure 2.9, in general, when the protein binds to the growth facets of a crystal, the growth in that particular direction is inhibited. On the other hand, the facets which are free of proteins continue to grow, thus changing the shape of the crystal. More specifically, in a study of CaOx monohydrate crystals and nephrocalcin, Deganello (1991) showed that nephrocalcin interacted specifically with the 101 plane of the crystals, thus blocking this growth site and inhibiting crystal growth in this particular direction. In summary, in contrast to its absorption on non-growth sites, binding of a macromolecule such as a protein to a specific growth site of the crystal can significantly affect growth.

**Aggregation**

In this process, already formed crystal adhere to each other and agglomerate to form larger masses. The difference between normal subjects and stone-formers is not the presence or absence of crystals per se, but more importantly, the size of their aggregates. In comparison to nucleation and growth, little is known about mechanisms by which aggregation can occur. Rumpf and Schubert (1977) have categorized the underlying mechanisms into 6 basic types: electrostatic attraction, Van der Waals forces, liquid bridging, capillarity, viscous binder effects and solid bridging. Of these, electrostatic forces have the highest energy and solid bridging has the lowest inherent energy. The same limited information can be applied to crystal aggregation during kidney stone formation (Brown and Purich, 1992).
Figure 2.9 Effect of protein adsorption on crystal growth
a: Protein adsorbed to growth facets A and B
b: Crystal shape changes because growth at A,B facets is inhibited

Figure 2.10 Schematic of one of the mechanisms by which crystal aggregation may occur.
a: CaOx crystals with negative surface charge
b: Protein with net positive charge
c: Aggregation by macromolecule bridging
A schematic of one of the mechanisms by which aggregation can occur in the presence of macromolecules like proteins is shown in Figure 2.10. Theoretically, as shown in the figure, when proteins, that have an overall positive charge can adsorb to the crystals and can mediate aggregation. This effect, however, can be changed significantly by factors like pH, which will affect the net charge on the protein. In addition, depending on the amount of protein adsorbed and other factors, the same protein may facilitate separation of the crystals.

Kok (1990) has demonstrated that once the crystals are formed, aggregation of crystals is a major element (Kok, 1991; Kok and Khan, 1994) and contributes to stone formation far more than crystal growth. This is due to the fact that in real time, crystal growth is too slow to cause any substantial increase in mass, whereas the aggregation can occur comparatively quickly forming large deposits, which occlude the tubules (Finlayson, 1978). These large masses can cause extensive damage to the tubular cells and start a vicious cycle of damage and dysfunction.

During stone formation, macromolecules and cells of the renal system contribute significantly in the regulation of this process. All the three stages of crystallization described above are constantly subjected to the various components of the urinary environment. This includes factors such as composition and pH of the urine, number and size of particles, macromolecules, and alterations in cell surfaces of the urinary epithelium. The salts involved in stone formation are extremely insoluble, and the driving force leading to crystallization of these salts is significantly influenced by the relative supersaturation (RSS) of the salt. Studies of Khan et al (1984) have shown that sub-
**Table 2.3**

Currently used *in vitro* methods for the study of crystallization

<table>
<thead>
<tr>
<th>Criteria Measured</th>
<th>Method</th>
<th>Total Assay Time</th>
<th>Reported by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleation</td>
<td>Turbidity</td>
<td>Particle Size</td>
<td>Up to 600 seconds</td>
</tr>
<tr>
<td>Growth</td>
<td>Depletion (Uptake) of labeled components ex: oxalate</td>
<td>Radioactivity Measurement</td>
<td>a) 40 minutes-24 hours</td>
</tr>
<tr>
<td>Aggregation</td>
<td>Particle Size</td>
<td>a) Light Scattering</td>
<td>a) Several minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b) Optical density</td>
<td>b) 8 minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Particle size)</td>
<td></td>
</tr>
</tbody>
</table>
cutaneous implants of potassium oxalate mini-osmotic pumps in rats resulted in CaOx crystals and high supersaturation for CaOx deposits. The higher the RSS, the greater the probability and rate of these processes (Brown and Purich, 1992).

In clinical terms, to treat stones successfully it is important to predict if recurrence is likely in a patient and to focus on inhibition of growth of existing stones, as well as prevention of formation of new stones (Kupin 1995). In experimental terms, from the discussion above it is clear that it is crucial to separate and analyze all three stages of crystallization. Table 2.3 shows current methods used to study these stages as separate phenomenon.

**Kidney Stone Composition**

During the formation of crystals and their aggregates, various inorganic (crystalline) as well as organic components of the urinary environment are incorporated in them, resulting in the final form: a stone. As reviewed by Kok, based on the type of crystals, more than 50 types of stones have been identified (Kok, 1991). Almost all stones contain more than one crystal type. For example, a mixture of calcium oxalate and calcium phosphate is quite common. Of the different type of crystals that can be found in stones, about 80% of the stones found in man contain calcium oxalate, making it the most common crystal type present in the stones.

The kidney stone matrix, which is a collective term for the organic phase associated with the stone, may include proteins (Khan et al., 1983), lipids (Khan et al., 1988), and cellular components such as membranes and so on. The matrix is present ubiquitously in all types of stones, regardless of their crystal type (calcium phosphate,
calcium oxalate, uric acid etc.). It has been demonstrated by Khan and Hackett (1993) that the organic matrix is closely associated with crystals during formation and growth. This suggests that the matrix has a crucial role in the formation of stones.

Typically, it has been seen that the matrix is a mucoprotein-polysaccharide complex which constitutes 2-3% of the total weight of the stone (Boyce and Garvey, 1956) of which 65% are mucoproteins (King and Boyce, 1959; Spector et al., 1976). Similar to other existing physiological biomineralization systems, acidic proteins are present in fairly high amounts (Butler, 1989).

By studying the composition of the matrix, a great deal of information can be obtained about the events leading to stone formation. For example, the mere presence of a component like protein in the matrix may suggest a function for that protein. However, a major question to be answered is whether the matrix actually participated in the crystallization process, or if it was incorporated in the crystals by chance. Consequently, the same question can be applied to the individual components of the matrix. Nevertheless, as will be seen in the next chapter, for most of the urinary proteins (THP, Opn, CMP) their identification in the stones has been an important clue for their involvement in stone formation. A detailed review of proteins currently under investigation for their involvement in stone formation is the subject of the next chapter.
CHAPTER 3
REVIEW OF LITERATURE

As mentioned in the previous chapter, urinary proteins have received considerable attention for their participation in kidney stone formation. This chapter will include a discussion about their occurrence, biochemical properties and basis for involvement in nephrolithiasis and other renal diseases, if any. Table 3.1 summarizes some of the characteristic features of seven proteins currently under investigation. Almost all of the proteins, with two exceptions, have been identified in stones, thus indicating that they exist as a part of the complex organic matrix. As shown in the table, all except prothrombin are synthesized in various segments of the nephron, and the sites of synthesis overlap in some cases. Note that their molecular weights range from 14 to 90 Kd and therefore the size of the protein does not seem to affect its ability to influence stone formation. An important biochemical feature that can be seen in all of the proteins in this list is the high percentage of acidic amino acids (aspartic and glutamic), especially in osteopontin, making it a classic acidic protein.

In the discussion presented below, although seven urinary proteins are included, emphasis will be given on details about Tamm Horsfall protein (THP), nephrocalcin (NC) and osteopontin (OPN), the three most prominent proteins currently under investigation.
### Table 3.1

Proteins currently under investigation for involvement in stone formation  
* Gamma-carboxy-glutamic acid

<table>
<thead>
<tr>
<th>#</th>
<th>Protein</th>
<th>Mw (Kd)</th>
<th>Site In The Nephron</th>
<th>Unique Feature</th>
<th>Primary Contributors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nephrocalcin</td>
<td>14 PT</td>
<td>TAL</td>
<td>Acidic AA, γCG* Acid</td>
<td>Coe/Nakagawa et al. 1987</td>
</tr>
<tr>
<td>2</td>
<td>Inhibitor</td>
<td>30 Not Known</td>
<td>Fragment of UTI?</td>
<td></td>
<td>Sorensen et al. 1990</td>
</tr>
<tr>
<td>3</td>
<td>Crystal Matrix Protein/Prothrombin</td>
<td>31 TAL</td>
<td>MD</td>
<td>γCG* Acid</td>
<td>Ryall et al. 1991/Stapleton et al. 1993</td>
</tr>
<tr>
<td>4</td>
<td>Uronic Acid Rich Protein</td>
<td>35 Not Known</td>
<td>Not Known</td>
<td>Not Known</td>
<td>Atmani et al. 1993a</td>
</tr>
<tr>
<td>5</td>
<td>Osteopontin</td>
<td>41.5 DT</td>
<td>CD</td>
<td>Aspartic Acid Rich (20%)</td>
<td>Worcester et al. 1992</td>
</tr>
<tr>
<td>6</td>
<td>Uropontin</td>
<td>55 PT</td>
<td>TAL</td>
<td>Aspartic Acid Rich (22%)</td>
<td>Shiraga et al. 1992</td>
</tr>
<tr>
<td>7</td>
<td>Tamm Horsfall Protein</td>
<td>90 TAL</td>
<td></td>
<td>Acidic (Asp 12%)</td>
<td>1955 onwards</td>
</tr>
</tbody>
</table>
Table 3.2

Data showing evidence of involvement of various proteins in the stone formation process.

<table>
<thead>
<tr>
<th>#</th>
<th>Protein</th>
<th>Found in Stones?</th>
<th>Inhibitor Of</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nephrocalcin</td>
<td>Yes</td>
<td>Growth, Nucleation, Aggregation</td>
</tr>
<tr>
<td>2</td>
<td>Inhibitor</td>
<td>Not Known</td>
<td>Growth</td>
</tr>
<tr>
<td>3</td>
<td>Crystal Matrix Protein/Prothrombin</td>
<td>Yes</td>
<td>Aggregation</td>
</tr>
<tr>
<td>4</td>
<td>Uronic Acid Rich Protein</td>
<td>Not Known</td>
<td>Growth</td>
</tr>
<tr>
<td>5</td>
<td>Osteopontin</td>
<td>yes</td>
<td>Nucleation</td>
</tr>
<tr>
<td>6</td>
<td>Uropontin</td>
<td>Yes</td>
<td>Growth</td>
</tr>
<tr>
<td>7</td>
<td>Tamm Horsfall Protein</td>
<td>Yes</td>
<td>Inhibitor/ Promoter</td>
</tr>
</tbody>
</table>
Additional information, especially for THP and OPN which are the subjects of this study is included in later chapters wherever relevant.

**Tamm Horsfall Protein**

**Occurrence**

Human urinary Tamm Horsfall protein (THP) was first described by Morner (1895) and was first characterized as the urinary component capable of inhibiting viral hemagglutination by Tamm and Horsfall (1950). Since then, it has been researched for more than 4 decades, for its normal biological function as well as for its involvement in stone formation. Independently, a similar glycoprotein named uromodulin was purified from the urine of pregnant women (Hession et al., 1987), which was later shown to have the same amino acid sequence as THP.

In humans, the daily excretion of THP ranges from 20-100 mg in adults, thus making it the most abundant protein in the human urine (Hunt and McGiven, 1978). In addition to humans, THP has been identified in other mammals such as hamster (Bloomfield et al., 1977), frog (Howie et al., 1991), cattle (Smagula et al., 1990) and isolated from rabbit (Marr AMS et al., 1971), cat (Rhodes et al., 1992) and rat (Kirchner and Bichler, 1976) urine. Recently, in a comprehensive study, Howie et al. (1993) have shown that out of the 48 species of vertebrate animals they examined, THP was present in mammalian kidneys and superficial skin layers of amphibians and fish, but was absent in birds and reptiles. They also concluded that although THP
Figure 3.1 Diagram showing the site of THP synthesis (arrows), in the thick ascending limb in the nephron. Note the gradual decrease in the level of synthesis along the nephron, as shown by transition from a darker to a lighter area. (Modified from Hogg and Kokko, 1979).
was conserved through evolution, it showed antigenic differences between amphibians and mammals.

Localization

Previously, it was documented by immunocytochemistry that in human and rat kidney, uromucoid (synonymous with THP) was localized in the distal tubules (Bichler and Kirchner, 1975). Later studies did not corroborate these observations, and a diagram of the nephron depicting the currently accepted site of THP synthesis in the kidney is shown in Figure 3.1.

The presence of THP in the kidney has been demonstrated by immunoperoxidase and immunofluorescent techniques in human kidney (Sikri et al., 1981a) hamster kidney (Sikri et al., 1981a), rat kidney (Hoyer et al., 1974; 1979a; 1979b) and rabbit medullary cell cultures (Drugge et al., 1989). Within the kidney, as shown in Figure 3.1, THP was specifically localized in the thick ascending limb (TAL) of the loop of Henle and its appearance at this site coincides with the development of TAL (Hoyer et al., 1974). THP is absent from the glomerulus, macula densa, proximal tubules, thin limbs of the loop of Henle, collecting ducts, blood vessels, convoluted tubules and the interstitium (Sikri et al., 1981a, Kumar et al., 1985). Due to its specific location in the TAL, it is used as a standard marker for identification of this segment (Ronco et al., 1987).

Immunocytochemistry (Howie, 1983) and northern blot analysis of various rat and human tissues showed that the primary site of synthesis for THP was the kidney.
Moreover, within the TAL, there was a gradation in the level of synthesis of THP along the length of the TAL, as demonstrated by in situ hybridization (Bachmann et al., 1990). The signal for THP mRNA was most intense in the cortex and outer medulla, very weak in the inner medulla, and was completely absent in the papilla. As it will be seen later in Chapters 5 and 6, this property (location) of THP has been used as a parameter for its examination in the nephrolithic rats. All other parts of the nephron were also negative for the mRNA. In summary, the location and site of synthesis of THP has been established unequivocally by a variety of techniques.

THP is a member of glycosyl phosphatidylinositol (GPI)-linked membrane protein family (Rindler et al., 1990), which consists of proteins associated with the cell membranes. Although the majority of the protein exists in the secreted form and is released into the tubular lumen, examination at the ultrastructural level has shown that THP is found on the basolateral and luminal aspects of the cell membrane. In addition, in the cell, it is present on the membranes of the Golgi apparatus and endoplasmic reticulum (Sikri et al., 1981a; and Bachmann et al., 1985), indicating active synthesis at these sites. It has been speculated that similar to other GPI-linked proteins (Thy-1 cell surface antigen, neural cell adhesion molecule (NCAM) and Qa) it is released from the renal-cell membrane by proteolytic or phospholipolytic cleavage. The major zymogen granule membrane protein (GP-2), which is also a GPI anchored protein shares a 53% identity and 85% similarity to THP (Hoops and Rindler, 1991).
The uromodulin/THP gene has been sequenced (Hession et al., 1987) and shown to have a coding sequence of 2.3 Kb and the protein is 639 amino acids in length, with 24 amino acid as the leader sequence. Because the coding sequence for THP contains an RGD motif similar to that found in the extracellular matrix proteins, it can be speculated that this may facilitate its binding to integrins on renal cells. It has been assigned to the chromosome 16 segment 16p13.11 (Jeanpierre et al., 1993).

Outside the kidney, THP has been detected in small amounts in the astroglial cells in the brain (Zalc et al., 1984). The occurrence of THP in other non-renal sites is somewhat dubious. Although some THP-cross-reactive material has been found in liver and the cerebrospinal fluid, there was no evidence of THP-mRNA at these sites (Hession et al., 1987). Similarly, small quantities of THP have been detected in the blood by immunoassays (Avis, 1977; Hunt et al., 1986). This may be explained by the fact that THP shows immunological cross-reactivity with red blood cell ghosts (Hartmann et al., 1981). This reactivity which may be due to the Sd blood group antigen on the red blood cells, which is a complex of β-linked N-acetylated galactoseamine (Donald et al., 1983).

Biochemical Properties

THP requires an alkaline pH to dissolve in water and it self aggregates and comes out of solution with an increase in ionic concentration (Stevenson et al., 1971). It can exist as different polymeric forms (Wiggins, 1987). The polymeric nature of urinary THP (molecular weight $7 \times 10^7$ D) was discovered by Curtain (1953), who
found that it could be dissociated into monomers by treatment with urea. THP can be precipitated from human urine by sodium chloride, usually by a single cycle of precipitation. The precipitate appears as a gel-like viscous polymer. In contrast, as found in this study and described by Hoyer (1974), several cycles of precipitation with NaCl and extraction with water are required to precipitate THP from rat urine. Several studies have shown that on reducing polyacrylamide gels THP migrates as a molecule of 90 Kd (Hunt and McGiven, 1978; Kumar and Muchmore, 1990).

Analysis of the amino acid composition of human THP has shown that unlike NC, it does not contain any unusual amino acids. Acidic residues such as aspartic and glutamic acids are in a slightly higher proportion than the other constituents (Flethcher et al., 1970a), which contribute to the low pI of 4.1. The amino acid composition of THP from two other mammals, hamster (Dunstan et al., 1974) and rabbit (Fabricius et al., 1989) is very similar, suggesting that the protein constituent is a conserved molecule.

Human THP is a highly glycosylated protein with a total carbohydrate content of 30% (Fletcher 1970b), and in contrast to the consistent nature of the protein component, shows variations between different species. For example, characterization of THP obtained from pregnant women by lectin-affinity chromatography indicated that there was a difference in the carbohydrate content between this protein and the salt-precipitated THP (Hession et al., 1987). A difference in the carbohydrate composition was also found between rabbit (Neuberger et al., 1972) and hamster THP (Bloomfield et al., 1977). THP digested extensively with pronase still retains most of
its immunosuppressive activity (Muchmore et al., 1987), indicating that the carbohydrate composition seems to be crucial to the normal biological functions. Furthermore, sialic acid residues and their type seem to be important for determining biological function such as the immunosuppressive activity. This is evident from the fact that in contrast to human THP with N-linked glycans, rabbit THP containing O-acetylated sialic acids and hamster THP with no sialic acid residues do not appear to inhibit viral hemagglutination.

Function

It becomes apparent from the discussion above that with its abundant occurrence in the system, a specific site of synthesis in the kidney and a fairly conserved structure, it was reasonable to assume that THP served an important biological function. Due to its abundance in human urine, at first THP was largely investigated for a normal physiological role, which was based mostly on its self-aggregating property that gave it a gel-like appearance. It was suggested that THP had a protective effect on the tubular cells, similar to the effect of mucin on the stomach lining (Kumar and Muchmore, 1990).

The specific and exclusive localization in the TAL suggested a role in renal salt and water transport (Bankir et al., 1989; Ronco et al., 1987), because sodium chloride movement occurs in this segment of the nephron. However, this concept may be somewhat contradicted by the observation that the synthesis of THP is independent of the intensity of transport in the TAL (Bachmann et al., 1991). Similarly, the
observation that chloride-transporting epithelia do not co-localize with THP (Budi-Santoso et al., 1987) further supports the idea that THP probably is not involved in NaCl transport.

An additional function for THP has been suggested, which is also based on its mucin-like physico-chemical properties. THP was suggested to promote bacterial (E. coli) infections by forming a pseudocapsule around the bacterial cells and making them resistant to phagocytosis (Kuriyama and Silverblatt, 1986). In contrast, by the same property, THP was shown to interfere with the attachment of E. coli to human kidney cells in tissue culture (Dulawa et al., 1988). The authors concluded that despite differences in the carbohydrate composition of THP isolated from normal subjects and diabetic patients, there was no significant difference in this anti-adherence activity.

It has been demonstrated that THP interacts with the cells and molecules of the immune system. For example, THP binds IgG with strong affinity (Rhodes et al., 1993), and interacts with cytokines such as tumor necrosis factor (TNF) (Sherblom et al., 1988), interleukins like IL-2 (Sherblom et al., 1989), and recombinant IL-1 in a pH-dependent manner (Moonen et al., 1988).

On human neutrophils, THP binds to receptors that specifically bind sialic acids (Thomas et al., 1993a). It can activate these cells in vitro to release reactive oxygen species and proteinases, which can cause tissue damage (Thomas et al., 1993b). In conflicting reports, THP has been shown to induce T cell proliferation (Hunt and McGiven, 1978) and also to inhibit lectin-induced proliferation and mixed
lymphocyte response (Franceschi et al., 1982). In summary, although these studies suggest that THP may have some type of immunomodulatory function, it needs to be investigated further.

In conclusion, it appears that THP has the potential to be involved in various functions. Although several functions have been suggested, it is intriguing that none of them has been singled out for a protein that is so much abundant in the human system.

THP and Nephrolithiasis

Although THP has been investigated in various renal pathological conditions as described later, studies on its involvement in nephrolithiasis have been a major focus of research. The first major evidence for its involvement in stone formation was the observation that it was identified in the core of renal stones, as a part of the matrix. The quantity ranged from 0.002-1.04 mg/gm of kidney stone (Grant et al., 1973). Then the question arose as to whether it was present as a bystander, or if it in fact had participated in stone formation in some manner. A later study by Fraij (1989), showed that when analyzed by SDS-PAGE, the stone matrix revealed the presence of THP and other proteins which together accounted for 40% of the mass of the matrix.

The effect of THP has been examined extensively on all three stages of crystallization and inconsistent results have been obtained. For example, when tested at the average physiological concentration of approximately 50 mg/L THP was shown to promote nucleation at pH 6.0 and an ionic strength of 0.15 (Yoshoika et al., 1989).
If the pH was raised to 6.5, the effect was reversed and THP inhibited nucleation. In a separate assay system using evaporated ultrafiltered human urine and at pH 5.3, the enhancement of nucleation was as high as 250% (Rose and Sulaiman, 1982).

The net effect of THP on crystal growth seems to be affected significantly by its concentration and the pH of the environment. At pH 6.5 and a concentration of 50 mg/L, THP inhibits growth by 38%. At a lower pH of 6.0, the inhibition is lowered to 3%. In addition to the pH, the concentration of THP is also an important factor that will determine its net effect. As shown by the observation of Worcester et al. (1988), at a low concentration of 8 mg/L, THP did not affect crystal growth.

Robertson and Scurr (1986) demonstrated that in a continuous crystallization system, THP promoted aggregation. A similar conclusion was reached by Yoshoika et al. (1989), when THP was tested in an assay system with whole urine. In contrast, in a spectrophotometry based assay, at 40 mg/L and pH 7.2, THP inhibited aggregation by 90% (Hess et al., 1989). Similar to its effect on growth, at a lower pH of 5.7, this effect was reversed and THP promoted aggregation (Hess et al., 1989). In contrast to this property, when an assay using ultra-filtered urine was employed, which probably removed other urinary components, THP prevented the formation of aggregates (Ryall et al., 1991). The experiments discussed here indicate that if different methods are used, the results of assessment of interaction of THP and crystals can vary considerably.

Quantitative comparison of THP excretion in normal subjects and stone-formers has yielded variable results. In several studies the THP excretion in stone
formers was found to be lower than the normal subjects (Bichler et al., 1976; Hess, 1994). In contrast, a separate study showed that the average excretion in both groups was similar (Lynn et al., 1982; Thornley et al., 1985). In summary, a definite correlation between stone disease and quantity of THP excretion has not been found.

An important clue to the role of THP in kidney stone formation was derived from the experiments of Hess et al. (1991b). In a comparative study, they found that the urine and THP from stone formers showed reduced inhibition of crystallization in *in vitro* assays. In fact, at the highest concentration studied, the THP from stone formers promoted aggregation (Hess et al., 1993). When physico-chemical properties of the two samples were compared it was found that the THP from stone-formers differed in a number of features and also exhibited self-aggregation. Essentially, therefore, THP was not available to interact with the crystals and could not interfere with the crystals. These observations indicated that there was possibly an intrinsic difference between the urinary components of the two populations, which could partially explain the basis for stone formation. It has been suggested that this abnormality may be inherited (Hess et al., 1991), but sufficient evidence is not available at this time.

Low citrate or hypocitrauria is common in stone-formers and it contributes to crystal aggregation in these patients (Kok et al., 1986). This observation was also reinforced by *in vitro* studies of Hess et al. (1993). They found that in a spectrophotometric assay, addition of citrate reduced the self-aggregation of THP from stone-formers and therefore reduced its promoting activity. These observations
emphasize the importance of other urinary factors such as citrate in the complex process of stone formation. All in all, it appears that the effect of THP on crystallization is modulated quite substantially by other urinary factors.

THP in Renal Diseases Other than Nephrolithiasis

As discussed by Hoyer and Seiler (1979), the involvement of THP has been examined in various pathological conditions with respect to the following factors 1) daily excretion, 2) formation of urinary casts, 3) abnormal localization in the tissue, and 4) reaction to THP as an antigen.

1) Although it appears that urinary excretion rate of THP is related to kidney function in patients with chronic renal disease (Thornley et al., 1985), no consistent conclusion can be drawn from independent studies. Schwartz et al. (1973) reported that urinary excretion of THP increased transiently prior to diminishing renal function. In another report on the study of various types of diseases, THP excretion was markedly reduced in patients with chronic renal failure and was within the normal range in children with Lignac-Fanconi syndrome (Grant et al., 1973). In patients with insulin-dependent diabetes (IDDM), excretion of THP is reduced (Torffvit and Agardh, 1993). In a separate study, the excretion of THP in the two groups has been reported to be similar (Rambausek et al., 1988).

2) THP is also a major component of the urinary casts (Fletcher et al., 1970a; Fletcher et al., 1970b; Hoyer, 1979). Analysis of the composition of urinary casts from the urine of nephrotic patients showed that their major component is THP
(Mcqueen 1962; Cohen, 1981). Similar findings were reported by Berdon et al. (1969), who discussed THP proteinuria in children with prolonged nephrograms and adults with multiple myeloma. Later it was demonstrated that THP specifically aggregated with cast-forming low molecular weight Bence Jones proteins (BJPs) \textit{in vitro}, thus indicating a mechanism of cast formation \textit{in vivo} (Sanders et al., 1990). Further examination of this phenomenon in an \textit{in vivo} experimental system showed that human cast-forming BJP coaggregated with THP and obstructed rat distal nephron (Sanders and Brooker, 1992). The functional importance of the carbohydrate moiety of THP was again emphasized when it was concluded that deglycosylated THP did not coaggregate with the BJPs (Huang et al., 1993).

3) A variety of renal conditions including acute and chronic interstitial nephritis (reflux and urinary obstruction), and medullary cystic disease have been linked to the extravasation of THP from the tubules. The appearance of THP as extratubular interstitial deposits was seen during rejection of renal allografts in patients without extrarenal obstruction (Cohen et al., 1984) as well as in obstructive uropathy in experimental rats (Dziukas et al., 1982). A comprehensive study of 247 bladder biopsies indicated that although THP was present at this site, it was of no clinical significance.

4) The presence of autoantibodies to THP was reported in children with urinary tract infections (Fasth et al., 1980), in interstitial cystitis (Neal et al., 1991; Stein et al., 1993), bladder tissue (Truong et al., 1994) and also in patients with end stage kidney disease (Work and Andriole, 1980). Maximum uptake of injected anti-
THP antibody was seen in rats (Ishidate et al., 1990). The formation of THP-anti-THP immune complexes in rats and mice (Fasth et al., 1986) indicated an autoimmune reaction, and suggests a role for THP in pathogenic processes in the kidney. However, the exact implication of this phenomenon is not clear.

Nephrocalcin

Occurrence

A 14 Kd glycoprotein rich in acidic amino acids was isolated from human urine by using DEAE-cellulose chromatography. In in vitro crystal growth assays, it inhibited CaOx crystal growth efficiently (Nakagawa et al., 1983; Worcester et al., 1988). It is synthesized in the proximal tubules and TAL has been and also has been s Its inhibitory properties were similar to a crystal growth inhibitor isolated from human embryonic kidney tissue culture media (Nakagawa et al., 1981). In human urine, the concentration of NC ranges from 5-16 mg/24 hrs (Nakagawa et al., 1983; Kaiser and Bock, 1989).

Biochemical Properties

Amino acid composition analysis has shown that in NC, the percentage of acidic amino acids is higher than in THP but lower than OPN. In addition, NC contains an unusual acidic amino acid, gamma-carboxyglutamic acid (Gla), which is
present as 2-3 residues/molecule (Nakagawa et al., 1983). A comparison of NC from the kidney tissue from 9 vertebrate species showed similar amino acid composition and a carbohydrate content ranging from 4-16% (Nakagawa et al., 1991). The amino acid sequence of nephrocalcin is not known. NMR-spectroscopy has demonstrated that NC can exist in at least 4 forms, which differ in their phosphate content. Apparently, the extent of phosphorylation is important for its interaction with crystals (Nakagawa et al., 1989).

Localization:

In addition to humans, NC has been localized in rat and other vertebrate species (Nakagawa et al., 1983; 1991). By immunocytochemistry, it was localized in the proximal tubules and TAL of human and rat kidney (Nakagawa et al., 1990) and mouse proximal tubule cell cultures (Sirivongs et al., 1989). Although the identity of this glycoprotein has been known for over 10 years and the protein has been localized in cells the site of synthesis has not been confirmed by localization of mRNA (for example by in situ hybridization).

The location of the NC gene is not known. Recently, a localization of the gene for osteocalcin (OC), a bone protein, in mouse, revealed that out of the three-gene cluster for OC, one of the genes is transcribed only in the kidney and not in the bone (Desbois et al., 1994). Moreover the protein encoded by this gene structurally resembles NC.
Function

In contrast to THP, NC has been studied only in the context of its involvement in stone formation and no other function for NC is known. The observation that NC excretion increases in pregnant women who become hypercalcemic (Davison et al., 1993) may indicate that the increase is due to the increased calcium in the system.

NC and Nephrolithiasis

In the urine of normal subjects and stone formers the free and protein bound Gla residues appear to be similar in amount (Colette et al., 1991). In contrast, a major difference between NC purified from normal subjects and stone-formers is that in the latter, it lacks the Gla residues and shows reduced film stability. As discussed in a review of nephrocalcin by Coe et al. (1994), the reason and significance of this observation is not clear at this time.

Similar to THP, NC from normal subjects and stone-formers has been compared in in vitro crystallization assays. For example, in a seeded crystal assay system, NC and urine from normal subjects showed inhibition of crystal nucleation (Asplin et al., 1991). At a concentration of 0.2 µM, the inhibition is 40%, which increases to 100% at 0.5 µM Furthermore, a structural study demonstrated that NC bound reversibly to CaOx crystals and the disruption of the oxalate group and water molecule by NC was modulated by its adsorption to certain faces of the crystals. Because the crystals showed one-sided growth in the presence of NC, it seems to have a binding affinity to specific faces of the crystals (Deganello et al., 1991).
In contrast to THP and albumin, in a seeded crystal system of metastably supersaturated solution, NC inhibited growth of CaOx crystals (Worcester et al., 1988). The growth inhibition data was linear when plotted in the form of Langmuir isotherm plots, indicating that NC adsorbed to the crystal surface and affected growth in a dose-dependent manner. In a separate comparative study of THP and NC, with CaOx dihydrate crystals, Deganello (1993) concluded that in contrast to THP, NC reduced growth and affected the crystal habit. The calcium-binding sites of NC have been recently identified (Mustafii and Nakagawa, 1994).

By using a spectrophotometric aggregation assay, identical to the one used for THP, it was determined that the effect of NC on aggregation was concentration-dependent. In this study, NC at 0.01 µM, had no effect on aggregation. In contrast, when the concentration was increased to 0.05 µM, the inhibition increased to 80%. In contrast to THP, the effect of lowering pH and increasing the ionic strength had negligible effect on NC (Hess et al., 1989).

**NC and Other Renal Conditions**

Although association of NC has been mainly restricted to CaOx lithiasis, it has also been found associated with non-stone conditions such as renal tumors, which contain cells of proximal tubule origin. For example, it was suggested that NC could be possibly used as a tumor marker, because cell cultures from primary and metastatic tumors produced nephrocalcin in the medium. Although NC from tumors cells was found to contain a higher amount of carbohydrate (Nakagawa et al., 1992), its
significance is not known. A recent study of renal cell carcinoma patients indicated that urinary NC in these patients is atypical and also in much higher quantity (Nakagawa et al., 1994) which decreased dramatically after tumor nephrectomy. It was not determined, however, whether there was a general proteinuria in these patients, or if only NC was in high quantities.

**Osteopontin (Uropontin)**

**Occurrence**

Osteopontin (OPN), a major bone phosphoprotein involved in bone mineralization, is a typical acidic glycoprotein with a high percentage of aspartic and glutamic acids. It was first purified from rat bone (Franzen and Heinegard, 1985) and was later identified in many other tissues and species.

In 1989, Shiraga et al. purified a protein from human urine by using an immunoaffinity column made with an antibody to the human urine fraction which was most inhibitory to CaOx crystal growth. The N-terminal amino acid sequence of this protein was identical to human OPN (Fisher et al., 1987). This urinary counterpart of osteopontin was termed uropontin. The concentration of OPN in mouse urine is 8 µg/ml (Worcester et al., 1992). The occurrence, structure, and functional properties of OPN have been reviewed by Butler (1989) and Denhardt and Guo (1993). OPN is a member of the “pontin” family of proteins and one of the principal functions of this protein was identified to be an “adhesive” between the bone cells and the bone
matrix, hence the name osteopontin. A partial list of various sources of osteopontin is given in Table 3.3.

From the table it is evident that there are significant differences in the molecular weights of OPN derived from different sources, which may be partly due to the different percent gels used in the analyses.

Localization

Localization studies in mouse kidney by immunocytochemistry and *in situ* hybridization have shown that the expression of OPN in the kidney appears to be somewhat heterogeneous (Lopez et al., 1993). OPN was detected in TAL and distal convoluted tubules and macula densa. It was prominent along the apical surface of the cells lining the lumen. OPN was absent in glomeruli, proximal tubules, thin limbs of the loop of Henle, collecting ducts, and interstitial cells. By fluorescence, confocal and electron microscopy, Kleinman et al. (1995) found that in rat kidney, OPN was primarily localized in the descending thin loop of Henle and in the calyceal fornyx of the papillary epithelium. The labeling was particularly intense along the luminal surfaces. These observations were confirmed by *in situ* hybridization.
Table 3.3

A partial list of various sources for osteopontin purification

<table>
<thead>
<tr>
<th>Source</th>
<th>Purification Method</th>
<th>% Gel</th>
<th>MW (Kd)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Rat and Bovine Bone</td>
<td>Extraction with guanidine-HCl/EDTA</td>
<td>8</td>
<td>60</td>
<td>Franzen and Heinegard, 1985</td>
</tr>
<tr>
<td>2 Rat Bone cells</td>
<td>Western Blot</td>
<td>10, 15</td>
<td>60, 56, 55,44</td>
<td>Kubota et al. 1989</td>
</tr>
<tr>
<td>3 Mouse Cortical Cells</td>
<td>Same as number 1</td>
<td>10-12</td>
<td>80</td>
<td>Worcester et al. 1992</td>
</tr>
<tr>
<td>4 Human urine</td>
<td>Immunoaffinity purification from inhibitory peak</td>
<td>16</td>
<td>50</td>
<td>Shiraga et al. 1992</td>
</tr>
<tr>
<td>5 Rat Kidney Cell Line</td>
<td>Immunoprecipitation</td>
<td>10</td>
<td>69</td>
<td>Singh et al. 1992</td>
</tr>
<tr>
<td>6 Transformed Rat</td>
<td>Immunoprecipitation</td>
<td>10</td>
<td>62, 69</td>
<td>Singh et al. 1992</td>
</tr>
<tr>
<td>7 Human Stones</td>
<td>Extraction</td>
<td>10</td>
<td>52</td>
<td>Kohri et al. 1993</td>
</tr>
</tbody>
</table>
The expression of OPN increases in pregnant mice and in glomeruli of aged mice indicating that age may affect physiological concentration of OPN (Waterhouse et al., 1992). Examination of a variety of human tissues showed that OPN is present on the luminal surface of the cells (Brown et al., 1992).

In studies with bone tissue, ultrastructural localization of OPN by using a specific antibody and protein A-gold labeling showed that it is always present in fairly high intensity along the "cement line" of the bone undergoing mineralization (Mckee et al., 1992; 1993; Chen et al., 1994). The gene for human OPN is located on chromosome 4 (Young et al., 1990).

**Biochemical properties**

OPN contains significantly high percentage of acidic amino acids (22%), and is considered a classic acidic protein. Differences between OPN from different cell types are seen not only in glycosylation, but also in the coding nucleotide sequences of the expressed genes. In a comparison of OPN from normal rat kidney cells (NRK) and osteoblast-derived cell lines (KNRK), Singh et al. (1992) found that OPN purified from conditioned media from NRK showed a band at 69 Kd whereas OPN from KNRK migrated at 62 and 69 Kd. In addition to this difference, the nucleotide sequence of the two samples differed. In the Madine Darby Canine Kidney (MDCK) cell line, a 20 Kd peptide related to OPN is synthesized. It has a 70% identity with the carboxy terminus of human OPN. Furthermore, it was shown that this 20 Kd protein was generated in the cell from a 60 Kd protein by proteolytic cleavage (Ullrich et al.,
In summary, it appears that OPN may exist as several forms, which may have different molecular weights and depending on its source of isolation.

**Function**

In contrast to THP, before OPN was considered to be a protein involved in nephrolithiasis, its normal physiological function had been clearly identified as being primarily involved in mineralization of bone (Denhardt and Guo, 1988; Bulter, 1989). In the bone, OPN is found in many types of cells and is an essential component for cell attachment and spreading of osteoblasts and fibroblasts to the bone (Butler, 1989). As discussed by Butler (1989), OPN is typically classified as an aspartic acid rich protein (AARP) and contains a cell-binding Arg-Gly-Asp-Ser (RGDS) sequence (Oldberg et al., 1986). The specific requirement of this sequence was confirmed when the attachment of cells was inhibited by RGD containing peptides.

Upregulation of OPN gene expression can be induced by 1,25-dihydroxyvitamin D3 in a dose-dependent manner, a response element for which has been identified in the gene for OPN (Noda et al., 1990). Similarly, transforming growth factor β (TGF-β) and interleukin were shown to regulate bone matrix protein synthesis (Noda and Rodan, 1989a; Jim et al., 1990).

Down-regulation of OPN protein was observed by treatment with dexamethosone (Yoon et al., 1987). Similarly, human parathyroid hormone suppressed the amount of OPN secreted by rat osteoblast-like cells and also reduced the level of mRNA synthesis in these cells (Noda and Rodan, 1989b).
**OPN and Nephrolithiasis**

A protein isolated from human urine, capable of inhibiting CaOx monohydrate crystal growth was identified by Shiraga et al. (1992). It was later shown to be have the same amino acid sequence as that of human osteopontin (Fisher et al., 1987). Therefore, it was called uropontin. In this discussion, the terms uropontin and osteopontin will be used interchangeably.

In normal subjects, the daily excretion of OPN is approximately $6 \times 10^{-8}$ M (Chalko et al, 1992). Data on the excretion of uropontin is not available. However, indirect evidence in mice shows that at least partially, under certain physiological conditions with increased calcium, OPN can account for a low incidence of stone formation (Waterhouse et al., 1992). The concentration of OPN has been shown to increase in response to conditions such as hyperoxaluria (Kohri et al., 1993) and bacterial infections (Patrarca et al., 1989).

OPN has been shown to be a major component of the organic matrix of stones, and is probably the source of the high percentage of acidic amino acids found in stones. Similar to THP, the content of OPN in stones has been quantitated. Calcium oxalate monohydrate stones contain an average of 800 µg OPN/100 mg of OPN and in contrast, dihydrate stones contain only 10 µg/100 mg of OPN (Hoyer and Daikhin, 1992). The reason for this difference is not apparent. The cDNA sequence of OPN extracted from stones has been found to be identical to uropontin (Kohri et al., 1993). *In situ* hybridization showed that in rats in which hyperoxaluria and CaOx deposits
were induced, there was increased signal for the protein as well as mRNA localization (Kohri et al., 1992).

Of the three stages of crystallization, the effect of OPN has been examined for involvement in crystal nucleation to some extent, but primarily on growth. Since OPN is a component of bones, it has been studied in the context of crystals other than CaOx. For example, as discussed by Gorski (1992), acidic glycoproteins from bone, including OPN, appear to associate with nucleation of calcium hydroxyapatite crystal formation. A specific study with bone sialoprotein (BSP) and OPN has shown that in the presence of OPN, hydroxyapatite components did not accumulate. In contrast, BSP appeared to enhance deposition of Ca and phosphate. These results, however, do not clarify the exact role of OPN in this process.

In the context of stone formation, similar to uropontin (from human urine), osteopontin from mouse cortical cells was shown to be a potent inhibitor of growth (Worcester et al., 1992). As discussed by Denhardt (1993), growth inhibition takes place by disruption of the crystal lattice.

Several properties of OPN may play an important role in modulating its effect on crystallization. For example, OPN can complex with osteocalcin (Ritter et al., 1992), and therefore may interfere with its reactivity with the crystals. It appears that various post-translational modifications, such as glycosylation, sulfation and phosphorylation may influence the effect of OPN on crystallization (Nagata et al., 1989).
OPN in Other Diseases

Brown et al. (1994) found that out of 76 carcinomas of various types examined, 71 tumors showed intense labeling for OPN mRNA by *in situ* hybridization. It was found that macrophages at the edge of these tumors and in necrotic areas labeled strongly for OPN mRNA. in these tumors. Although in most carcinomas tumor cells did not label for RNA (except kidney and endometrium), they stained positive for the protein. These findings suggest a role for OPN in processes like invasion and metastasis.

A study by Singh et al. (1990) has established that OPN has high affinity for mouse macrophages. They found that a) macrophages bound with high affinity to OPN and b) OPN induced macrophage-rich infiltrate when inoculated in mice. Increase in OPN mRNA in angiotensin-induced tubulointerstitial nephritis was observed by Giachellie et al. (1994). This increase was specifically associated with infiltration and accumulation of monocytes.

In summary, OPN is a unique protein among the other urinary proteins discussed in this section, because it has a specific normal biological function. Although it has been discovered recently, information on its role in stone formation has accumulated rather quickly, probably partly due to the availability of the protein and gene sequence.
Uronic Acid-Rich Protein (UAP)

Atmani et al. (1993a; 1993b) reported the purification of a previously unknown glycoprotein from human urine by gel chromatography. This 35 Kd glycoprotein contained 8.5% carbohydrates and a high percentage of uronic acid and inhibited CaOx crystallization *in vitro*.

A comparison of UAP isolated from normal subjects and stone-formers showed that UAP from stone formers showed reduced inhibitory activity in the crystallization assay. Comparative biochemical analysis of UAP from the two groups showed that the amino acid content of the two samples was similar. In contrast, the stone-former’s protein contained significantly decreased amount of sialic acid (Atmani et al., 1994). The authors have speculated that this difference in structure may partly be responsible for the lower inhibitory activity. The distribution of this protein in the kidney is not known.

Uronic Acid Rich Fractions

A study on uronic acid rich fraction (Shirane et al., 1989) isolated from stone formers urine showed that this fraction was rich in hyaluronic acid and acidic amino acid rich proteins, and promoted CaOx aggregation *in vitro*. However, no data is available from normal subjects for comparison.
Crystal Matrix Protein (CMP)

A comprehensive review on crystal matrix protein has been recently published by Stapleton and Ryall (1994). In a study to determine which proteins might associate with early stages of crystallization, Doyle et al. (1991) noted that the predominant protein was a 30 Kd glycoprotein, which they named crystal matrix protein (CMP). The authors concluded that most urinary proteins except CMP did not play a direct role in crystallization. Immunohistochemical staining showed that CMP, a potent inhibitor of crystal aggregation, was primarily present in the kidney in the TAL and the distal convoluted tubules (Stapleton et al., 1993a). Sequence comparison with other proteins revealed a 81% identity with the 11 N-terminal amino acids of prothrombin (Stapleton et al., 1993b). Detailed characterization and analysis of CMP has been reported by Stapleton (1994). In an independent study, the association of CMP with CaOx crystals has been also established by Suzuki et al. (1994). The location of this protein in the TAL of the kidney coincides with the primary location of THP, however, there is no information on any other normal functional properties at this time.

Prothrombin is a 30 Kd protein found in the human plasma which contains 10 residues of Gla in the first 32 amino acids Western blot studies on human osteopontin using an antibody to human prothrombin revealed that this antibody reacted with
OPN, indicating a similarity between the two proteins (Stapleton et al., 1993). In the same study it was found that a comparison of amino acid sequences of prothrombin and CMP revealed that there was 81.8% identity in the 11 N-terminal amino acids of these two proteins. Furthermore, identification of prothrombin by anti-CMP antibody on Western blots confirmed that CMP is the activated peptide of human prothrombin (Suzuki et al., 1994).

Renal Lithostatine

In an isolated report, a protein similar to lithostatine found in the pancreatic juice was identified in the kidney. Although this protein was localized in the proximal tubule and the TAL, and inhibited calcium carbonate crystal growth *in vitro*, it was immunologically not related to nephrocalcin (Verdier et al., 1992).

From the discussion of the seven urinary proteins presented above the following facts become evident:

1. In addition to the clinical data, most of the information regarding the effect of these proteins on modulation of crystallization has been obtained via *in vitro* studies.

2. All proteins, except THP have shown a single type of effect on crystallization, namely of inhibition. The proteins interact with crystals during either their nucleation or growth.

3. In contrast, experiments with THP demonstrate several possible effects: it could possibly inhibit, promote or have no effect on crystallization.
4. It appears that THP most likely interacts with the crystals during the aggregation phase of crystallization. Furthermore, this effect can be modified significantly by other urinary factors, such as pH, ions like calcium and sodium, and other molecules like citrate.
CHAPTER 4

COMPARATIVE ANALYSIS OF HUMAN AND RAT TAMM HORSFALL PROTEIN

Since this study utilizes rat as the *in vivo* model to assess the participation of THP in stone formation, it was imperative to investigate and characterize the protein in normal untreated rats. Secondly, under normal physiological conditions, rats tolerate considerably higher (three-fold higher than human) oxalate levels, and yet rarely form kidney stones. Therefore, it was of interest to know if rat THP had any peculiar properties. In addition to the examination of human THP, a second focus of the experiments described below was detailed biochemical analysis and quantitation of THP excretion from normal male Sprague Dawley rats.

As the review of literature indicates, a substantial part of the information on THP has been derived from studies on human protein by *in vitro* studies. Since its first identification in human urine, numerous reports on various properties of human urinary THP have been published (Friedmann and Johnson, 1966; Fletcher et al., 1970a; Hunt and McGiven 1978). The average excretion, molecular weight, and amino acid and carbohydrate composition have been well documented. In these studies, a common technique used for the purification of THP was precipitation with sodium chloride, which was based on the method originally described by Tamm and Horsfall (1950). Although a single precipitation with sodium chloride was found to be sufficient for precipitation of
most of the THP from the urine, some studies have used a method which includes several precipitation steps. In a different method, Serrafini-Cessi (1989) bound the THP from the urine to diatomaceous filters and then eluted it by desorption with water. In this study, a single cycle of precipitation was used to purify THP from human urine, as described by Hunt and McGiven (1978). The rationale for re-examination of the biochemical properties of human THP was as follows: 1) First, because an animal model was employed to study THP under conditions simulated for human stone disease, it was only logical to compare proteins from human and normal rat under non-disease conditions. 2) Secondly, experiments with human THP provided a basis for trials and establishment of various analysis techniques for the glycoprotein, which were later used for analysis of rat THP. 3) Lastly, as most researchers in this field would agree, in contrast to human samples, the time and effort required for collection of rat urine and its purification are significantly higher. Therefore, experiments with human THP were used as pilot experiments for the analysis of rat protein.

In contrast to human THP, information on purification and properties of rat THP in the literature is very limited. For example, Kirchner and Bichler (1976) first isolated "uromucoid" from rat urine by using an affinity column made with an antibody to human THP and characterized it by polyacrylamide disc gel electrophoresis. Antibody made to this preparation localized the protein in distal tubules in the kidney, an observation which could not be confirmed by later studies (Hoyer 1974; Bachmann, 1990). The method of purification of rat THP used in this study is based directly on the technique of Hoyer (1974; personal communication) who first described the necessity of repeated cycles of
extraction with water and precipitation with salt, for complete extraction of the protein from urine. Other studies have only made cursory references to the method of sodium chloride precipitation, excluding details of the procedures used. In summary, it appears that although some data on rat THP are available, detailed biochemical characterization has never been reported.

**Materials and Methods**

**Purification and Characterization of Human Urinary THP**

Urine was collected from a total of five healthy donors (one each male, female, pregnant female, a stone-former with one incidence of stone and a recurrent stone-former) over a 24 hour period at 4 °C. THP was isolated by the method of Hunt and McGiven (1978) as follows: The precipitation step included addition of sufficient sodium chloride so as to attain a molarity of 0.58 M. After stirring for 30 minutes at room temperature, the urine was centrifuged and the supernatant was discarded. The pellet was washed with cold 0.58 M sodium chloride (in distilled water), re-centrifuged, and the supernatant, which also contained most of the pigments, was discarded. Next, the gel-like viscous THP was dissolved in alkaline distilled water (pH 9.0), centrifuged, and the non-soluble contaminants were discarded. THP was re-precipitated from the solution and stirred overnight at 4 °C to obtain a homogeneous suspension. After extensive dialysis with distilled water (at least 100 times the volume of the sample solution) over 48 hours, it was lyophilized and stored frozen until further use.
All human THP samples were analyzed by gel electrophoresis and their identity was confirmed with Western blotting. Amino acid analysis was performed on all samples. In this chapter, only data from the normal subjects will be discussed. Data from stone-formers urine will be discussed in Chapter 5, with the data for THP from nephrolithic rats. The normal human male THP was subjected to all other analyses, for comparison with rat THP.

Urine Collection from Rats

Male Sprague Dawley rats weighing 125-150 gm were housed in metabolic cages and were given food and water ad libitum. Urine was collected at 4°C, with 0.02 % sodium azide, either separate for each rat or as a pooled sample and stored frozen at -20°C until further use.

Purification of Rat THP

THP was purified from the pooled urine from either male or female Sprague Dawley rats by a method described by Hoyer (1974) with slight modifications (personal communication). All solutions contained 0.02% sodium azide to prevent protein degradation by bacteria. Urine volume was measured and sufficient sodium chloride was added to make it 0.58M. THP was allowed to precipitate at 4°C for 7-10 days. The urine was then centrifuged at 7000 rpm for 25 minutes and the supernatant was discarded. The pellet was washed once with 0.58M sodium chloride in distilled water. THP was extracted from the pellet with water (pH 9.0) at 4°C overnight. It was then precipitated by
making it 0.58M with sodium chloride by adding one-fifth volume of 3.5M sodium chloride. This sequence of extraction/precipitation was repeated four to six times until no more precipitate was formed. All precipitates were pooled and stirred overnight to obtain a homogeneous solution. The final product was then dialyzed, re-precipitated, dialyzed again, freeze-dried and stored at -20° C until further use. Although gel electrophoresis and amino acid analysis was performed on THP from male and female rats, for the sake of consistency, in all other comparisons and experiments, only the male rat THP was used.

**SDS-PAGE analysis:** Electrophoresis of THP (10 µg) was performed on a 10% denaturing polyacrylamide gel according to the method of Laemmli (1970). The gels were stained with 0.2% Coomassie brilliant blue R250 for 10 minutes to visualize the proteins. Purity of the preparations was determined by SDS-PAGE (of 1µg protein per lane) followed by the sensitive silver staining method by using a silver staining kit obtained from BioRad (Melville, NY).

**Amino Acid Analysis**

For amino acid analysis, after electrophoresis, the gel was blotted on to an Immobilon membrane (Millipore, USA) overnight in 10 mM MES buffer with 20% methanol (pH 6.0). The membrane was stained with 0.2% Coomassie R250 for 5 minutes and de-stained with 50% methanol and 10% acetic acid for 15 minutes. The proteins were analyzed for amino acid composition by the Protein Core Laboratory at the University of Florida, by the acid hydrolysis method.
Detection of Carbohydrates

The presence of carbohydrates was detected by periodic acid Schiff staining of 10 µg of THP as described by Zacharius et al. (1969).

Carbohydrate Analysis

THP was analyzed for monosachharide composition at the Complex Carbohydrate Research Center, a National Institute of Health Resource Laboratory located at the University of Georgia, Atlanta, Georgia. Briefly, the trimethylsilyl (TMS) derivatives of the methyl glycoside were obtained followed by gas chromatography (GC) and then combined GC and mass spectrometry (MS). Inositol was added as an internal standard before derivatization.

Western Blotting

The gels were blotted overnight on nitrocellulose membrane at 4°C, in tris-glycine buffer with 20% methanol. The membrane was blocked with 2% bovine serum albumin (BSA) in tris-buffered saline with 0.1% Tween-20 (TBST). After incubation with the polyclonal antibody (at a dilution of 1:10,000) it was washed three times (10-15 minutes each) with TBST. Next it was incubated with the goat anti-rabbit alkaline phosphatase conjugate (Hyclone, Logan, UT) at a dilution of 1:5000 for 30 minutes. After three washes with TBST, color was developed with NBT and BCIP (alkaline phosphatase kit, BioRad, Melville, NY) for 5-15 minutes.
Lectin Binding Assay

The type of sialic acid residues present in the rat THP were characterized and compared with human THP by application of digoxigenin-labeled lectins (Boehringer Mannheim, Indianapolis, USA). (SNA, specific for Neu NAc \( \alpha(2\rightarrow6) \) Gal/GalNAc) and MAA (specific for Neu NAc \( \alpha(2\rightarrow3) \) Gal). After gel electrophoresis, the proteins were blotted on nitrocellulose overnight. The non-specific binding sites were blocked by incubation in 5% non-fat dry milk in PBS for 30 minutes. The membrane was washed twice in TBS for 10 minutes each and once with buffer 1 (1 mmol/L each MgCl\(_2\), MnCl\(_2\) and CaCl\(_2\), pH 7.5 in TBS). The membrane was incubated in digoxigenin-labeled lectin at a final concentration of 1 mg/ml in buffer 1 (for one hour), followed by three washes for 10 minutes each in TBS. This was followed by incubation with anti-digoxigenin antibody (150U/0.2 ml in TBS) for 60 minutes. The unbound antibody was removed by three washes of 10 minutes each in TBS. The lectins were visualized by using NBT and BCIP as substrates, at a final concentration of 1.875 mg and 3.75 mg respectively in 10 ml of buffer 2 (100 mM Tris-HCl, 100 mM NaCl and 50 mM MgCl\(_2\), pH 9.5), according to the manufacturer's directions.

Preparation of Anti-Rat THP

A polyclonal antibody to the THP isolated from male rats was made in rabbits, in collaboration with Kel Farms, Alachua, Florida, as follows: Animals were pre-bled from the ear vein (15 ml) for pre-immune sera at least 14 days prior to injection of the antigen. They were injected with follows: 300 \( \mu \)g of the purified THP in saline was emulsified
with 0.5 ml of complete Freund’s adjuvant by passing through a syringe (total volume of the mixture = 1.0 ml). This mixture was injected at 2 sites intra-muscularly and at 3 sites subcutaneously (0.2 ml each). Next, at day 30, a second dose of 200 µg of the protein was injected in a similar manner. After day 44, at 2-3 week intervals, 100 µg protein was injected. Rabbits were bled periodically and the antibody titer was checked with western blotting. Finally, anti-THP antiserum was collected and stored frozen at -20° C until further use.

Characterization of Antibody

A polyclonal antibody to rat THP was a kind gift from Dr. John Hoyer (Children’s Hospital, Philadelphia, PA). The newly generated antibody (described above) was compared with Hoyer’s antibody by ELISA for its specificity, and reactivity as follows: 96-well plates were coated overnight with 1 µg/ml of purified rat THP. After blocking the non-specific binding sites with 2% BSA-PBS, either Hoyer’s or the new antibody was added as two fold serial dilutions in the range of 1:5000 to 1:640,000. After appropriate incubation and washes, a second antibody (alkaline phosphatase conjugated goat-anti-rabbit-IgG) was added and finally color was developed with the NBT-BCIP substrate. The plates were read at 405 nm in a microplate reader.

Antigen Capture Assay for Quantitation of Rat Urinary THP

The method for quantitative determination of rat urinary THP was based on the technique described by Reinhart et al. (1989), with modifications. Urine was collected
from rats individually in separate tubes over a 10 day period and stored frozen at 20 °C until further use. Samples at 1, 3, 5, 7, and 9 days were used for the antigen capture assay. IgG was purified from the anti-rat polyclonal THP antibody (described previously) by passing through a sepharose B column conjugated to protein A (Sigma Chemical Co., St. Louis, MO). It was further biotinylated for use as a second antibody in this assay.

A standard curve was run with each individual experiment. A stock was made from the purified protein (1 µg/ml) followed by 6 two-fold serial dilutions (up to 0.0156 µg/ml). All washes were performed with 200 µl PBS containing 0.2% Tween 20 and 0.02% sodium azide. Based on previous pilot experiments, three dilutions (1:320, 1:640 and 1:1280) of each urine sample were run in duplicates. Between each step, plates were washed 3-4 times with TBST. Plates were coated with the purified IgG overnight at a concentration of 5µg/ml (50 µl/well) in PBS. After washes, the non-specific binding sites were blocked with 1% BSA (in PBS containing 0.1% azide) overnight at 4°C. Urine samples (or dilutions of purified rat THP) were added to the wells and incubated at 37°C for 30 minutes. After washes, biotinylated anti-rat THP was added to each well at a dilution of 1:1000 and incubated for 30 minutes at 37°C. Following washes, plates were incubated with a 1:1000 dilution of streptavidin conjugated to alkaline phosphatase (Fisher Scientific Co., USA) at 37°C for 30 minutes. Excess antibody was removed by washes, and the plates were incubated with the alkaline phosphatase substrate (Sigma Chemical Co, CA) at 37°C for 30 minutes and the plates were read at 405 nm on a microplate reader.
Calculations were performed with the statistical software Sigma Plot (Jandel Scientific Co, CA). The average daily THP excretion was calculated based on 20 separate samples over 9 days (samples taken at day 0, 3, 5, 7 and 9).

Results

Purification of Rat THP

A photograph of the arrangement of metabolic cages for rat urine collection is shown in Figure 4.1. and a typical precipitation profile for THP from the collected urine is shown in Figure 4.2a. As seen in this Figure, several cycles were necessary to remove all the THP from the urine. Approximately 10-11 mg of THP could be obtained from 500 ml of pooled normal rat urine.

Purification of Human THP

A single precipitation appeared to be sufficient to precipitate most of the THP from the human urine. Although there was probably some loss of sample during the processing, a substantial amount of THP could be isolated from a 24 hour urine collection (e.g: 25 mg in one sample). On a 10% polyacrylamide gel, all THP samples showed a band at approximately 90 Kd. On Western blots, sheep anti-human THP antibody (The Binding Site, CA), confirmed the identity of these preparations (data not shown). For the sake of comparison and consistency, data presented below refers to THP purified from the normal male subject and from normal male rats.
Figure 4.1 Arrangement of metabolic cages showing a setup for rat urine collection. Urine flowing through the tubing (T) is collected in either individual containers or as pooled samples, in the ice chest (IC).
Figure 4.2 Purification and analysis of normal rat THP. (a) Typical precipitation cycles showing the viscous gel-like precipitates (arrows). (b) Rat and human THP stained for protein (Coomassie blue) and carbohydrate (PAS, purple). Note that both the proteins appear similar. (c) A silver stained gel showing bands of rat (R) and human (H) THP. (d) Identification of THP in rat urine (RU, 10 µl) by the polyclonal antibody to rat THP (dilution 1:10000).
Staining for Protein and Carbohydrate

When stained with Coomassie blue for protein and periodic acid schiff (PAS) method for carbohydrates, both human and rat THP samples showed staining of similar intensity. At this level of analysis, this indicated that the protein (blue) and carbohydrate (purple) contents of these samples were very similar (Figure 4.2b).

Gel Electrophoresis

Both human and rat normal male THP showed a molecular weight of approximately 90 Kd. The purity of the THP preparations was confirmed when single bands were seen in gels stained with the sensitive silver staining technique as shown in Figure 4.2c.

Western Blotting

Both human and rat THP preparations showed single bands at approximately 90 Kd, when stained with their respective antibodies, indicating purity of the preparations. The anti-rat THP antibody specifically detected THP in 10 µl of normal rat urine, when used at the dilution of 1:10,000 (Figure 4.2d).

Amino Acid analysis

A comparison of the amino acid analysis of human and rat THPs is shown in table 4.1. The amino acid composition of human and rat samples indicated that although there were some variations in the constituents, the contents were similar. It is evident that
Table 4.1

A comparison of amino acid composition urinary THP from different sources. Rabbit* (Marr et al., 1970) and Human* (Fletcher et al., 1970c) values are taken from the literature for comparison with data obtained in this study.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Rabbit*</th>
<th>Human*</th>
<th>Human Male</th>
<th>Human Female</th>
<th>Rat Male</th>
<th>Rat Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>10.17</td>
<td>10.90</td>
<td>11.24</td>
<td>10.36</td>
<td>11.43</td>
<td>12.07</td>
</tr>
<tr>
<td>Thr</td>
<td>8.96</td>
<td>7.63</td>
<td>7.34</td>
<td>6.10</td>
<td>8.06</td>
<td>8.42</td>
</tr>
<tr>
<td>Ser</td>
<td>8.13</td>
<td>7.86</td>
<td>7.44</td>
<td>8.71</td>
<td>8.54</td>
<td>8.82</td>
</tr>
<tr>
<td>Glu</td>
<td>9.79</td>
<td>8.44</td>
<td>10.71</td>
<td>11.72</td>
<td>12.82</td>
<td>12.81</td>
</tr>
<tr>
<td>Pro</td>
<td>3.99</td>
<td>4.26</td>
<td>5.93</td>
<td>5.08</td>
<td>5.46</td>
<td>2.96</td>
</tr>
<tr>
<td>Gly</td>
<td>8.56</td>
<td>8.41</td>
<td>10.50</td>
<td>14.43</td>
<td>9.39</td>
<td>8.83</td>
</tr>
<tr>
<td>Ala</td>
<td>4.89</td>
<td>6.79</td>
<td>7.08</td>
<td>6.14</td>
<td>4.82</td>
<td>3.71</td>
</tr>
<tr>
<td>Val</td>
<td>7.03</td>
<td>6.40</td>
<td>4.08</td>
<td>4.40</td>
<td>5.40</td>
<td>5.81</td>
</tr>
<tr>
<td>Met</td>
<td>1.54</td>
<td>2.05</td>
<td>1.85</td>
<td>0.80</td>
<td>1.75</td>
<td>1.66</td>
</tr>
<tr>
<td>Ile</td>
<td>3.05</td>
<td>2.46</td>
<td>2.73</td>
<td>3.06</td>
<td>3.39</td>
<td>3.68</td>
</tr>
<tr>
<td>Leu</td>
<td>7.96</td>
<td>7.58</td>
<td>8.78</td>
<td>8.45</td>
<td>8.47</td>
<td>9.05</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.39</td>
<td>3.83</td>
<td>4.92</td>
<td>4.95</td>
<td>5.59</td>
<td>5.07</td>
</tr>
<tr>
<td>Phe</td>
<td>3.49</td>
<td>3.14</td>
<td>6.63</td>
<td>5.07</td>
<td>4.94</td>
<td>4.52</td>
</tr>
<tr>
<td>Lys</td>
<td>2.06</td>
<td>2.65</td>
<td>2.89</td>
<td>3.63</td>
<td>3.20</td>
<td>5.41</td>
</tr>
<tr>
<td>His</td>
<td>2.75</td>
<td>2.67</td>
<td>2.58</td>
<td>2.22</td>
<td>1.71</td>
<td>1.68</td>
</tr>
<tr>
<td>Arg</td>
<td>3.78</td>
<td>4.49</td>
<td>5.18</td>
<td>4.80</td>
<td>4.91</td>
<td>5.42</td>
</tr>
</tbody>
</table>
Table 4.2

Carbohydrate composition of normal human and rat THP. All values expressed in %. Total %: This number is based on mass determination and represents total % of carbohydrates in a given THP sample. The % values of the individual constituent sugars indicate their quantity in a total of 100 % carbohydrate moiety. GlcNAc: Glucoseamine, GalNAc: Galactoseamine, SA: Sialic Acid.

<table>
<thead>
<tr>
<th></th>
<th>Total %</th>
<th>Glucose</th>
<th>Fucose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>GlcNAc</th>
<th>GalNAc</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>18.1</td>
<td>1.5</td>
<td>2.3</td>
<td>15.7</td>
<td>16.5</td>
<td>34.1</td>
<td>5.9</td>
<td>24.1</td>
</tr>
<tr>
<td>Rat</td>
<td>19.3</td>
<td>2.0</td>
<td>3.1</td>
<td>12.6</td>
<td>22.5</td>
<td>33.0</td>
<td>1.2</td>
<td>25.5</td>
</tr>
</tbody>
</table>
acidic amino acids (aspartic and glutamic) were present in higher amounts than the basic amino acids (arginine and lysine), which are known to contribute to the low pI (4.1) of THP.

Carbohydrate Analysis

Detailed analysis of the carbohydrate composition of rat and human THP is given in Table 4.2. As shown in the table, human THP showed 18.1% of total carbohydrate complex type N-linked, highly branched oligosachharides (based on the ratio of galactose to mannose) and highly sialated (based on the equal amounts of Galactose and sialic acid). Normal rat THP showed a total of 19.3% carbohydrates, also with highly branched oligosaccharides. It appears that although most of the components were similar, galactoseamine appeared to be considerably lower in the rat sample. The significance of this observation is not clear at this time.

Lectin Binding Assay

Human as well as rat THP samples bound the two lectins specific for sialic acids in a similar fashion, indicating that their sialic acid content was similar.

Characterization of Anti-THP Polyclonal Antibody

As shown in Figure 4.3, the new polyclonal antibody generated (which was also used for the Western blots shown in Figure 4.2d) showed high activity and compared well with Hoyer’s antibody.
Figure 4.3 Comparison of Hoyer's antibody (H) and the anti-rat THP antibody made for this study (UF). Note that the new antibody shows higher reactivity with the purified rat THP in ELISA.
Quantitation of Rat Urinary THP by Antigen Capture Assay

The three dilutions of urine (1:320, 1:640 and 1:1280) gave comparable results. Therefore, for all calculations, the dilution of 1:1280 was selected as a precaution to avoid interference by other factors in the assay. Although there was a minor variation in readings from plate to plate, standard curves from all plates were similar and consistent. An example of a typical standard curve is shown in Figure 4.4.

As shown in Table 4.3, statistical analysis of 20 separate urine samples over a 9 day period showed that THP values ranged from a minimum of 552 µg/day to 2865 µg/day with a mean of 422 µg (at 99% confidence level). When converted to mg/L, the daily excretion of rat THP translated into a range of 34.5 ± 38.6 to 180 ± 38.6 mg/L with a mean of 104.9 mg/L. These values are based on average rat urine volume of 16.5 ml per day, calculated from urine volume measured throughout the experiment.

The THP values of three rats are shown in Figure 4.5. As it can be seen from the graph, there was considerable variation in the daily excretion of THP in a single rat over 9 days. For example, rat #2 showed a value of 2000 µg on day 0, followed by 1900, 1600, 2150 and 2400 µg at days 3, 5, 7, and 9 respectively.

Discussion

As stated in the introduction of this chapter, analysis of human THP was used as a tool for standardization of various techniques for characterization of the glycoproteins. A comparison of the biochemical data of human THP from the literature and from this study
Figure 4.4 A typical standard curve from one of the microplates for antigen capture assay.
Table 4.3

Quantitative measurements of rat urinary THP excretion

<table>
<thead>
<tr>
<th></th>
<th>Avg/24 hours</th>
<th>Avg/ml</th>
<th>Avg/100 gm/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1679.54</td>
<td>119.18</td>
<td>46.60</td>
</tr>
<tr>
<td>Standard. Deviation</td>
<td>617.85</td>
<td>48.01</td>
<td>16.27</td>
</tr>
<tr>
<td>Standard Error</td>
<td>145.62</td>
<td>11.31</td>
<td>4.35</td>
</tr>
<tr>
<td>95 % Confidence</td>
<td>307.2583</td>
<td>23.87</td>
<td>9.39</td>
</tr>
<tr>
<td>99 % Confidence</td>
<td>422.10</td>
<td>32.80</td>
<td>13.10</td>
</tr>
<tr>
<td>Minimum</td>
<td>552.96</td>
<td>36.86</td>
<td>14.74</td>
</tr>
<tr>
<td>Maximum</td>
<td>2865.60</td>
<td>229.24</td>
<td>75.16</td>
</tr>
<tr>
<td>Sample Size</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 4.5 A graph showing daily THP excretion of four rats over a nine day period. Note that there is a significant day-to-day variation in the quantity of THP excreted.
showed that their composition was basically similar. THP migrated at 90 Kd and did not contain any unusual amino acids. The total carbohydrate content observed in this study was slightly lower than some reports, which can be attributed to sample variation and differences in the techniques of analysis. The overall similarity of data on human THP obtained in this study and that from the literature established the validity of the analysis techniques used.

As discussed in the previous chapter, in vertebrates other than man, a comprehensive study of various tissues of 48 vertebrate species has shown that THP appeared early in phylogeny in the skin, gills and later in the kidney. Furthermore, the results of this study suggest that although it is conserved through evolution, THP is antigenically different in amphibians and mammals (Howie et al., 1993). However detailed biochemical analysis of rat THP has not been described previously. The objective for examination of normal rat THP was three-fold: 1) Since in spite of markedly high oxalate levels than man, rats rarely form spontaneous kidney stones (Khan and Hackett, 1985), the analysis of normal rat THP itself could possibly provide information about structure-function relationship, with respect to the involvement of THP in CaOx lithiasis. Therefore assuming that THP was involved in stone formation, it was of interest to examine if the THP from normal rats had any peculiar properties which could influence its interaction with crystals. 2) THP has been shown to be a phylogenetically conserved molecule in mammals. Detailed analysis of rat THP and a comparison with the human glycoprotein was necessary to test if rat THP showed similar properties. 3) Lastly, because this study primarily utilized a rat model of nephrolithiasis to study THP, it was
imperative to first purify and characterize normal rat THP. In addition, this information would provide a standard for analysis of THP from nephrolithic rats described in chapters 5 and 6.

Antibody affinity purification (Kirchner and Bichler, 1976), and single precipitation (similar to human THP) by sodium chloride (Wirdham and Milner, 1985) for purification of rat THP has been reported. In the latter case, neutralization of surface charges by the salt leading to changes in structural conformation is the major element in precipitation of a protein (Englard and Sifter, 1990). In addition, the concentration of the salt necessary to precipitate a protein depends on the net number and distribution of charges exposed on the surface. In this study, it was found that several attempts to purify rat THP by a single step of sodium chloride precipitation failed. This observation agreed with that described by Hoyer (1974). Therefore, it was possible that there was an intrinsic physico-chemical difference in human and rat THP, which could explain the single precipitation method not being effective for rat THP. This hypothesis was further examined by detailed biochemical analysis of rat THP.

In general, the quantity of total THP precipitated from a batch of rat urine depended upon the starting volume. However, even though an identical precipitation procedure was used for all samples, the profile of THP precipitation differed with each sample. In some cases, most of the THP precipitated in the first cycle, and in others, maximum THP precipitated in the later cycles. To precipitate all of the THP from each sample, the cycle was repeated at least 4-5 times as suggested by Dr. Hoyer (personal communication).
A comparison of THP from several species of mammals (excluding rat) has shown that the protein component of THP in these animals is very similar. From a comparison of the amino acid composition of rat and human THP it was evident that although there were minor differences in the content of some amino acids, basically the composition of THP from human and rat urine was very similar. Both contained the acidic amino acids (aspartic and glutamic) in slightly higher amounts (10-12%) than the other constituents. However, in contrast to OPN which contains 22% acidic amino acids (Butler, 1989), this percent is not sufficiently high to classify THP as a typical acidic protein.

As demonstrated by the results, although the percentage carbohydrate contents of rat and human THP was similar, some differences in the composition were apparent. For example, the content of the sugar galactoseamine was significantly lower (1.2% versus 5.9%) than human THP. Similar to human THP, and in contrast to rabbit THP, rat THP showed N-linked glycan chains. Moreover, in contrast to hamster THP which lacks sialic acid, sialic acid content of rat THP, was similar to that of human THP. These observations confirm that although there may be a difference in the carbohydrates, the protein content of THP is remarkably similar within different species.

In the literature, the values of THP excretion in mammals can be found expressed as different units. For humans, the average daily THP excretion is 20-100 mg, based on an average daily urine volume of 1-1.5 liters. In the study by Kirchner and Bichler (1976) uni-dimensional agar Laurell technique revealed that the average daily THP excretion in rats was 197±56 units. Wirdham and Milner (1985) used the radioimmunoassay
technique and quantitated THP in serum (concentration in plasma: 43-240 ng/ml), urine and various tissues of normal Wistar rats. They used urine diluted to 1:500 in distilled water and concluded that normal excretion of THP in rats was 473±34 mg/day in males and 383±23 mg/day in females. In addition, there was a correlation in the amount of THP excretion and body weight in females but not in males. In a previous study Wirdham and Milner (1984) they reported that release of THP by rat kidney cortex slices into media was increased from 22.6±0.6 to 30.9 ng/mg of tissue, when the sections were incubated with frusemide, a diuretic.

In another study, Bachmann et al. (1985) used radiolabeled antibody to determine excretion of THP in Brattleboro rats, a strain with hereditary hypothalamic diabetes insipidus. In control rats, the urinary THP excretion was 367± 41 µg/day /100 gm body weight. The authors concluded that rate of THP synthesis was linked to neither the process of urine concentration nor the ion transport activity in the TAL. By using a similar method, urinary THP in cats was found to be 49.2±35.5 µg/ml (Rhodes et al., 1992).

The antigen capture assay used for quantitative determination of rat THP in this study is similar to that for human THP as described by Reinhart et al. (1988) this study. After several experiments were performed to standardize the assay, it was determined that using highly diluted (1:1280) urine samples would eliminate interference of other urinary proteins in the assay. This approach made the assay extremely sensitive and consistent, as shown by the uniformity of standard curves obtained from all assays. A comparison of THP excretion values in other mammals with the values for rat seen in this study
indicates that in general rats excrete higher amounts of THP. The higher values of THP excretion observed in this study could be attributed to a combination of the particular strain of rats, type of assay and extreme sensitivity of the assay. In comparison with the range of human THP excretion (20-100 mg/day), rats excrete an average of 422 µg THP per day. If the average value for rat urinary THP excretion is calculated for a volume of 1.5 liters (approximate daily urinary volume for humans), daily THP excretion for rats is approximately 50 mg, a value very similar to the human THP value.

In conclusion, analysis of rat THP indicated that it did not contain any unusual biochemical characteristics. A comparison of information rat and human THP revealed that the biochemical features of rat THP parallel that of human THP. Therefore, it could be assumed that rarity of calcium oxalate precipitation in rats was not due to the structural properties of the glycoprotein alone, which emphasized the necessity to examine other factors.
CHAPTER 5

ANALYSIS OF TAMM HORSFALL PROTEIN IN A SEMI-ACUTE ACCELERATED MODEL FOR NEPHROLITHIASIS

As discussed in Chapter 2, urine is a complex mixture of water, ions, salts, lipids, proteins, and other metabolites, all of which can influence the process of calcium oxalate crystallization leading to stone formation. Of these components, proteins, which are a major constituent of the urinary stone matrix (Boyce, 1956; Khan et al., 1983), have the intrinsic ability to interact with crystals. Therefore they can modulate crystallization effectively in a positive or a negative fashion. It is apparent from the literature review that the majority of the structural as well as functional information on THP in relation to stone formation has been derived from human THP, and therefore, via in vitro studies. Because these studies have yielded controversial results, it is not clear whether THP is indeed involved in any fashion in stone formation. Therefore, without any preconceived notion as to whether THP is involved in this process or not, the experimental approach with the rat nephrolithiasis model in general was based on the following rationale:

1. In the nephrolithiasis model used in this study (Chapters 5, 6), the stone-forming conditions were simulated by induction of hyperoxaluria. Since rats have oxalate metabolism very similar to humans, they provide an ideal in vivo system to study processes involved in stone formation. 2. Secondly, since THP is synthesized at a
precisely specific location in the nephron, examination of its localization under stone-forming conditions would be in an important tool for investigating its participation in this process. 3. Biochemical analysis of THP purified from the stone-forming rats could provide valuable information on the structure-function relationship of this glycoprotein.

Because CaOx stones are most common in man, in this study, the examination of THP (described in this chapter and Chapter 6) was performed in the context of deposition of CaOx crystals in rat kidneys. As discussed by Khan and Hackett (1985), and one can use various protocols to induce either acute or chronic stone-forming conditions in in vivo models. All protocols are based on the presence of hyperoxaluria, a primary requirement for deposition of crystals. Hyperoxaluria, in turn, can be induced either indirectly by using any oxalate precursor, or by direct load of oxalate salts via different routes. The following combinations have been well documented:

**Acute Hyperoxaluria** a) Intraperitoneal sodium oxalate injections (Khan et al., 1979; Khan, 1991)

**Semi-Acute Hyperoxaluria** a) A combination of ammonium oxalate and ammonium chloride in diet (Gregory et al., 1984) b) Ethylene glycol and ammonium chloride in drinking water (Boeve et al., 1993)

**Chronic Hyperoxaluria** a) Ethylene glycol in drinking water (Lyon, et al., 1966) b) Sodium oxalate in diet (Gregory et al., 1984) As indicated in the introduction, this study has utilized two slightly different models of stone formation in rats. Although the models differ in some features and use a distinctly different perspective for analysis of
THP, the primary aim of the two protocols is identical: to induce hyperoxaluria and CaOx deposits in the kidney.

This chapter describes the investigation of THP under conditions in which the crystal deposition was accelerated by deliberate lowering of the urinary pH. A primary advantage of this protocol was the short-time course of crystal deposition. This allowed the precise tracking of crystal deposition in the kidney and facilitated the daily urine collection and examination throughout the experiment. Data in the literature and previous experience with this model had indicated that crystal deposition could be accelerated by adding ammonium chloride (AC) in combination with ethylene glycol (EG). In this section, experiments were designed to obtain the following information:

1. time course of crystal deposition,

2. precise determination of when the crystals were first visible in the kidney,

3. obtaining a microscopic profile of crystals formed,

4. examination of localization of THP under these conditions,

5. purification and biochemical analysis of THP, and

6. quantitative determination of THP excretion during crystal deposition

**Materials and Methods**

**Induction of CaOx Deposits:**

For the accelerated induction of crystal deposits (semi-acute model), rats were given 0.75% ethylene glycol and 2% ammonium chloride (to lower the pH) for 10 days.
The two control groups included feeding with regular water and with 2% ammonium chloride only. In this model, animals were sacrificed at regular intervals of 4, 6, 8, and 10 days. At sacrifice, the kidneys were fixed in either 10% formaline or half strength Karnovsky’s fixative (Karnovsky, 1965) for 24-72 hours and processed for histology by standard techniques.

Examination of Bladder Aspirates by Scanning Microscopy:

In order to obtain a profile of crystal deposition during the 10 day period, at the time of sacrifice bladder aspirates were collected on 0.2µ polycarbonate filters (Solution Consultants, Atlanta, USA) by using a vacuum manifold. The dried filters were sputter-coated with carbon and examined under a Hitachi 4000 scanning electron microscope.

Analysis of THP

Protocols for Purification of THP, gel electrophoresis and amino acid analysis were same as those described in the previous chapter for normal rat THP.

Immunohistochemical Staining

The antibody and goat serum dilutions were made in 2% Bovine serum albumin (BSA) in phosphate buffered saline (PBS). In this protocol, one of the following antibodies were used at a dilution of 1:100 in this protocol: 1) A polyclonal antibody to rat THP was kindly provided by Dr. John Hoyer (Children’s Hospital, Philadelphia, PA) the specificity for which has been shown previously (Hoyer et al., 1974). 2) Antibody
made at the University of Florida, described in Chapter 4. Non-immune rabbit serum was used as the negative control.

Paraffin was cleared in xylene and the sections were re-hydrated through a graded alcohol series. After two rinses in PBS of 5 minutes each, the endogenous peroxidase activity was removed by incubation in 3% hydrogen peroxide for 30 minutes at room temperature. The slides were washed twice in PBS and treated with 0.1% trypsin (Lipshaw Chemicals) for 10 minutes at 37°C. The non-specific binding sites were blocked by incubation with normal goat serum (3 drops in 2% BSA-PBS) for 5 minutes. The sections were then incubated with the primary antibody (anti-THP 1:100) for 30-60 minutes at room temperature. After rinses with PBS, sections were incubated with goat anti-rabbit-Horseradish peroxidase conjugate (Fisher Scientific Co. Atlanta, GA) at a dilution of 1:200 for 10 minutes. The color was developed with 3,3'-diaminobenzidine (DAB, Sigma Chemical Co.) as the substrate. After counter-staining with hematoxylin and re-hydration, slides were mounted with Permount (Fisher Scientific Co, Atlanta, GA).

Antigen Capture Assay

Quantitative determination of THP excretion was performed with individual urine samples from the rats at days 1, 3, 5, 7, and 9. The protocol was identical to the one described for the normal rats in Chapter 4. The day before the start of the treatment was designated day zero (baseline collection). Therefore data for day 3 indicated treatment for 2 days and so on.
Results

Crystal Deposits

At sacrifice, crystal deposition was seen at one or more of the following areas: cortex, cortico-medullary junction, papilla (either in tubules or external), papillary tip, and fornices. Due to the use of AC, in contrast to the chronic model described later, crystal deposition was accelerated and the deposition in the papilla was much more frequent. Examination of kidney sections over the 10 day period showed that typical crystal deposits (Figure 5.1) which appeared after day 5 and were present thereafter. From here on, the terms pre-crystal (up to day 5) and post-crystal (after day 5) will be used to differentiate data before and after crystal deposition. Crystal aggregates showed morphological features similar to those described by Khan (1994). The deposits ranged in size (longest axis) from approximately 10-110 microns. Kidneys from normal rats and from ammonium chloride-fed rats showed normal morphology without crystals.

Profile of Crystal Deposition

Examination of the bladder aspirates revealed that at day 4, single crystals were present with a few small aggregates (Figure 5.2a). The number of aggregates increased gradually and by day 10, most of the crystals were present in the form of large, numerous aggregates (Figure 5.2b). Based on the morphology, calcium oxalate monohydrate as well as dihydrate crystals were visible.
Figure 5.1 Typical birefringent crystal deposits (arrows) in the papilla of a rat treated with ethylene glycol and ammonium chloride.
Figure 5.2 Scanning electron micrographs of bladder aspirates of stone-forming rats. (a) Single crystals seen at day 4. (b) Large aggregates of calcium monohydrate and dihydrate at day 10.
Biochemical Analysis of THP

After several experiments it became clear that the quantity of THP that could be purified from the nephrolithic rats was small, and not sufficient for complete biochemical analysis. Therefore only partial characterization such as gel electrophoresis and amino acid composition analysis was performed. The glycoprotein, purified from several separate pooled urine samples migrated at approximately 90 Kd, similar to the normal rat THP described in Chapter 4. Amino acid analysis data for samples from various time periods during the experiments and from THP of two stone formers is shown in Table 5.1. Values of normal rat and human THP are repeated for comparison.

The following conclusion can be made from the data in this table:
1. In general, although there were minor differences in the values of individual amino acids, the amino acid composition of the THP from the nephrolithic rats was similar to that of the normal rats. As seen in the table, in comparison to normal THP, stone former’s THP did not show any striking differences.
2. Similarly, there were no significant differences in the amino acid composition of THP purified from the pre and post-crystal stages.

Localization of THP

Control Rats The specificity of the anti-THP antibody was demonstrated by a comparison of sections incubated with non-immune serum (Figure 5.3a) and the antibody (Figures 5.3b-d). Kidney samples from control rats showed THP showed a typical normal pattern of distribution in the cortex and the medulla, with intense staining in the
Table 5.1

A comparison of amino acid composition of normal rat THP and THP purified from urine samples at various time points during the experiment.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Normal (Pooled)</th>
<th>Pre-Crystal</th>
<th>Post-Crystal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days 2+3</td>
<td>Day 4</td>
<td>Days 5+6</td>
</tr>
<tr>
<td>Asp</td>
<td>10.95</td>
<td>11.18</td>
<td>11.25</td>
</tr>
<tr>
<td>Thr</td>
<td>7.61</td>
<td>8.22</td>
<td>8.19</td>
</tr>
<tr>
<td>Ser</td>
<td>9.73</td>
<td>9.16</td>
<td>9.40</td>
</tr>
<tr>
<td>Glu</td>
<td>12.07</td>
<td>11.73</td>
<td>11.52</td>
</tr>
<tr>
<td>Pro</td>
<td>4.78</td>
<td>5.12</td>
<td>5.11</td>
</tr>
<tr>
<td>Ala</td>
<td>4.76</td>
<td>4.51</td>
<td>4.67</td>
</tr>
<tr>
<td>Val</td>
<td>5.40</td>
<td>6.46</td>
<td>5.69</td>
</tr>
<tr>
<td>Met</td>
<td>0.83</td>
<td>0.59</td>
<td>0.69</td>
</tr>
<tr>
<td>Ile</td>
<td>3.22</td>
<td>3.23</td>
<td>3.16</td>
</tr>
<tr>
<td>Leu</td>
<td>7.81</td>
<td>8.13</td>
<td>7.96</td>
</tr>
<tr>
<td>Tyr</td>
<td>4.49</td>
<td>4.81</td>
<td>4.90</td>
</tr>
<tr>
<td>Phe</td>
<td>4.72</td>
<td>5.38</td>
<td>5.35</td>
</tr>
<tr>
<td>His</td>
<td>4.72</td>
<td>5.27</td>
<td>5.24</td>
</tr>
<tr>
<td>Lys</td>
<td>2.77</td>
<td>2.36</td>
<td>2.56</td>
</tr>
<tr>
<td>Arg</td>
<td>4.70</td>
<td>4.59</td>
<td>4.85</td>
</tr>
</tbody>
</table>
Figure 5.3 Immunoperoxidase localization of THP in normal rat kidney. (a) Non-immune serum control is negative. (b) Specific staining can be seen in the thick ascending limbs (TAL, arrows). (c) Higher magnification micrograph of TALs showing intense stain in the lining cells. (d) THP is absent in the papillary region.
TALs (Figure 5.3b). Examination at a higher magnification revealed that the cytoplasm of the cells lining the TALs was intensely positive for THP (Figure 5.3c). As expected, the papilla was negative for THP (Figure 5.3d).

The second control group, with NH₄Cl, the staining was normal at day 4. At day 10, THP appeared in very few papillary tubules and was of very low intensity when compared to that of the nephrolithic rats.

Nephrolithic Rats In the EG-NH₄Cl-treated rats, the appearance of THP in the papilla coincided with the formation of crystal deposits in the kidney. At day 4 (when no deposits were seen in the sections), there was no abnormal THP staining. In contrast, at day 6, when few crystals were seen in the sections, some papillary tubules stained positive for THP. At day 8, in rats with few deposits, the THP staining in the papilla remained somewhat comparable to day 6. In rats with increased deposits, the number of papillary tubules stained had increased significantly.

The most atypical staining for THP was observed in the papilla of rats with 10 days of EG treatment. THP was found associated with the crystal deposits in the papilla. In addition, some of the tubular lumen contained deposits of the dark matrix-like material. Some of these were associated with the crystals (Figure 5.4a), others were found in tubules without crystals (Figure 5.4b).

Quantitative Determination of THP Excretion

The data for THP excretion of the stone-forming rats is shown in Table 5.2 and Figure 5.5. The results show that similar to the day to day variation seen in normal rats,
Figure 5.4 Immunoperoxidase localization of THP in the papilla of nephrolithic rats. (a) THP associated with crystals (arrows). (b) THP deposit in some tubular lumen, without crystals (arrows).
Table 5.2
Urinary THP excretion of four nephrolithic rats. Note that no specific trend of either increase or decrease in the THP secretion levels as a function of treatment time can be detected.

<table>
<thead>
<tr>
<th></th>
<th>Rat#1</th>
<th>Rat#2</th>
<th>Rat#3</th>
<th>Rat#4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>954.91</td>
<td>1428.53</td>
<td>1344.92</td>
<td>969.52</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>84.09</td>
<td>496.94</td>
<td>830.42</td>
<td>444.98</td>
</tr>
<tr>
<td>Std. Error</td>
<td>42.05</td>
<td>222.24</td>
<td>371.37</td>
<td>199.00</td>
</tr>
<tr>
<td>95% Conf. Int.</td>
<td>133.78</td>
<td>617.02</td>
<td>1031.07</td>
<td>552.50</td>
</tr>
<tr>
<td>99% Conf. Int.</td>
<td>245.15</td>
<td>1022.97</td>
<td>1709.39</td>
<td>915.98</td>
</tr>
<tr>
<td>Days Tested</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Minimum</td>
<td>852.48</td>
<td>841.73</td>
<td>827.90</td>
<td>285.69</td>
</tr>
<tr>
<td>Maximum</td>
<td>1049.47</td>
<td>2179.20</td>
<td>2816.64</td>
<td>1440.00</td>
</tr>
</tbody>
</table>

Figure 5.5 Graphical representation of the data listed in Table 5.2. The data clearly illustrate the absence of a systematic trend in THP secretion levels. Note that all the rats were given identical treatment.
there was increase/decrease in the quantity of THP excreted. However, a definite one-directional trend of increase/decrease was not seen. Although there was a day to day variation in the amount of THP excreted by the stone-forming rats, a specific trend of increase or decrease was not seen.

Discussion

Proteins have been long known to be important components of biological crystallization systems. Due to their inherent property of binding crystals, they can act as efficient modifiers of the crystallization processes (Addadi and Weiner, 1985). In pathological crystallization such as the urinary stone, they are a major constituent of the stone matrix (Boyce and Garvey, 1956; Boyce and King, 1963; Boyce, 1968) and may modulate the formation of stones. The specific association between organic matrix and crystals has been demonstrated by the electron microscopic studies by Khan and Hackett (1993), which confirmed previous observations of others in more detail at the ultrastructural level.

Oral administration of EG induces a low grade but persistent hyperoxaluria, leading to characteristic CaOx deposits in the kidney (Rushton and Spector, 1982; Khan and Hackett, 1982), and resembles stone formation in humans. The quantity of CaOx crystals deposited in the kidney is proportional to the concentration of ethylene glycol used (Blood, 1965). As demonstrated by the immunoperoxidase technique at the light
microscopic level, the matrix like THP was closely associated with the crystals, and showed abnormal localization by appearing in the renal papilla.

Lyon et al. (1966) demonstrated that by using a combination of ethylene glycol and ammonium chloride in drinking water, the crystal deposition could be accelerated. A major advantage of this protocol is that due to the lowering of pH and increase in urinary calcium by use of ammonium chloride, the crystal deposition process is accelerated considerably. This short-term accelerated model allowed us to precisely track the course of crystal formation by examining the bladder aspirates by scanning electron microscopy and deposition in the kidneys by light microscopy. A similar model has been used by other researchers (Boeve et al., 1993; De Bruijn et al., 1993) who noted that crystal deposition occurred in various parts of the nephron.

The administration of ammonium chloride such as used in this study will induce imbalance of the acid-base resulting in acidosis of the urinary environment. A similar condition is not uncommon in stone-forming patients, for example in renal calculi with renal tubular acidosis (Konnack et al., 1982; Osther et al., 1989). One of the criteria that was considered during evaluation of this model was whether the use of ammonium chloride itself caused significant damage to the renal tubules. Although the literature indicates that there is some effect of ammonium chloride itself, the effect appears to be reversible within the limits of the experiment. For example, in a study on the effect of ammonium chloride, it has been shown that in rats the metabolism of components such as amino acid remains unaffected (Galicek et al., 1981). In a separate study with rabbit TALs, monitoring of intracellular pH showed that although there was initial intracellular
acidification of the TAL, it was followed by a recovery to a steady state value (Von Recklinghausen et al., 1992). Nevertheless, in order to avoid any interference of this factor, the use of ammonium chloride was eliminated in the chronic model, described in the next chapter.

It was found that although hyperoxaluria and crystalluria were present at day 2, crystals were visible in the kidney at day 6 and persisted thereafter. Positive staining for THP in the papilla was seen only at day 6, concurrent with the crystal deposition. In contrast, prior to crystal deposition (day 4), no THP was found in the papilla. This indicated that the process of crystal deposition and appearance of THP in the papillary tubules occurred somewhat simultaneously. As the extent of deposition increased, as indicated by larger aggregates in the bladder aspirates and larger and more numerous deposits in the kidneys), significantly more number of papillary tubules stained positive for THP.

Quantitative determination of THP excretion in normal subjects and stone formers has been reported in several studies (Bichler et al., 1973; Grant et al., 1973; Samuell, 1978), and majority of the studies have shown that there is no correlation between the two. However, the issue of the relationship between stone formation and amount of THP excretion does not appear to be settled. As discussed by Hess (1994) two separate studies which used radioimmunoassay as the detection method, revealed that stone-formers had lower rates of THP excretion.

The following conclusions can be made based on the data in this section: 1) Hyperoxaluria followed by deposition of CaOx crystals in the kidneys induced
accumulation of THP around the crystals in the cortex and the medulla. Within 10 days, 80-90% of the rats showed considerable crystal deposition. 2) By SEM analysis of bladder aspirates, it was confirmed that the aggregates were actually present in situ in the urine, during deposition in the kidney. 3) Biochemically, THP during pre-crystal and post-crystal deposition stages was very similar to normal THP, as was also seen in the case of THP from normal human subjects and stone-formers. Moreover, there was no correlation between THP excretion and crystal deposition. 4) In addition, THP also appeared in the papillary tubules, an otherwise abnormal site for its localization. It was present in association with the crystals and also as what appeared to matrix-like deposits in some tubular lumen, where crystals were not visible.

Due to the low urinary volume that could be collected from these animals, sufficient THP could not be obtained so as to perform complete biochemical analysis and also for use in functional assays. These experiments were performed with THP purified from the chronic model as described in the next chapter.
CHAPTER 6

ANALYSIS OF TAMM HORSFALL PROTEIN IN A CHRONIC NEPHROLITHIASIS MODEL

As seen in the previous chapter, although the interaction of THP and crystals was examined in vivo, due to the severity and the short time range of the model, detailed analyses of THP under stone-forming conditions could not be performed. Administration of ethylene glycol either in the diet (Blood, 1965) or drinking water (Robinson et al., 1990) without any other additives over a few weeks results in consistent hyperoxaluria and consequent deposition of crystals. It has been documented that in male Sprague Dawley rats, 0.5% ethylene glycol for up to 90 days did not result in any calculi. In contrast, if the dose was increased to 1%, there was a low degree of crystal deposition. In this study, based on this information and previous experience in the laboratory a dose of 0.75% (volume/volume) ethylene glycol was used which was expected to induce moderate crystal deposition (Robinson et al., 1990).

The common feature in this and the previous model was induced hyperoxaluria, although the conditions were less severe due to omission of ammonium chloride. The experiments described in this section were designed to obtain the following information: 1) One of the questions to be addressed was if THP-crystal association was similar to that found in the other model. 2) Secondly, because the previous model had shown that THP
was found in the papilla, an abnormal site, it was necessary to examine if the appearance of THP was due to mere deposition or actual synthesis at those sites. 3) Thirdly, further biochemical characterization of the glycoprotein purified from these rats, which could not be performed in the previous model. 4) Comparison of the effect of THP from normal and nephrolithic rats in \textit{in vitro} aggregation assays. 5) In addition, to examine the possibility of more than one protein being simultaneously associated with stone formation, the localization of a second protein, OPN, was examined.

\textbf{Materials and Methods}

\textbf{Induction of CaOx Deposits in Rat Kidneys}

Male Sprague Dawley rats weighing 120-125 grams were housed in metabolic cages three days prior to the start of the experiment, to acclimatize the rats. They were administered 0.75\% ethylene glycol in water \textit{ad libitum} and the control rats were given water. Rats were sacrificed at 42 days. The processing of kidney tissue was similar to that described in the previous chapter. In addition, tissue blocks from rats treated with ethylene glycol for up to 12 and 24 days were obtained from Paula Scott (University of Florida) and examined in a similar manner.

\textbf{Biochemical analysis of THP from Nephrolithic rats}

Methods used for urine collection, purification (purified from urine pooled for 1-2 weeks) gel electrophoresis, staining for protein and carbohydrates, amino acid
composition analysis, and carbohydrate analysis of THP were identical to that described for normal rats in Chapter 4.

**Analysis of Urine Composition**

In order to examine the ionic composition of urine during stone-forming conditions, individual urine samples were collected from 11 rats at day 42, and analyzed individually by ion chromatography.

**In situ Hybridization for THP-mRNA:**

*Synthesis of RNA probe specific for rat THP* Based on the rat THP gene sequence, forward (bases 67-91) and reverse primers (bases 632-608) were selected with the aid of a computer program. Rat kidney mRNA was made by using a mRNA isolation kit from In Vitrogen (San Diego, CA). Rat THP cDNA was synthesized by polymerase chain reaction (PCR) using a cDNA cycle kit from In Vitrogen and the primers described above. As expected, a product of approximately 600 base pairs (bp, range expected 550-650 bp) was obtained. Lower molecular weight primer dimers were removed by using a purification kit from Promega Corporation (Madison, Wisconsin). The purified PCR product was then cloned in the PCR II vector using a TA cloning kit from In Vitrogen (San Diego, CA). Briefly, after ligation and transformation of competent cells, colonies with the correct insert were identified based on their color in the presence of X gal as the substrate. Positive colonies appeared white due to the inactivation of the lac Z gene and negative colonies were blue. DNA minipreps were made (Magicprep Kit from Promega, Madison, Wisconsin) from positive and negative colonies and analyzed on 1.3% GTG-
Nusieve agarose gels (FMC, Rockford, ME). Finally, a large amount of the plasmid with the correct insert was purified from cultures by using a maxiprep column from Quiagen Inc. (Chatsworth, CA). The identity of the relevant insert was confirmed based on its size and by using a restriction enzyme (ScaI) with a site exclusively within the insert. In addition, the segment was sequenced (reaction by using a Sequenase Kit, from United States Biochemical, (Cleveland, Ohio) and the sequence was compared with the rat THP gene to confirm its identity.

After extracting the DNA with phenol-chloroform, the plasmid was linearized with either HindIII or XbaI. The linearized templates were used to synthesize DIG-labeled sense and antisense probes by *in vitro* transcription by using T7 and SP6 polymerases, respectively, in a kit from Boehringer Mannheim (Indianapolis, IN). The probes were checked and their concentration was estimated by using a dot blot assay according to the manufacturer's recommendations.

*In situ* hybridization on tissue sections A survey of current literature on *in situ* hybridization revealed that there was a variety of protocols were available. Out of these, attempts to use DIG-labeled oligonucleotide primers were unsuccessful. The technique for *in situ* hybridization was based on the method described by Viaene and Baert (1994). Several experiments were performed to perfect the technique and optimize conditions for the following conditions:

1. concentration of probe,

2. temperature and time of hybridization,

3. antibody concentration,
4. time for antibody incubation, and

5. color development with the substrate.

Based on the numerous experiments with the parameters listed above, an optimum protocol was developed for tissue sections.

Paraffin sections (similar to those used for immunohistochemistry) were used for in situ hybridization. First, paraffin was cleared in xylene and sections were dehydrated and equilibrated in PBS. The sections were permeabilized with proteinase K (Boehringer Mannheim, Indianapolis, IN) at a concentration of 10 mg/ml for 5-10 minutes at 37°C. After a wash with PBS, the sections were incubated in a pre-hybridizing solution for 60 minutes at 37°C. The pre-hybridizing solution consisted of 0.02% Ficoll, 0.02% BSA, 0.02% PVP, 5% dextran sulfate, 500 µg/ml yeast tRNA, 500 µg/ml salmon sperm DNA (denatured by boiling in a 95°C water bath for 5 minutes), 53.5% formamide and 0.195% DEPC-treated water. For hybridization, DIG-labeled RNA probe was added (5 ng in 30 µl per section). The sections were incubated for 60 minutes at 37°C. The sections were washed (2 washes each for 10 minutes each) with 53.5% formamide and 46.5% 2X or 0.2X SSC. The sections were incubated in Genius buffer 1 (made according to the directions for Genius Kit # 4, Boehringer Mannheim, Indianapolis, IN). Next, non-specific binding sites were blocked by incubating the sections in Genius buffer 2 (Boehringer Mannheim) for 60 minutes. This was followed by incubation with alkaline phosphatase conjugated anti-DIG-alkaline phosphatase antibody (1:1000) for 30 minutes. After washes with Genius buffer 1, the sections were equilibrated in Genius buffer 3 for 1 minute. The color was developed with NBT and BCIP (45 and 35 µl/10 ml respectively)
for 15-30 minutes. The reaction was stopped with Genius buffer 4. Sections were counter-stained with either Neutral red or Fast green.

**In vitro Aggregation Assays:**

The effect of Tamm Horsfall protein on calcium oxalate monohydrate (COM) crystal aggregation was examined by *in vitro* aggregation assays based on the method of Hess et al. (1989). The assays were performed in rat artificial urine (RAU), the composition for which is shown in Table 2.2. Essentially, RAU is a solution of various salts in appropriate amounts, so as to give the correct ionic composition of normal rat urine. Therefore, it was the medium of choice for performing the assays, as opposed to a buffer with only one or two ions, as used in other studies. In addition, it does not contain any proteins or other organic components, and therefore the effect of a single protein can be studied without the interference of other proteins. The composition of RAU was based on values published by Shevock et al. (1993) and the pH was adjusted to 6.3.

The assays were performed in RAU with either normal citrate concentration (16 mM) or with lower citrate concentration (9 mM), which was the same as found in the urine of nephrolithic rats. The entire assay was performed in spectrophotometer at 37°C. To obtain a uniform and stable population of crystals, COM crystals were aged for 6 months in distilled water, harvested and dried. Crystal slurries were made by suspending COM in RAU at a concentration of 0.7 mg/ml and was stirred overnight at 37°C. A 1.5 ml aliquot was transferred to a quartz cuvette in a cell holder which was kept at 37°C and measurement of optical density was started. In order to obtain a base line control curve, the slurry was stirred at 850 rpm for 180 secs and then the stirring was stopped. The fall
in OD (due to aggregation of crystals) was monitored for 300 seconds. During the assay, samples were examined visually for turbidity, and also by scanning electron microscopy.

To study the effect of proteins on aggregation, the slurry was incubated with the protein for at least one hour with constant stirring. Concentrations ranging from 5-100 mg/L were used in pilot experiments. Based on those results, a concentration of 15 mg/L was selected and several curves were generated for each protein at this concentration. The following proteins were tested:

1. normal rat THP,
2. THP from nephrolithic rats, and
3. albumin (as the negative control).

The aggregation of crystals was monitored by using exactly the same procedure as for the baseline. The percent of crystal aggregation \( P \) and the percent inhibition with added protein \( I \) was calculated as follows:

\[
P = \frac{T_s}{T_{sc}} \times 100
\]

where \( T_s = \) slope of the linear regression of \( OD_{620} \) Vs time, in the presence of THP

\[
T_{sc} = \text{turbidity slope of the control}
\]

Therefore, percent inhibition \( I \) in the presence of THP = \( 100 - P \).

To maintain consistency, for each run, extreme care was taken to ensure that only the linear portion of the slope was taken into account for calculations.

**Immunohistochemical Staining**
The technique used for immunohistochemical staining was identical to the method described in Chapter 5. In addition to THP, a polyclonal antibody to OPN (a gift from Dr. Elaine Worcester, VA Hospital, Milwaukee,) was used to localize OPN.

Results

Crystal Deposits

Previous experiments in the laboratory indicated that consistent hyperoxaluria was present by day 12, at the first time point of sacrifice (data not shown). Examination of kidneys after 12 and 24 days showed very few typical birefringent renal CaOx deposits: out of 6 rats, 4 rats showed only 1 deposit per kidney. In contrast, after 42 days, the incidence of renal deposits had increased significantly. All rats showed deposits (Figure 6.1), which ranged from only a few to more than 50 per kidney. They were localized in areas similar to that described in the semi-acute model described in Chapter 5: the crystals were seen in the cortex, medulla and the papilla, however, they were less prevalent in the papilla than seen in the semi-acute model. Kidneys from control rats showed normal morphology without crystals.

Biochemical Analysis

Analysis of THP purified from the nephrolithic rats by SDS-PAGE, Coomassie blue and PAS stain showed that THP from normal and nephrolithic rats was very similar
Figure 6.1 Cross section through the kidney of a rat treated with ethylene glycol for 42 days. Typical birefringent crystals can be seen (arrows).
to normal rat THP described in Chapter 4. Results of amino acid and carbohydrate analysis are described in Tables 6.1 and 6.2.

Amino Acid Analysis

Results of the amino acid composition analysis are shown in Table 6.1. As indicated in the table, there were no major differences in the amino acid contents of the THP from normal and nephrolithic rats.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Normal Rat</th>
<th>Nephrolithic Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>11.43</td>
<td>11.81</td>
</tr>
<tr>
<td>Thr</td>
<td>8.06</td>
<td>8.44</td>
</tr>
<tr>
<td>Ser</td>
<td>8.54</td>
<td>8.98</td>
</tr>
<tr>
<td>Glu</td>
<td>12.82</td>
<td>11.86</td>
</tr>
<tr>
<td>Pro</td>
<td>5.46</td>
<td>4.85</td>
</tr>
<tr>
<td>Gly</td>
<td>9.39</td>
<td>8.91</td>
</tr>
<tr>
<td>Ala</td>
<td>4.82</td>
<td>3.98</td>
</tr>
<tr>
<td>Val</td>
<td>5.40</td>
<td>6.43</td>
</tr>
<tr>
<td>Met</td>
<td>1.75</td>
<td>1.4</td>
</tr>
<tr>
<td>Ile</td>
<td>3.39</td>
<td>3.5</td>
</tr>
<tr>
<td>Leu</td>
<td>8.47</td>
<td>8.98</td>
</tr>
<tr>
<td>Tyr</td>
<td>5.59</td>
<td>5.04</td>
</tr>
<tr>
<td>Phe</td>
<td>4.94</td>
<td>6.58</td>
</tr>
<tr>
<td>Lys</td>
<td>3.20</td>
<td>3.05</td>
</tr>
<tr>
<td>His</td>
<td>1.71</td>
<td>2.33</td>
</tr>
<tr>
<td>Arg</td>
<td>4.91</td>
<td>3.78</td>
</tr>
</tbody>
</table>
Carbohydrate Analysis

The carbohydrate composition analysis is shown in Table 6.2. As shown in the table, although the carbohydrate composition of the THP from normal and nephrolithic rats was largely similar, there were some differences. For example, sialic acid content was lower in the nephrolithic rats and galactoseamine content was lower in normal rats.

Urinary Composition

Table 6.3 shows the average urinary ionic concentration values (based on the data from 11 rats), for nephrolithic rats, at 42 days of EG treatment. As seen in this table, there was a significant difference in the oxalate and citrate concentrations between the normal and nephrolithic rats. The oxalate was five-fold higher and the citrate was 1.7-fold lower. In addition to normal citrate, this concentration of citrate was later used in the aggregation assays.

Characterization and Quantitation of RNA Probe

Analysis of the cloned fragment digested with the appropriate restriction enzymes revealed a 600 bp fragment. Based on the results of the dot blot assay, the concentration of the probe was adjusted and approximately 5 ng probe was used per section. The sequence comparison of the probe and a segment of the rat THP gene revealed a close match (Figure 6.2).
Table 6.2

Carbohydrate composition of THP isolated from normal and nephrolithic rats: All values expressed in %. Total %: This number is based on mass determination and represents total % of carbohydrates in a given THP sample. The % values of the constituent sugars indicate individual percentage of the carbohydrate moiety. GlcNAc: Glucoseamine, GalNAc: Galactoseamine, SA: Sialic Acid.

<table>
<thead>
<tr>
<th></th>
<th>Total %</th>
<th>Glucose</th>
<th>Fucose</th>
<th>Mannose</th>
<th>Galactose</th>
<th>GlcNAc</th>
<th>GalNAc</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>19.3</td>
<td>2.0</td>
<td>3.1</td>
<td>12.6</td>
<td>22.5</td>
<td>33.0</td>
<td>1.2</td>
<td>25.5</td>
</tr>
<tr>
<td>Rat</td>
<td>15.5</td>
<td>2.8</td>
<td>3.1</td>
<td>15.2</td>
<td>23.2</td>
<td>33.8</td>
<td>2.4</td>
<td>19.6</td>
</tr>
</tbody>
</table>
Table 6.3
A comparison of urinary ionic composition of normal and nephrolithic rats

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>Oxalate</th>
<th>Citrate</th>
<th>NaCl</th>
<th>KCl</th>
<th>MgSO₄</th>
<th>NaH₂PO₄</th>
<th>Na₂SO₄</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Rat*</td>
<td>3.39</td>
<td>1.05</td>
<td>16.8</td>
<td>149.0</td>
<td>26.7</td>
<td>21.4</td>
<td>35.8</td>
<td>29.3</td>
<td>6.3-8.2</td>
</tr>
<tr>
<td>EG-Rat**</td>
<td>2.06</td>
<td>4.11</td>
<td>9.7</td>
<td>133.6</td>
<td>211.0</td>
<td>13.3</td>
<td>18.5</td>
<td>28.6</td>
<td>6.3-6.7</td>
</tr>
</tbody>
</table>

*Shevock et al, 1993
** Values obtained from ion chromatography of urine samples from nephrolithic rats
In situ Hybridization

The sense probe did not show any hybridization on the sections (Figure 6.3a) In the normal kidneys, the antisense probe detected THP-RNA in the cortex and outer medulla as expected (Figure 6.3b) indicating specific hybridization. A closer examination of that TALs (Figure 6.3c) revealed an intense purple precipitate in the cells lining the TAL, indicating active site of synthesis. In contrast, as expected, THP-RNA was absent in the papilla, indicating that THP was not synthesized in these cells.

In the nephrolithic rats, the distribution of mRNA remained similar to normal. The observation was confirmed by examining sections from kidneys in which crystals as well as protein was positively present in the papilla. This indicated that although THP was deposited in the papillary tubules, it was not synthesized at these abnormal sites.

Effect of THP on In Vitro Crystal Aggregation

Numerous pilot experiments were performed to obtain optimum control curves and for all analyses, the curves were normalized for comparison. A typical control curve with an explanation for various regions is shown in Figure 6.4. After transferring the slurry to the cuvette, an initial optical density was noted (a). After the stirring was stopped at 180 seconds (b), there was a lag period before sedimentation commenced, as designated by the region between band c. The curve showed a characteristic 'knee' followed by a slope. For each curve, the linear part of the slope (area between d and e) was analyzed and used for calculations.
Figure 6.2 Matching of the DNA template used to synthesize the RNA probe with the rat THP gene sequence. Similarity index = 99.444%, total bases not opposite a gap = 179 Number of gaps = 1, bases opposite gaps = 1, Length of the overlapping region = 180, Number of unambiguous matching bases = 179, Number of ambiguous matching bases = 0.
Figure 6.3 In-situ hybridization for THP-RNA in normal rat kidney. (a) Sense probe showing absence of signal. (b) In the same area as "a", anti-sense probe shows specific detection of THP-RNA in the TALs (arrows). (c) Higher magnification of TALs showing that the cells lining them actively synthesize THP.
Figure 6.4 A typical control curve showing various regions of analysis. a: Initial optical density, b: Stirring stopped at 180 seconds c: 'Knee' indicating start of sedimentation, d--e: Linear region of slope

Figure 6.5 Effect of rat THP (15 mg/ml) on crystal aggregation
Figure 6.6 A visual comparison of aggregation assay with and without THP. In contrast to the control run (Cont), the sample with added protein (RTHP) appears turbid at the end of the assay due to inhibition of sedimentation.
Figure 6.7 Scanning electron micrographs of slurry samples from aggregation assays. (a) Aged crystals used in this assay. (b) Typical aggregates from a control run. (c) Higher magnification micrograph of an aggregate in (b) and (d) A higher magnification micrograph showing crystals coated by THP.
After experimentation with various concentrations of rat THP ranging from 5-100 mg/L, the concentration of 15 mg/L was selected for all further experiments. This concentration is similar to the normal urinary excretion in rat and human. A comparison of the aggregation assay without protein (control) and with 15 mg/L rat THP showed that addition of the protein inhibited aggregation by approximately 45% (Figure 6.5). THP from nephrolithic rats showed similar inhibition and albumin did not have any effect, indicating the effect of THP was specific. The effect of rat THP could be seen clearly by a visual comparison of the samples with and without protein as shown in Figure 6.6. As shown in this figure, in the cuvette in which the assay was performed, the supernatant appears clear, due to the complete sedimentation of the particles. In contrast, in the sample with added rat THP, the supernatant was turbid because the aggregation and consequently the settling of particles was inhibited. On a closer examination by scanning electron microscopy, it was apparent that the crystals were coated with THP (Figure 6.7).

Similarly, in the low-citrate urine, the effect of normal and nephrolithic rat THP was similar to RAU. Both proteins inhibited aggregation by approximately 45%.

**Immunostaining for THP and OPN**

The distribution of THP in normal kidney has been discussed in Chapter 4. Normal staining for OPN is shown in Figures 6.8 and 6.9. As seen in Figure 6.6, some but not all nephrons stained positive for OPN. In addition, as shown in Figure 6.7, intense staining for THP was seen at the calyceal fornices on the papillary surface.
Figure 6.8 Immunoperoxidase localization of osteopontin in normal rat kidney. Positive signal can be seen in some nephrons (arrows) in the outer medullary region.

Figure 6.9 Immunoperoxidase localization of osteopontin in normal rat kidney. Note the intense staining in the calyceal fornices (arrows) of the papillary region.
Figure 6.10 Osteopontin was localized around crystals (arrows), in areas similar to where THP was also found.

Figure 6.11 THP was associated with crystals (arrows). In this photograph, positive staining in TAL indicates normal distribution of THP.
Figure 6.12 THP localization in a nephrolithic rat kidney. Note the intense deposits (arrows) in some papillary tubules, while they are absent in others (X).

Figure 6.13 Osteopontin localization in a nephrolithic rat kidney. The localization is similar to figure 6.9, with some tubules with deposits (arrows) and some without (X).
Nephrolithic rats. In samples with few and small deposits, the distribution pattern of THP and OPN was largely similar to the controls, with only occasional staining of papillary tubules. In contrast, the staining for both THP and OPN was significantly enhanced in rats with extensive (50 or more) crystal deposits. In these samples, in addition to normal staining pattern in the TAL, THP was found closely associated with the crystal deposits, similar to that described earlier. In contrast to OPN, this deposition however, was not visible consistently in all sections examined. OPN was seen consistently and more prominently around the crystal deposits and was present at the same sites (Figure 6.10) as THP (Figure 6.11) consistently in all sections examined (Figure 6.11). In addition, there was a general increase in the signal for OPN in the outer medullary region.

Examination of the papillary region of these kidneys revealed an abnormal and striking presence of the darkly stained matrix-like THP and OPN in some of the tubules, mostly seen without crystals. In comparison to control rats and rats with very few deposits, there was a remarkable increase in both the frequency and intensity of THP (Figure 6.12) and OPN (Figure 6.13) staining in this area.

Discussion

A rat model of nephrolithiasis, such as the one used in this study (with experimentally induced hyperoxaluria) closely mimics stone formation in humans and therefore is a valuable tool in stone research, as discussed by Khan and Hackett (1985). In
the experiments described in this section, a 0.75% concentration of ethylene glycol was used which induces hyperoxaluria, a pre-requisite for formation of crystal aggregates in the kidney. Such a treatment over several weeks (for example 42 days, as used in this study) results in a low intensity, slow crystal deposition process, the frequency and incidence of which increases towards the end of the treatment (as seen in this study and other ongoing experiments in our laboratory). In this study, the deposits typically ranged from none to extensive deposition of more than 50 per kidney examined. In contrast to the time-based short term study performed in the semi-acute model, chronic model facilitated the analysis and specially the localization of THP during a gradual course of crystal deposition, under the following conditions: 1) before appearance of crystal deposits in the kidney, 2) after appearance of at least a few crystal deposits, and 3) after extensive crystal deposition.

In a study with stone-forming rats in which hyperoxaluria was induced by feeding glycolate, Kohri et al. (1993) observed that the mRNA for OPN increased significantly, however, the localization of protein was not examined. In the present study, once it was observed that THP appeared at an abnormal site (renal papilla), the next logical step was to examine if it was merely deposited at that site, or was synthesized at these sites. In situ hybridization revealed that in spite of crystal deposits as well as THP being present in the papilla, THP-mRNA was absent. Therefore, it can be concluded that THP was deposited in association with crystals and not synthesized in the papillary tubules.

The importance of crystal retention as a prerequisite to development of kidney stone has been established by Khan and Hackett (1991). As described in the time-based
study (Chapter 5), it was obvious that THP appeared in the papilla only after (or at the same time) crystals were deposited in the kidney. These data taken together suggest that THP probably travels with the urinary flow, and adsorbs to the existing crystals. Deposition of THP in response to general renal injury, as has been observed in other renal conditions, is also not unlikely.

Most of the functional information on THP has been obtained from studies on human THP, and therefore by *in vitro* studies. These studies have used either urine processed by different methods, or different buffers as the assay media and demonstrated that the effect of THP on crystal aggregation can vary depending on the experimental conditions. For example, Rose and Sulaiman (1982) by using the technique of rapid evaporation, concluded that THP induced CaOx crystallization in ultrafiltered urine. In contrast, in a solution of calcium and oxalate, a 5 mg percent solution of THP had no effect on crystallization and indicated that THP had no role in CaOx crystalluria (Sophasan et al., 1980). Grover et al. (1990) noted that by the evaporation technique, THP (35 mg/L) promoted CaOx crystal deposition. In contrast, by the oxalate load method, THP reduced the size of precipitated CaOx particles significantly. In another study, in undiluted ultrafiltered human urine, THP significantly inhibited crystal aggregation (Ryall et al., 1991). In summary, there is evidence that the method of assay is of critical importance in determining the effect of THP on crystallization.

Hess (1989) used a variety of buffers in a seeded assay system and demonstrated that human THP inhibited CaOx aggregation. This property of THP, was however negatively affected by an increase in NaCl and lowering the pH. In a separate study, it
was shown that addition of 3.5M citrate reversed the property of promoting CaOx aggregation by THP from stone-formers to that of inhibiting aggregation (Hess et al., 1993). In later studies Hess has shown that urinary components like citrate can modulate crystal aggregation significantly. For example, addition of 5.5M citrate can change the negative effect of a stone formers THP to an inhibition range of 35%. This indicates that the contribution of other urinary factors is extremely important in this process.

Burns and Finlayson (1980) described a recipe for artificial human urine and suggested that to facilitate compatibility between assays performed at various laboratories it should be used as a standard reference. It has been used in various crystallization assays (Miller et al., 1977; Robertson and Scurr, 1986; Rodgers and Wandt, 1991). Artificial urine provides an ideal medium to test the effect of macromolecules such as proteins on the crystallization processes. In contrast to buffers which usually contain some, but not all ions, artificial urine contains a mixture of various salts resulting in a solution with the complete ionic composition of normal urine. The advantage of this system is that because it is devoid of any organic components, the effect of individual factors such as proteins can be studied without interference with other proteins.

To complement the in vivo analysis of THP in the rat nephrolithiasis model, in vitro assays were performed in artificial rat urine. Its composition was based on that described by Shevock et al. (1993). To study the effect of citrate on the interaction of THP and crystals, THP samples were also tested in urine in which the citrate concentration was lowered to the citrate levels found in nephrolithic rats.
The assays used in this study which were based directly on Hess's assay, although performed in rat artificial urine instead of buffers, revealed that THP inhibited aggregation. This observation confirmed that THP interacted with the aggregation stage of crystallization. Since the biochemical composition of normal and nephrolithic rat THP was found to be very similar, it was not surprising to find that they interacted with the crystals in a similar manner. Although Hess (1993) found that addition of citrate increased the inhibitory potential of THP, in this study, lowering of citrate did not affect the inhibitory potential of THP.

Examination of normal kidneys of control rats showed the expected pattern of THP staining in the cortex and the medulla, with no stain in the papilla. OPN staining was evident in some nephrons, specifically in the thin limb of Henle and in the area of the calyceal fornix. Similar localization was reported by Kleinman et al. (1995) by fluorescent, confocal and electron microscopy. In the literature, the reports on the precise site of synthesis of OPN in the kidney are conflicting. Kohri et al. (1993) could not localize OPN in the normal rat kidney by immunofluorescent methods. In addition, they found that pre-treatment with acid-urea showed the distal tubules to be only weakly positive. In a comprehensive study on OPN localization in normal mouse kidney, Lopez et al. (1993) have reported that heterogeneity exists in the expression of OPN in different nephrons. They have shown by immunostaining and in situ hybridization that in the mouse kidney, OPN is present in the TAL and distal tubules only and the site of expression shifts to the proximal part of the tubules with increase in age. The above
information and observations from this study indicate that OPN is selectively expressed in some nephrons.

In the nephrolithic rats, the major observation was that the intensity of positive staining for the two proteins corresponded to the degree of crystal deposition. When few small deposits were seen in the kidney, there was no significant difference in the THP and OPN staining pattern when compared to controls. In contrast, in samples with extensive crystal deposits, the abnormal localization of the two proteins was strikingly exaggerated. The crystals appeared to be embedded in the darkly stained matrix-like material, positive for both THP and OPN. In addition, the number of papillary tubules in which they appeared and their staining intensity was remarkably higher in kidneys of these rats. It can be concluded from these observations that the abnormal staining (around the crystals and in the papilla) and the intensity of staining corresponded to the extent of crystal deposition in the kidney.

Although THP was seen around deposits, it was not consistently visible in all sections, which represented different planes of the kidney tissue. It is possible that THP bound largely to the crystal surface, and therefore was visible via staining only if the section was through a particular plane containing both the crystals and THP. As a second possibility, being surface-bound (as opposed to internal), in some areas THP could have been affected by the various steps during the staining procedure and washed off. In contrast, the staining for OPN was intense and consistent. It can be speculated that OPN was present within the crystals, as has been shown previously by Mckee and Khan (1994) by electron microscopy. Therefore, probably OPN was much more stable and was
retained and stained consistently in the different sections examined. Because normally the expression of these two proteins is primarily restricted to the TAL (additionally in DCTs for OPN, as discussed earlier), alteration in their staining pattern and increase in their expression indicates an important physiological event.

A primary question under current investigation regarding the protein-crystal interaction in kidney stone formation is whether the proteins promote or inhibit the process of crystallization. In vitro studies have shown that as a general rule, organic substances (e.g. proteins) when added to crystallization systems almost always slow the rate of crystallization and additionally may also change crystal morphology (Weiner and Addadi, 1991). Glycoproteins, especially acidic molecules associated with various mineralized tissues can be found on the interface of the matrix and crystals or actually within the crystals, and therefore are thought to be effective modulators of crystallization (Gorski, 1992). It has been observed that they show a stereochemical affinity to certain faces of crystals which is essential to their interaction with the crystals (Addadi and Weiner, 1992) OPN is a classic example of an acidic protein with 22.3% aspartic and 17.0% glutamic acid residues (Butler, 1989) and based on its sequence information is thought to mediate metal binding (Boskey et al., 1993) By in vitro experiments, it has been shown specifically that it does not initiate hydroxyapatite crystals nucleation (Hunter et al., 1993) but in contrast binds rapidly to these crystals (Kasugai et al., 1992) and inhibits their formation and growth. As discussed previously, there is convincing evidence that OPN can modulate CaOx crystallization effectively in vitro by inhibiting crystal growth (Shiraga et al., 1992; Worcester et al., 1992) Furthermore, separate
experiments have shown that the mechanism for growth inhibition of CaOx crystals is by disruption of the growing crystal lattice (Denhardt and Guo, 1993). Based on the properties of OPN discussed above and this study, it appears that the interaction of OPN with CaOx crystals is extremely specific and it could possibly function as a modifier of crystallization *in vivo*.

In contrast to OPN, THP cannot be classified as a typical acidic protein although it contains higher amounts of acidic amino acids than the other amino acids (unpublished data from our laboratory). At present, there are no reports to indicate that a correlation exists between the type of structural components of THP and its interaction with crystals. Typically, in the case of THP this interaction appears to be specifically influenced by other urinary factors, including pH, ionic strength and citrate (Hess et al., 1989a; 1993). As described in the introduction, although the reports on the outcome (positive or negative) of effect of THP on *in vitro* crystallization are contradictory, it is generally agreed that THP is mainly involved in the aggregation of formed crystals. The observations made in this study suggest that THP is likely associated with the crystal surface, and therefore could effectively mediate aggregation of crystals.

The following conclusions can be made from experiments described in this section:

1. Biochemically, there were no significant differences in the THP from normal and nephrolithic rats. 2. *In vitro* assays demonstrated that the two THPs showed similar inhibitory effect on crystal aggregation in *in vitro* assays, in the presence of normal as well as low citrate. This indicates that THP can potentially inhibit aggregation *in vivo*. 
3. The association of THP as well as OPN with the crystal deposits suggests that several factors play a simultaneous role in stone formation. Based on these results and the literature, it appears that OPN probably associates with an early stage of crystallization and THP at the later stage of aggregation.
CHAPTER 7
DISCUSSION

Before this project was undertaken, a literature survey on THP indicated that there was a prominent controversy regarding its participation in stone formation. Although its actual identification in stones did indeed suggest a role in the crystallization process, later studies did not clarify whether it promoted or inhibited the formation of stones. Research on human THP, mostly by in vitro experimentation had yielded controversial results. One of the reasons for the apparent inconsistency in the data obtained is possibly the use of in vitro methodology only, as well as the diversity of these techniques. In this study, a combination of in vivo as well as in vitro methods eliminated such disparity and facilitated a more complete analysis of THP.

Detailed biochemical analysis of normal rat THP showed that it did not contain any unusual components and its composition is largely similar to that of human THP. Therefore, it could be assumed that rarity of calcium oxalate precipitation in rats was not due to the structural properties of the glycoprotein per se and it was necessary to examine other factors.

One of the parameters examined in this analysis in the in vivo nephrolithiasis model was the localization of THP and its mRNA. It was seen that the crystals were surrounded by the organic matrix like THP, and THP also showed abnormal localization
as deposits in the papillary tubular lumen. This demonstrated that THP indeed associated with the process of crystallization and that the normal localization of THP, but not its normal site of synthesis was altered under stone-forming conditions. On the other hand, in normal and nephrolithic rats, there was no difference in the biochemical properties and urinary excretion of THP.

The specificity of THP-crystal association was further confirmed by in vitro crystal aggregation assays in rat artificial urine. THP inhibited aggregation efficiently, but at similar concentrations, albumin, another urinary protein did not show any effect. A comparison of THP from normal and nephrolithic rats further revealed that both THPs inhibited aggregation in a similar manner, in the presence of normal or low citrate. The in vivo and in vitro data taken together indicated that THP associated with crystals and it had the potential for modulating crystallization. However, it seemed likely that THP was not the exclusive protein/factor interacting with crystals in vivo.

Therefore, localization of OPN, a comparatively more recent candidate under current scrutiny for its role in kidney stone formation was also examined. Its conspicuous presence around the crystals at sites similar to THP, and deposition in papillary tubules indicated that in addition to THP, OPN also associated with crystals. This indicated that at least two proteins, THP and OPN simultaneously associated with crystallization.

Based on the data from this study and information from the literature, it is likely that THP and Opn probably associate with different stages of crystallization and the following scenario can be speculated: 1) The deposition of THP around the crystals and in the tubules could be a purely physical phenomenon. Urinary THP travels with the urine
and adheres to the already existing crystals in the renal tubules. The viscous nature of THP may even promote retaining additional crystals in this meshwork. Thus, THP, most likely associates with the aggregation step of the crystallization process. 2) In contrast, Opn, which has been shown to be an inhibitor of crystal nucleation, probably associates with the crystals during early formative phase.

Kidney stone formation is a multifactorial disease and it appears that much more is known about the factors and conditions that favor its promotion, rather than those which cause its inhibition. One of the difficulties encountered in understanding the complex process of stone formation is probably the sheer number of factors that may affect this process at any one time. Among the various modulators of the process of crystallization, proteins have the intrinsic ability to bind crystals and therefore can associate with any of the three stages of crystallization. Each protein, tested separately by different research groups and under different conditions, appears to be a reasonable candidate, and consequently has been claimed to play the important role in this process. In contrast, the results of this study have shown for the first time that more than one urinary protein interacts simultaneously with crystals during the stone-forming process.

The author believes that the conclusions made from this study have certainly taken the research on kidney stone formation a step further. However, as for any other multifactorial disease that perplexes mankind, still much remains to be learned about the complex processes leading to kidney stone formation.
Future Directions

In order to further elucidate the involvement of THP in crystallization, one could examine a nephrolithiasis model in which either a) THP has been infused in vivo or b) has been completely eliminated by creating a "knock-out" model in which THP gene has been eliminated.

As far as the in vitro experimentation is concerned, from the results of this and other studies it has become clear that in the research for stone formation, a unification of certain assay systems is necessary. For example, for proteins, one can design a 'reconstitution' assay, in which the proteins can be added in succession (or removed) to determine a combined effect. In addition, further studies on the structural aspects of the proteins (e.g. sialic acids) and contribution of other urinary components like citrate and cellular membranes are warranted.
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Jayashree A. Gokhale was born in Poona, India, and was raised in Calcutta where she attended school. After getting her Bachelor’s degree in Microbiology from the University of Poona, she obtained her Masters degree in Applied Biology at the Cancer Research Institute at Bombay. She joined the Laboratory of Electron Microscopy at the Department of Microbiology and Cell science, where she obtained another Master’s degree. She joined the graduate program at the Department of Pathology in the fall of 1989. She is married to Atul Gokhale, who is a metallurgist by profession.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Saeed R. Khan, Chair
Associate Professor of
Pathology and Laboratory Medicine

Raymond L. Hackett
Professor of
Pathology and Laboratory Medicine

Wayne T. McCormack
Assistant Professor of
Pathology and Laboratory Medicine

K. J. Kao
Professor of
Pathology and Laboratory Medicine

Maurice S. Swanson
Assistant Professor of
Molecular Genetics and Microbiology
This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1995

[Signature]
Dean, College of Medicine

[Signature]
Dean, Graduate School