Scanning Microscopy

Volume 5 | Number 3 Article 11

7-31-1991

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Scanning Microscopy, Vol. 5, No. 3, 1991 (Pages 689-696)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/91$3.00+.00

# THE ROLE OF TAMM-HORSFALL GLYCOPROTEIN AND NEPHROCALCIN IN CALCIUM OXALATE MONOHYDRATE CRYSTALLIZATION PROCESSES

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(Received for publication May 6, 1991, and in revised form July 31, 1991)

## Abstract

Theoretical considerations as well as clinical observations suggest that the aggregation of nucleated crystals is the most dangerous step in the formation of calcium oxalate (*CaOx*) renal stones. The effects of 2 major urinary glycoproteins, **Tamm-Horsfall glycoprotein (THP) and Nephrocalcin (NC)**, on calcium oxalate monohydrate (COM) crystal aggregation *in vitro* are studied. At low ionic strength (IS) and high pH (within urinary limits), THP is a powerful crystal aggregation inhibitor (90% inhibition at 40 mg/l). Decreasing pH to 5.7 and raising IS to 0.21 increases THP viscosity, thereby lowering THP crystal aggregation inhibition. Upon addition of calcium (5 mmol/l), some THPs are no more soluble and promote crystal aggregation (up to 70%). In the presence of citrate (5 mmol/l), which is only slightly inhibitory (14%), the promoting effect of THP is reversed into aggregation inhibition (up to 55%). There is evidence for a molecular abnormality in THPs from severe recurrent *CaOx* stone formers, since they exhibit increased polymerization and reduced solubility. The 14 kD (kilodalton), Gla-containing glycoprotein NC also strongly inhibits crystal aggregation. However, NC isolated from urines of recurrent *CaOx* stone formers and from *CaOx* renal stones are 10 times less inhibitory. Both are structurally abnormal in that they lack Gla and are less amphophilic.

**Key Words**: nephrolithiasis, calcium oxalate, urinary inhibitors and promoters, Tamm-Horsfall glycoprotein, Nephrocalcin.

## Introduction

Renal stone formation occurs as a consequence of crystallization within the urinary tract. Crystallization has two major aspects (19): a **thermodynamic** one including high urinary supersaturation during which crystal nucleation occurs, and a **kinetic** one comprising rates of nucleation, growth and aggregation (agglomeration) of crystals.

Calcium oxalate crystalluria is a common feature in recurrent renal stone formers as well as in healthy people (10, 33). Under identical conditions of dietary and fluid intake, however, healthy controls excrete only single calcium oxalate crystals 3-4 $\mu$m in diameter, whereas recurrent calcium stone formers pass large calcium oxalate crystals (10-12 $\mu$m diameter), often fused into polycrystalline aggregates 20-300 $\mu$m in diameter (31). Furthermore, inhibition of the formation of large calcium oxalate crystal aggregates *in vitro* by 5 vol% human urine is reduced in recurrent calcium stone formers (32), and renal stones contain highly aggregated crystals (23). These observations clearly suggest that calcium oxalate **crystal aggregation** may be more important for renal stone formation than crystal nucleation and growth. This is supported by theoretical considerations: calcium oxalate crystal growth alone might be too slow to produce clinically significant particles within renal tubules (9), whereas crystal aggregation occurs within seconds and is, therefore, considered to be much more dangerous for the formation of large crystalline particles within the urinary tract (4).

There are two main reasons why the urinary compounds that modify calcium oxalate crystal aggregation are not well defined. First, there is some confusion in the literature about the definitions of "crystallization" and "inhibitory activity" (33). In many studies on "crystallization", it is not completely clear whether authors refer to nucleation, growth or aggregation of crystals. On the other hand, the term "inhibitor" is being used for compounds that act as chelators of calcium or oxalate as well as for molecules binding to the surface of preformed calcium oxalate crystals (33). Whereas chelators clearly reduce free ion activity and supersaturation, inhibitors do not influence supersaturation, since they bind

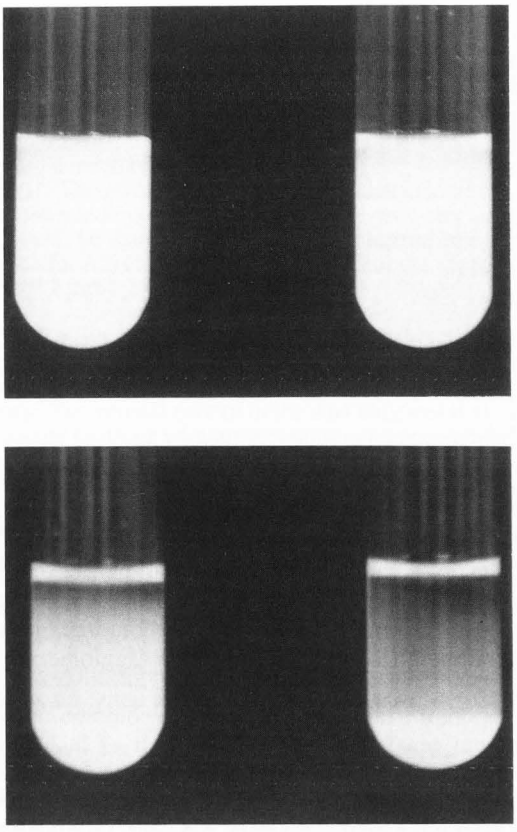

**Figure 1.** Spontaneous sedimentation of COM crystals. COM crystal suspensions (0.8 mg/ml) were incubated overnight at 37°C under constant stirring (1100 rpm) in 16 x 125 mm glass tubes and then kept standing at room temperature. At time zero (upper panel), there was no visible difference between control crystals (right) and crystals pre-incubated with 16 mg/l of normal human THP. After 60 minutes (lower panel), particles in the control suspension had settled almost completely, whereas settling was clearly retarded in the presence of THP (left).

**Table 1.** Basic forces determining crystal aggregation in stirred suspensions*.

| Aggregating | Disaggregating |
|---|---|
| ▸ Van der Waals<br>▸ Viscous binding<br>Foreign molecules = "glue"<br>↓<br>▸ Solid bridges<br>⟹ Stabilization | ▸ Electrostatic repulsion<br>⟶ Zeta potential<br>▸ Shear force<br>Turbulence of aqueous suspensions (stirring) |

*Adapted from reference 20.

to crystal surfaces and block growing sites ("crystal poisoning") at very low (usually micromolar) concentrations (2). On the other hand, some molecules act as promoters, probably by providing preformed surfaces for heterogeneous nucleation, epitaxial growth and aggregation of crystals (2). Because compounds can have varying effects on different crystallization processes, the term **"modifier"** should be used (43), and it should always be stated whether a specific molecule inhibits/promotes nucleation, growth or aggregation of crystals (33).

The second reason, why urinary modifiers of calcium oxalate crystal aggregation are poorly understood, is the variety of assay systems/conditions applied for measuring crystallization processes. In the case of crystal aggregation, measurements usually have been made under conditions of supersaturation with respect to calcium oxalate, so simultaneous nucleation and growth might have altered crystal aggregation kinetics (4, 7, 32, 36, 39, 44).

During our own experiments focused on growth of calcium oxalate monohydrate (COM) crystals in a seeded system (unpublished), we observed that spontaneous sedimentation of crystals suspended at equilibrium solution concentrations was retarded after preincubation with urinary proteins (Fig. 1). Since average sedimentation velocity of particles falling through a liquid is directly proportional to average particle size (17), more slowly settling particles must be smaller. In a saturated solution, crystals can not nucleate nor grow. Since crystal aggregation also occurs at saturation or even undersaturation (20), smaller particles in an equilibrated crystal suspension must be less aggregated.

Table 1 summarizes the basic forces by which crystal aggregates in solutions are held together (8): the attractive van der Waals forces favor particle aggregation, whereas the electrostatic surface potential is repulsive (8, 20). The process of viscous binding implies that crystal foreign molecules are attached to crystal surfaces and act as a glue between particles, thereby promoting crystal aggregation (20). If crystalline material connects several particles (solid bridge formation), the aggregate becomes more stable (20). In a crystal suspension, constant stirring (shear force) basically favors disaggregation (20); as expected, slower stirring produces more aggregated particles (4). Based on these observations, we have measured COM crystal aggregation *in vitro* by spectrophotometrically monitoring the sedimentation of crystals that have been pre-aggregated by slow stirring in an equilibrated suspension (13). It has to be acknowledged, however, that under saturated conditions, macromolecular modifiers possibly might affect COM crystal aggregation differently from supersaturated conditions (20). This paper reviews recent work on two major urinary glycoproteins, Tamm-Horsfall glycoprotein and Nephrocalcin, with regard to their effects on calcium oxalate crystallization processes *in vitro*.

**Table 2.** THP inhibition of calcium oxalate crystallization processes *in vitro**.

| | Nucleation | | Growth | | Aggregation | |
|---|---|---|---|---|---|---|
| % inhibition | +19 ⟺ -250 | | +38 ⟺ 0 | | +90 ⟺ -35 | |
| [THP] mg/l | 50 | 35 | 50 | 8 | 40 | 40 |
| pH | 6.5 | 5.3 | 6.5 | 5.7 | 7.2 | 5.7 |
| IS | 0.15 | | 0.15 | 0.15 | 0.10 | 0.21 |
| Osmol. mOsm/kg | | 1250 | | | | |
| Ref. | 18 | 36 | 18 | 50 | 13 | 14 |

*The most contradicting findings, taken from the literature, are listed. Lower part depicts physico-chemical conditions at which experiments were performed. Negative inhibition values = promotion. IS = ionic strength; Osmol. = Osmolality; Ref. = reference.

### Tamm-Horsfall Glycoprotein

The pathophysiology of Tamm-Horsfall glycoprotein (THP), also called uromucoid, has been reviewed extensively (16, 21, 34). Briefly, THP is produced primarily by the kidneys, where it is specifically localized to the epithelial cells of the thick ascending limb of Henle's loop and the most proximal part of the distal tubule (21). Biochemically, THP is a glycoprotein; its carbohydrate content amounts to 30%. In its monomeric form, THP isolated from urine by the original salt precipitation method has a molecular weight (MW) of 80 kD (21). The THP gene recently has been cloned and sequenced; the mature protein contains 616 amino acids. A very similar protein, uromodulin, has been isolated from pregnancy urines by lectin adherence; its protein backbone is identical to THP. During pregnancy, the protein's carbohydrate structure may be altered; in fact, uromodulin contains more unprocessed high mannose chains and is at least 10 times more immunosuppressive *in vitro* than salt-precipitated THP (21). The exact function of THP remains enigmatic (16, 21, 34); recent studies suggest that THP might be a specific ligand for cytokines in the kidney (21).

Because THP is present in various amounts in renal stones (12), it has been proposed to play a role in **renal stone formation**. Several studies did not find a difference between normals and calcium oxalate stone formers in the daily urinary excretion of THP, averaging 40-50 mg (3, 12, 38, 47), with the exception of patients with distal renal tubular acidosis in whom THP excretion seems to be significantly lower (46). The many *in vitro* studies investigating the effects of THP on calcium oxalate crystal nucleation, growth and aggregation seem to have revealed very contradicting results. Table 2 depicts the most conflicting values currently available from published studies. Kitamura and Pak reported 19% inhibition of calcium oxalate **crystal nucleation** by normal human THP at 50 mg/l, pH 6.5, 1 mM $CaCl_2$ and ionic strength (IS) 0.15 (18). At otherwise identical conditions, but pH 6.0, Yoshioka *et al.* found promotion of nucleation by 25% (50). The addition of THP (only 1 mg/l) to synthetic urine in a mixed suspension, mixed product removal analytic system with very high calcium (6.76 mM) and oxalate (1.03 mM) concentrations at pH 5.7 promoted nucleation by 183% (6). Finally, upon addition of 35 mg THP to 1 liter of extremely concentrated urine (evaporated to 1250 mOsmole/kg) at pH 5.3, nucleation of amorphous calcium oxalate crystals increased by 250% (35)!

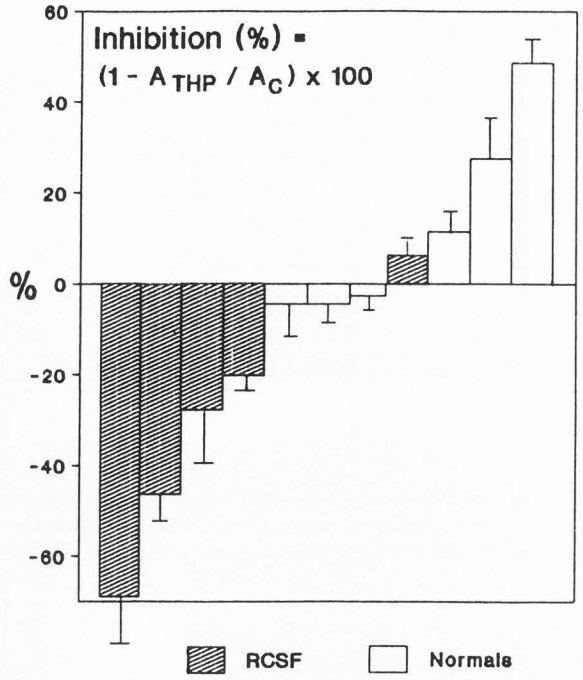


**Figure 2.** COM crystal aggregation inhibition by normal and stone former THP. COM crystal aggregation inhibition (%) is measured at pH 5.7, 200 mM NaCl and 5 mM $CaCl_2$. A = crystal aggregation; $A_c$ = aggregation in control experiments (no inhibitor added, 0% inhibition); $A_{THPP}$ = aggregation in the presence of 40 mg/l of THP from 6 normal men (normals) and 5 recurrent calcium oxalate renal stone formers (RCSF). Values are mean ± SEM. Negative inhibition values = promotion of crystal aggregation.

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Most authors agree that THP is a weak inhibitor of calcium oxalate crystal **growth** *in vitro*. At pH 6.0, IS 0.15, 1 mM $CaCl_2$ chloride and 0.2 mM sodium oxalate, 50 mg/l of normal THP inhibited by 3% (50); at the same IS, but pH 6.5 and only 0.44 mM calcium and oxalate, respectively, 50 mg/l THP from stone formers inhibited by 38% (18). In a study performed at pH 5.7, IS 0.15, 1 mM calcium and 0.2 mM oxalate, crystal growth was not affected by 8 mg/l of THP (49).

Measuring calcium oxalate **crystal aggregation (agglomeration)** *in vitro* in the presence of 5 vol. % human urine before/after removal of THP by antibody precipitation, Felix *et al.* (7) could not detect an inhibitory effect of THP. Scurr and Robertson, however, found 22% aggregation inhibition by 48 mg/l of isolated THP at pH 6.0, IS 0.15, 2 mM calcium and 0.4 mM oxalate (39). Upon addition of oxalate to metastably supersaturated ultra-filtered human urine in the presence of isolated THP (50 mg/l), predominantly small crystals of calcium oxalate monohydrate instead of aggregated calcium oxalate dihydrate "envelopes" are formed (37). Using the newly developed spectrophotometric method at pH 7.2, IS 0.1 and only equilibrium concentrations of calcium and oxalate, e.g., 0.14 mM each, we found about 90% aggregation inhibition by normal human THP at 40 mg/l (13). In a subsequent study, however, lowering pH to 5.7 and increasing IS to 0.21 at otherwise unchanged conditions revealed 35% promotion of aggregation by 40 mg/l of THP isolated directly from calcium renal stones (14).

These apparently paradoxical influences on the various crystallization processes may be explained by the well-known **physicochemical properties** of THP: increasing the concentrations of the protein itself (22), divalent cations like calcium and magnesium (5, 4, 6), sodium (22, 45) and hydrogen ions (22) all induce increased polymerization (self aggregation) of THP molecules, leading to reversible gel formation (increased viscosity) (11). Scanning electron microscopy studies revealed that THP molecules formed elongated fibers about 20 nm thick and up to 1000 nm long in solutions containing NaCl (100 mmol/l) or $CaCl_2$ (1 mmol/l); when NaCl plus $CaCl_2$ were present, however, thick bundles of fibers, as found in hyaline casts from human urine, could be seen (48). It is obvious from table 2 that less THP inhibition or even promotion of calcium oxalate crystallization processes occurred in those studies performed at lower pH and higher IS.

Studying the effects of increasing IS from 0.01 to 0.21 at pH 5.7 on individually purified THPs (40 mg/l) from normal men and male patients with severe recurrent idiopathic calcium oxalate stone disease (> 20 stones), we found reduced COM crystal aggregation inhibition in all proteins, but more markedly in stone former THPs (15). This was not simply due to lower solubilities of patient THPs at the experimental conditions (15), but seems to be the consequence of a **molecular THP abnormality** in patients with severe calcium oxalate nephrolithiasis: stone former THP exhibits an increased tendency to polymerize at lower pH and higher IS, as evidenced from viscosity measurements and molecular weight determinations (15). Studies on THPs of the family members of one severe stone former indicate that this abnormality might be **inherited**. The molecular basis of this apparent structural abnormality, however, remains unknown (15).

Most recently, we studied the effects of high physiological **calcium and citrate** concentrations on COM crystal aggregation inhibition by individual THPs. After equilibrating COM crystals in 200 mM NaCl and 5 mM $CaCl_2$ at pH 5.70, the rate of crystal aggregation was identical to the one originally described without additional calcium (13). Urinary THPs of 6 healthy men and 5 severe male idiopathic calcium stone formers (10-200 stones) were studied by adding low amounts of highly concentrated aqueous THP solutions to a final assay concentration of 40 mg/l. Normal THPs (values mean ± standard error of mean, SEM) inhibited by 13.7 ± 8.8% (range -3.0% to 50.1%), stone former THPs by -31.4 ± 12.6% (range -68.0 to 7.4%, $p < 0.01$ versus normal THPs), e.g., most stone former proteins actually promoted crystal aggregation (Fig. 2). Citrate at 5 mmol/l, without any effect on crystal aggregation if no

additional calcium was present (13), inhibited by 14.3 ± 3.82 % (n=10). When we restudied the 2 most promoting stone former THPs in the presence of 5 mM citrate, however, promotion was reversed into inhibition, e.g., inhibition changed from -68% and -45% (Fig. 2, far left) to +55% and +34%, respectively (not shown), clearly above the slight inhibition by citrate alone. Similar results were obtained with studies in whole urine of our most severe stone former (200 stones). Crystal aggregation inhibition was measured after incubating unfiltered fresh 24 hour urine with COM crystals for 36 hours; inhibition was -54%, e.g., whole urine promoted crystal aggregation. Since urinary calcium was relatively much higher than citrate (4.64 versus 1.88 mmol/l), citrate was added to a final concentration of 6.88 mmol/l; remeasuring crystal aggregation revealed true inhibition of 88%.

Most authors agree that the **mechanism**, by which urinary macromolecules inhibit crystal aggregation *in vitro*, is their binding to the crystals, which induces a more negative surface charge (Zeta potential) on the crystal surface (8, 39). In comparison with glycosaminoglycans and RNA, however, THP produces a less negative Zeta potential on COM crystals (39). Furthermore, two studies have demonstrated that upon raising THP concentrations within physiologic urinary limits, e.g., above $10^{-7}$ M, Zeta potential does not become any more negative, e.g., the potential curve flattens off at about -20 mV (13, 39). Our own studies did not reveal Zeta potential differences between THPs from healthy men and severely recurrent stone formers, nor was COM crystal aggregation inhibition by physiologic THP concentrations correlated to Zeta potential values (15).

We found, however, a negative linear correlation between crystal aggregation inhibition and intrinsic viscosity of THP at pH 5.7 and 200 mM NaCl, e.g., THPs with lower viscosities allowed for more crystal aggregation inhibition (14, 15). In accordance with others (22, 40), our most recent data obtained at pH 5.7, 200 mM NaCl and 5 mM $CaCl_2$ suggest an additional role of THP solubility: measured at room temperature as described elsewhere (15), THP solubility (mean ± SEM) was 61.0 mg/l for normal and 34.8 mg/l for stone former THP ($p < 0.01$). When adding 40 mg of dry stone former THP to 1 liter buffer solution (pH 5.7, NaCl 200 mM, $CaCl_2$ 5 mM), visible hair-like precipitates were regularly detectable.

Altogether, there is strong evidence for a dual role of THP in COM crystal aggregation. At higher pH and lower IS, THP is a powerful aggregation inhibitor. Upon lowering pH and raising IS within physiologic urinary limits, increased polymerization of THP molecules occurs. This most possibly increases attractive viscous binding forces on COM crystal surfaces. Since the repulsive Zeta potential is not increased any further, the overall forces between crystals become more attractive, allowing for more crystal aggregation (reduced inhibition). If in addition high physiological calcium concentrations are present, certain THPs even may form insoluble precipitates on which crystals preferably aggregate (promotion of aggregation). This seems to be reversed by equimolar concentrations of citrate, suggesting a higher affinity of calcium ions to citrate than to THP. As our recent data indicate, some stone former THPs have an increased tendency of polymerization and lower solubilities than normal THPs. These findings, however, do not exclude that, in a given clinical situation, an extreme difference in the chemical composition of urine between stone formers and normals might in itself produce various THP effects irrespective of molecular THP abnormalities.

### Nephrocalcin

In 1978, Nakagawa *et al.* (24) isolated an acidic glycoprotein from human urine which strongly inhibited the growth of COM crystals *in vitro*. Subsequently, the same glycoprotein was also purified from human kidney tissue culture medium (25). Since further characterization demonstrated that the protein contained gamma-carboxyglutamic acid (Gla) (26), it later was named nephrocalcin (NC), in analogy with osteocalcin, the Gla-containing bone protein (28). In its monomeric form, NC has a molecular weight of 14 kD; it contains calcium and magnesium ions that are likely to be involved in the formation of aggregates of higher molecular weights in human urine (26). NC is rich in acidic amino acids and contains 10 weight% of carbohydrates (26); it does not contain any uronic acids (30). The glycoprotein shows a strong surface activity as evidenced by a very high collapse pressure at air-water interfaces, suggesting strong amphophilic properties and a highly organized two-dimensional structure of NC (26).

There are several lines of evidence for a renal origin of NC. To date, NC has been isolated from kidney tissue of nine vertebrae species (30). Primary cultures of mouse proximal tubule cells produce NC (42). Renal cell cancers, which are believed to originate from proximal tubule cells, seem to produce NC as well, as evidenced from the purification of NC from the culture medium of renal carcinoma cells (41). Immunohistochemically, NC could be localized to the proximal tubule and the thick ascending limb of Henle's loop in mouse as well as in human kidneys; there was no cross-reactivity with THP (29). Daily urinary excretion of NC in humans amounts to about 20 mg; there seems to be no difference between normals and renal stone formers (M. Netzer, personal communication).

NC isolated from normal human urine is a strong inhibitor of **COM crystallization processes** *in vitro*. Using a constant composition assay, Asplin *et al.* recently demonstrated that NC (as well as 20 % dialyzed human urine) inhibited the secondary nucleation of calcium oxalate on COM crystals as well as crystal growth (1). By means of a seeded crystal growth system, NC purified from normal human urines and from human kidney tissue culture medium repeatedly has been shown to strongly inhibit COM crystal growth at concentrations between $10^{-9}$ and $10^{-6}$ M (25-28, 49). Our own studies

demonstrated that normal NC at concentrations between $5 \times 10^{-8}$ and $1 \times 10^{-6}$ M also is a powerful inhibitor of COM crystal aggregation (13). Opposite to THP, increasing ionic strength or lowering pH of the solution did not affect the inhibitory activity of normal NC at physiologic concentrations (13).

Considering the **mechanism** of NC inhibition, it is important to note that patients with recurrent calcium oxalate nephrolithiasis produce a structurally defective NC (27); NC with the same abnormalities has also been isolated from human calcium oxalate renal stones (28). Both stone former and stone NC lack gamma-carboxyglutamic acid, are less amphophilic (formation of less stable air-water interfacial films) and induce a less negative Zeta potential on COM crystal surfaces (13, 27, 28). Functionally, stone former as well as stone NC are weak crystal aggregation inhibitors at physiologic concentrations (about 10-fold less effective than normal NC); however, increasing their concentrations above the physiologic range raises inhibitory activity to normal levels (13). Since progressively increasing the concentrations of these defective NCs improves their inhibitory activity yet does not alter crystal surface charge (13), we conclude that their weaker amphophilic properties, possibly due to the lack of gammacarboxyglutamic acid, may be responsible for the loss of COM crystal aggregation inhibition. One might speculate that conformational changes in the defective forms of NC, when bound to COM crystals, allow for relatively more viscous binding on the crystal surface. However, promotion of crystal aggregation, as found in abnormal THP, has never been demonstrated for defective NC.

**Acknowledgements**

The technical assistance of Mrs. L. Zipperle is greatly acknowledged.

Dr.Hess is a grant recipient of the Swiss National Science Foundation (No.32-26428-89).

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### Discussion with Reviewers

**W.G. Robertson**: Why under certain conditions does THP appear to exert an inhibitory effect on agglomeration yet, according to the author, does not show any relationship with Zeta potential?
**Author**: The extent of crystal aggregation (agglomeration) *in vitro* is determined by attractive and repulsive forces that are balanced against each other (Table 1). As demonstrated by your group (40) and our own results (13), increasing THP concentration within physiologic limits does not change Zeta potentials. Therefore, given constant stirring rates in our system, the overall repulsive force remains constant, and increased THP inhibition (less crystal aggregation) must be the consequence of reduced attractive forces. We find that THPs with low viscosities, e.g., less polymerized molecules, inhibit crystal aggregation more markedly than THPs with high viscosities (14, 15). One explanation would be that low viscosity THP molecules, compared to more viscous THP polymers, produce less viscous binding, which would lead to a relative increase of net particle repulsion, e.g., more crystal aggregation inhibition.

**W.G. Robertson**: Is there any relationship between the structure of NC and that of part of the THP molecule as was suggested by one group?
**Author**: I cannot answer this question, since the primary structure of NC is still under investigation.

**S.R. Khan**: What happens to the calcium oxalate zeta potential in the presence of NC?
**Author**: As demonstrated by our own studies (13), NC also induces a negative Zeta potential on COM crystals. Similar to THP, increasing NC concentrations within physiologic urinary limits does not raise any further the negative Zeta potential, e.g., the potential curve flattens off at about -18 mV. Defective NCs, isolated either from stone former urines or directly from CaOx renal stones, induce a less negative Zeta potential of about -13 mV on COM crystal surfaces (13).

**M. Akbarieh and R. Tawashi**: Increasing THP concentration, increasing the IS and changing pH could lead to increased aggregation (polymerization!) of the protein. What critical precaution the author suggests in the preparation and purification that could minimize the effect of these variables?
**Author**: We routinely purify THP from human urines by the classical method of repeated salt precipitation, modified, however, by using 4 M urea in 0.02 M sodium phosphate, pH 6.8, and gel filtration (Sepharose 4B) as a final purification step (13). This minimizes the effect of pH and IS; in addition, increasing the urea concentration is known to decrease THP viscosity (16, 21).

**N. Akbarieh and R. Tawashi**: The author speculates that the structurally defective forms of NC present in stone former urines and renal stones lead to viscous binding of COM crystals. If this is true, does the production of less negative charge on COM crystal surface really matter?
**Author**: As mentioned above, it is purely speculative to state that defective forms of NC might allow for more viscous binding on COM crystal surfaces. This was done in analogy to THP, where increasing concentrations within the physiologic range also do not raise the negative Zeta potential yet inhibitory activity changes (see also my answer to first question by Prof. Robertson). Therefore, I agree, that the negative Zeta potential does not seem to contribute really to the differences in COM crystal aggregation inhibition that we observe, at least not under our experimental conditions and at the (physiologic) concentrations of both THP and NC we have chosen for our studies.

**D.J. Kok**: Is there a correction applied for the difference in density between single crystals and their aggregates?
**Author**: I am aware of the fact that the density of crystals and their aggregates does not have to be identical; however, no such correction was applied in our *in vitro* system.

**D.J. Kok**: It has been shown before that the effect of citrate on crystal agglomeration depends on the type of complexes formed in solution. It can be increased by the presence of magnesium or by changing the calcium concentration. What evidence does the author have that this is not the case here?
**Author**: Magnesium was never present in our assay solutions. We can, however, demonstrate an increased inhibitory effect of citrate upon formation of calcium-citrate complexes. Previously, when calcium was present only at equilibrium concentration (0.14 mM) in our system (13), we had not found any effect of millimolar citrate concentrations on COM crystal aggregation. The experiments presented in this paper were performed with 5 mM calcium and, therefore, allowed for calcium-citrate complex formation; in fact, we demonstrate 14.3% inhibition by 5 mM citrate. Our most recent studies (unpublished) indicate a 45% increase of aggregation inhibition by normal THPs after addition of citrate, considerably more than with citrate alone. This additional increase must be related to THP itself, possibly because calcium is mainly chelated by citrate and can no longer induce conformational changes of THP molecules (polymerization) to the same extent.

**D.J. Kok**: The structural changes in THP and NC may represent two different groups of calcium oxalate stone formers or there may be some (cellular?) aberrance connecting both. Does the author have any information supporting one of these hypotheses?
**Author**: I do not have any information on either of the two hypotheses.