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Dissolved Urate *Salts Out* Calcium Oxalate in Undiluted Human Urine In Vitro: Implications for Calcium Oxalate Stone Genesis

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Summary

Hyperuricosuria has long been documented as a predisposing factor to calcium oxalate (CaOx) stone pathogenesis. However, its mechanism is still without sound scientific foundation. Previously, we showed that hyperuricosuria, simulated by the addition of dissolved sodium urate, promotes the crystallization of CaOx. In the present study, we demonstrate that the urate's effect on the crystallization is attributable to its salting out CaOx from solution. Furthermore, analysis of urines revealed that their metastable limit decreased with increases in the product of the prevailing concentrations of calcium and urate: this has implications for CaOx stone genesis. We also outline anti-salting out strategies for future research for the prevention and/or treatment of CaOx calculi.

Introduction

Evidence linking derangements of uric acid (UA) metabolism to calcium oxalate (CaOx) stone formation dates back to 1967, when Yu and Gutman [1] observed urinary calculi composed of CaOx or a mixture of CaOx and calcium phosphate (CaP) in gouty patients. Later, Smith et al. [2] reported that hyperuricaemia and hyperuricosuria were associated with CaOx calculi. This finding was then strengthened by empirical observations of Dent and Sutor [3]. The most striking evidence for this association came from the apparent success of allopurinol, a drug that reduces urinary output of urate, in reducing CaOx stone recurrence in patients whose only demonstrable abnormality is hyperuricosuria [4-11]. With time, more workers reinforced the notion of a connection between UA excretion and CaOx stone formation and suggested that "hyperuricosuric calcium oxalate" stone disease was a separate clinical entity. In an attempt to explain this apparent association of hyperuricosuria with CaOx stone formation, several theories have been proposed [12-15]. However, to date, a plausible rationale for these observations is still without sound scientific foundation. One of these theories, which requires dissolved urate and can lay its claim to being both the oldest and the most ignored, is "salting out" [15]. Briefly, the term salting out refers to a decrease in the solubility, or, alternatively, an increase in the activity coefficient, of a nonelectrolyte with increasing concentrations of electrolyte [16]. For the purpose of the definition, electrolytes and nonelectrolytes are salts that have high and low solubilities, respectively. The concept of salting out is well known to physicochemists/biochemists and is routinely used in preparative chemistry/biochemistry to separate through precipitation the less soluble product from its reaction mixture [17, 18]. It is also used in biochemistry for the isolation of DNA [19, 20], purification of proteins [21, 22], and colorimetric assays of free fatty acids [23]. Mathematically, the influence of an electrolyte on the solubility of a nonelectrolyte in water can be expressed by the physical equation for gases, commonly known as the Setschenow equation [15], given below:

$$\log s_o/s = \log fc = k\, c_s$$

where s_o and s are the solubilities of the nonelectrolyte in water and electrolyte solution, respectively; c_s is the concentration of the electrolyte; fc is the activity coefficient of the nonelectrolyte; and k is the salting constant. A positive value for this constant indicates salting out and a negative value indicates salting in. In the former scenario, since s_o is constant, it follows from the equation above that the amount of electrolyte added is directly proportional to the amount of nonelectrolyte precipitated.

In 1963 at a meeting of the American Chemical Society in New York, Stern et al. reported that UA interferes with the solubility of CaOx, an observation they later published [24]. During the next few years, Kallistratos and coworkers [15] reported that addition of dissolved urate to inorganic solutions containing calcium and oxalate ions, and to human urine, caused precipitation of CaOx. These authors attributed this ability of the urate to the principle of salting out. It is interesting to note that the authors presented no evidence to suggest that all the dissolved urate added remained in soluble phase. This is vital because addition of dissolved urate would increase concentration of the salt. This, in turn, would reach the formation product and, hence, could very well result in precipitation of microscopic particles of urate. This particulate urate could then have either directly [12, 13] or indirectly [14] promoted CaOx crystallization, as has already been documented in the literature. Also, the authors did not determine the salting constant nor did they study the relationship of the urate added to the amount of CaOx precipitated. It is therefore perplexing how in the absence of these important parameters, the authors could attribute the urate's effect to the principle of salting out. Because of the potential significance of the findings of Kallistratos and coworkers [15], we have repeated and extended their studies and have shown that the addition of dissolved sodium urate (NaU) to human urine does indeed promote crystallization of CaOx [25]. We have also shown that such a promotory

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Table 1. Physical and Salting Constants of the Type B Urines

Sample Number	pH	Cs (mol/liter)	So $(mol/liter)^2$	S $(mol/liter)^2$	Log So/S	*k*
1	6.10	0.0032	3.25×10^{-6}	1.938×10^{-6}	0.2245	70.156
2	6.12	0.0046	2.39×10^{-6}	1.496×10^{-6}	0.2045	44.456
3	6.12	0.0048	2.169×10^{-6}	1.616×10^{-6}	0.1278	26.625
4	6.15	0.0046	2.670×10^{-6}	1.695×10^{-6}	0.1973	42.891
5	6.12	0.0030	2.968×10^{-6}	1.468×10^{-6}	0.3057	101.900
6	6.21	0.0036	1.766×10^{-6}	1.142×10^{-6}	0.1893	52.583
7	6.25	0.0040	1.883×10^{-6}	1.343×10^{-6}	0.1467	36.675
8	6.32	0.0040	2.338×10^{-6}	1.458×10^{-6}	0.2050	51.250
9	6.28	0.0034	1.913×10^{-6}	1.073×10^{-6}	0.2511	73.852
10	6.26	0.0039	2.3725×10^{-6}	1.4698×10^{-6}	0.2079	53.307

effect of the urate cannot be attributed to the formation of its microscopic particles in the urine samples [26, 27]. In the present investigation, we reveal that the salting constant in urines exhibiting a promotory effect of dissolved urate on CaOx crystallization is a positive number and that the higher the amount of dissolved urate added, the higher the tendency of the urine to crystallize CaOx, until a saturation with the urate is reached. Collectively, this constitutes the first presented evidence indicating that the urate's effects result directly from its ability to salt out CaOx from solution. We also outline anti-salting out strategies for future research for the prevention and/or treatment of CaOx stones.

Results and Discussion

As mentioned in our previous studies [25-27], while increasing the concentration of dissolved urate in urines, two types of samples were noted. The more concentrated urines, designated "type A," spontaneously deposited CaOx dihydrate crystals simply upon the addition of dissolved urate alone. The dilute samples "type B," on the other hand, did not precipitate any crystalline material; in these, CaOx crystallization was induced by the oxalate load technique [28].

Determination of the Salting Constant, *k*, in Undiluted Human Urine In Vitro

Table 1 shows pH, molar increase in urinary concentration of urate obtained in the samples spiked with the salt (c_s), and products of urinary concentrations of calcium and oxalate, at which precipitation of CaOx occurred in urines enriched with dissolved urate (s) and their control counterparts (s_o). Computation of these values into the Setschenow's equation revealed that the salting constant, k, for the urines ranged from 26.625 to 101.9. Most importantly, its numerical value was a positive number in every urine sample tested. This suggests that in accordance with the Sestchenow equation, dissolved urate salts out CaOx from urine.

Ideally, as the name indicates, the numerical value of the salting constant should remain constant: it should not change from one sample to another. However, the results of the present study suggest that the converse is true. This can perhaps be attributable to the fact that the present study was carried out in undiluted human urine, the real medium in which stones form, and not in an aqueous inorganic solution as used in other physicochemical studies. It is remarkable that the physicochemical characteristics of urine are quite different from that of aqueous inorganic solutions even when designed to mimic urine as closely as possible. Also, human urine contains large numbers and quantities of low and high molecular weight components, some of which are well-documented inhibitors of CaOx crystallization [29]. This is further complicated as the concentrations and relative amounts of urinary components vary from one urine specimen to another.

Figure 1 shows crystalline material deposited, in response to the titration against sodium oxalate solution, in control and in the urate-enriched portions of one of the type B urine samples. It shows that the crystalline deposits in both the portions consisted exclusively of CaOx monohydrates. However, the crystals deposited in the samples spiked with dissolved urate were markedly smaller, more numerous, and more highly aggregated than those deposited in the control sample. Urate crystals were not seen. The deposition of large numbers of small crystals of CaOx monohydrates in the test samples is almost certainly attributable to the urate enhancing the rate of CaOx nucleation. This, in the absence of epitaxy [27] and glycosaminogycans depletion [26], once again is consistent with the urate's ability to salt out CaOx from urine.

Determination of Relationship between Dissolved Urate Added and the Type and Amount of CaOx Precipitated

Both the type A urines spontaneously precipitated CaOx crystals simply on the addition of dissolved urate alone; no crystals were detected in the control samples. Figure 2A shows a typical scanning electron micrograph of the crystals deposited in one such urine sample. It shows the exclusive precipitation of aggregates of CaOx dihydrate crystals: urate crystals were never observed.

Figure 2B shows the volume of CaOx crystals deposited in response to increasing quantities of dissolved urate added in one of the type A urine samples. After an initial lag, there was a linear relationship between the amount of the urate added and the volume of crystalline CaOx deposited until a saturation point (8.6 mmol/l in this case) with the NaU was reached.

The type B urines did not spontaneously precipitate CaOx crystals and were titrated against sodium oxalate solution to determine the empirical metastable limit with respect to CaOx precipitation. Figure 3A shows a typical

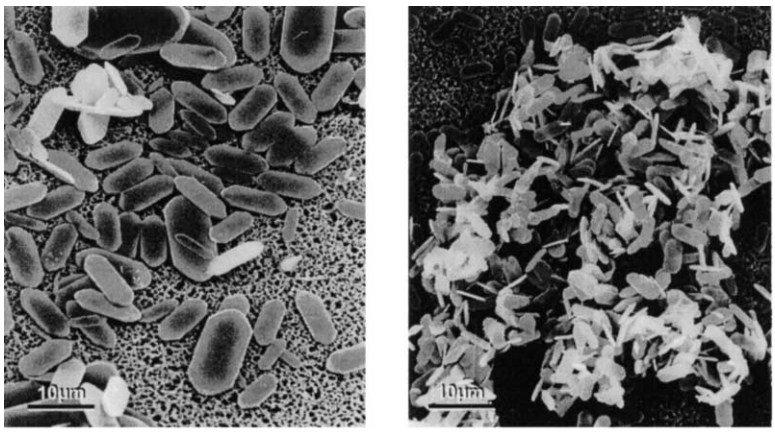

Figure 1. Crystals Precipitated in a Type B Urine Sample

Typical scanning electron micrograph of the crystals precipitated in response to the titration against sodium oxalate solution from a control type B urine sample and from the same sample to which dissolved urate had been added. It shows that although the crystalline deposits in both the portions consisted exclusively of calcium oxalate monohydrates, the ones deposited in the sample spiked with the urate were markedly smaller, more numerous, and more highly aggregated than those deposited in the control sample. Also, urate crystals were not seen.

scanning electron micrograph of the crystals deposited in one of these samples after the titration. It shows that the crystalline deposits consisted exclusively of numerous small CaOx monohydrates clustered into large aggregates; once again, urate crystals were never observed.

Figure 3B shows a plot of the amount of oxalate required to induce detectable spontaneous CaOx crystal nucleation against the urate concentration in the spiked aliquots of one the type B urine samples. There was an inverse correlation between the empirical metastable limit and the amount of dissolved urate added until a saturation point (8.8 mmol/l in this case) with the NaU was reached.

The dose-response curves presented reveal that irrespective of the type of urine, the higher the amount of dissolved urate added, the higher the tendency of the urine to crystallize CaOx, until a saturation with the urate was reached. This observation, in the absence of epitaxy [27] and glycosaminogycans depletion [26], fits very well with the Setschenow equation, which predicts that the higher the concentration of electrolyte (urate), the higher the precipitation of nonelectrolyte (CaOx). This, once again, is in accordance with the urate's ability to salt out CaOx from urine. More importantly, scanning electron microscopic examination of the material precipitated in these samples revealed that they exclusively deposited CaOx: dihydrates in type A and monohydrates in type B urines. Urate crystals were never observed.

Determination of Relative Supersaturations of the Control Urines and in Portions of the Same Urines Supplemented with Dissolved Urate

Figures 4A–4C show relative supersaturations of the type B control and test urines with respect to UA, NaU, and CaOx, respectively. They reveal that while the addition of dissolved NaU to the urines increased their median relative supersaturation of UA from 0.69485 to 1.8794 ($p \leq 0.01$) and of NaU from 5.7052 to 15.8355 ($p \leq 0.01$), it did not affect the relative supersaturation of CaOx. The median relative supersaturation of CaOx was 1.73055 and 1.67575 (nonsignificant) in the control and test samples, respectively. This is despite the fact that, as mentioned above, the addition of dissolved urate alone caused the spontaneous precipitation of CaOx crystals in the type A urines and significantly lowered the metastable limits with respect to CaOx precipitation in the type B urines. This suggests that the EQUIL computer program, which is commonly used to calculate urinary relative supersaturations and to predict the probability of mineral crystallization, does not take into account parameters of the salting out. Hence, it cannot be used in its present form to study the effect of hyperuricosuria on CaOx relative supersaturation. This lack of correspondence between the computed and the observed results can perhaps be attributable to the fact that the program calculates ionic concentrations of calcium and oxalate involved in known soluble complexes from their total concentrations [30] and does not take into account the possibility of salting out. However, the program perhaps may be able to be modified by introducing the salting constant, *k*, to determine the ionic concentrations of calcium and oxalate.

Determination of Relationship between the Urinary Concentrations of Calcium, Oxalate, and Urate That Induced the Precipitation of CaOx

In this experiment, various combinations of calcium, oxalate, and urate that induced precipitation of CaOx were explored. Figure 5 shows one such combination in which the product of the urinary concentrations of calcium and urate was plotted as a function of the total oxalate concentration in the urine at the metastable limit. The most significant relationship between the variables was obtained from the plot shown by logarithmic transformation to give the equation

$$y = 2.0503 + -1.072*\text{LOG}(x)\ R\text{^}2 = 0.792$$

The graph demonstrates that the amount of oxalate required to elicit spontaneous CaOx crystal formation decreased with increases in the product of the prevailing concentrations of calcium and dissolved urate. This suggests that the urate's effect depends upon the prevailing concentrations of calcium and oxalate. It follows, therefore, that in patients passing urine rich in calcium and oxalate, even relatively low increases in concentrations of dissolved urate may cause precipitation of CaOx crys-

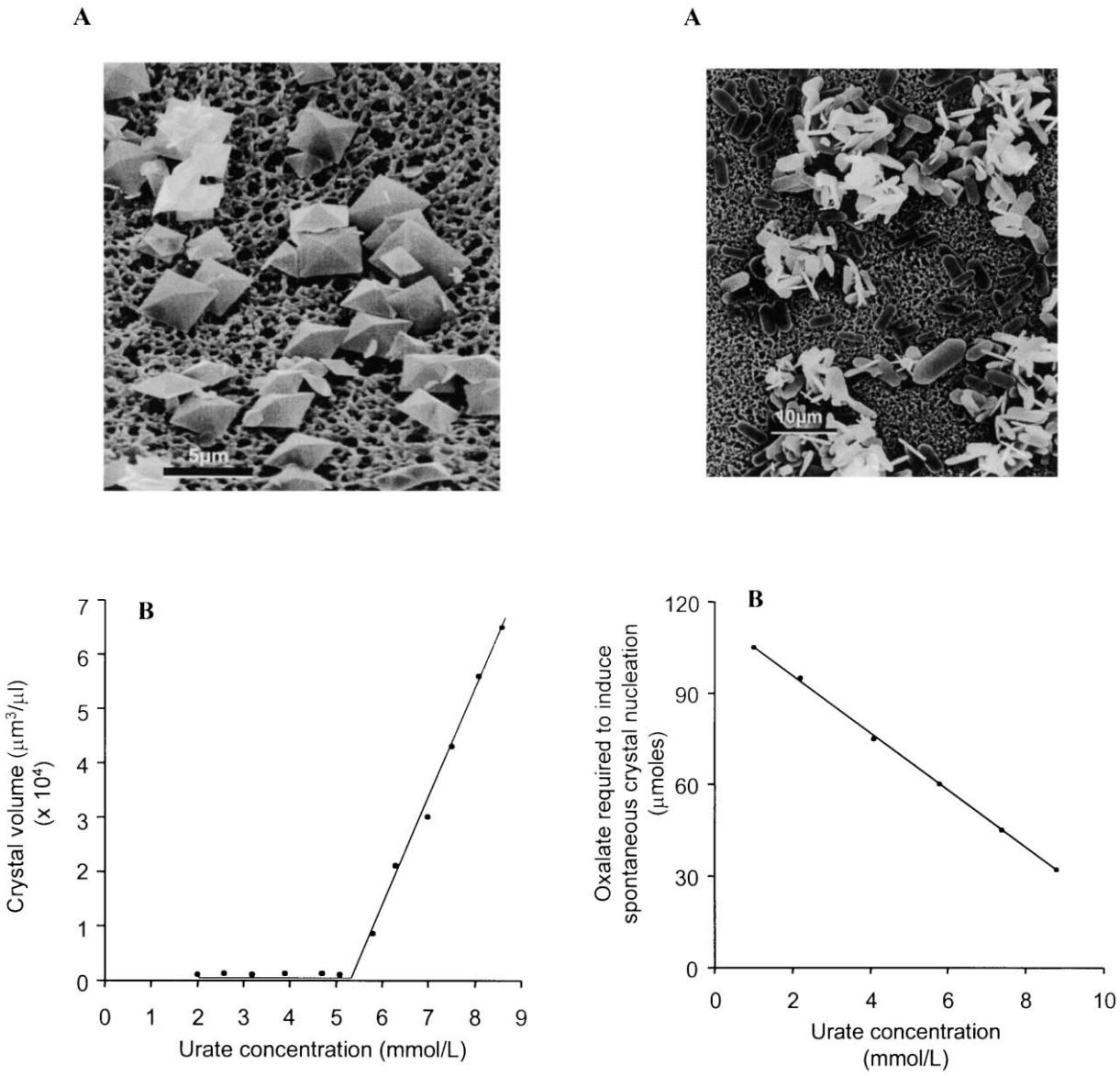

Figure 2. The Urate Dose-Response Curve and the Crystals Precipitated in a Type A Urine Sample

(A) A typical scanning electron micrograph of calcium oxalate dihydrate crystals precipitated in response to increasing the urate concentration in a type A urine sample. No urate crystals were observed. (B) Relationship of the volume of calcium oxalate crystals precipitated to increasing quantities of dissolved urate added in portions of a type A urine sample. It shows that after an initial lag, there is a linear relationship between the amount of dissolved urate added and the volume of crystalline calcium oxalate deposited until a saturation point (8.6 mmol/l in this case) with the sodium urate is reached.

Figure 3. The Urate Dose-Response Curve and the Crystals Precipitated in a Type B Urine Sample

(A) A typical scanning electron micrograph of calcium oxalate monohydrate crystals precipitated in response to the titration against sodium oxalate solution in a dissolved urate-enriched type B urine sample. It shows that the crystalline deposits consisted exclusively of numerous small calcium oxalate monohydrates that are clustered into large aggregates; once again, urate crystals are not observed. (B) Relationship of the amount of oxalate required to induce detectable spontaneous calcium oxalate crystal nucleation to increasing quantities of dissolved urate added in portions of a type B urine sample. It shows that there is an inverse correlation between the empirical metastable limit and the amount of dissolved urate added until a saturation point (8.8 mmol/l in this case) with the sodium urate is reached.

tals. This inference is supported by results of dose-response curves of type A urine samples presented in this study, which showed that even when dissolved, urate was well within the physiological range; small increments in its concentration caused the precipitation of CaOx. This could explain why there has been such a lack of the unequivocal demonstration of "hyperuricosuria" in stone formers [reviewed in 31-33] and why Ettinger et al. [11] observed only a slight reduction in CaOx stone recurrence in patients treated with allopurinol. Had they selected hyperuricosuric and hypercalciuric patients rather than normocalciuric, they may have observed a higher reduction of CaOx stone recurrence on treatment with allopurinol. Thus, in addition to lowering urinary urate, the management of hyperuricosuric or normouricosuric CaOx stone-formers should also include

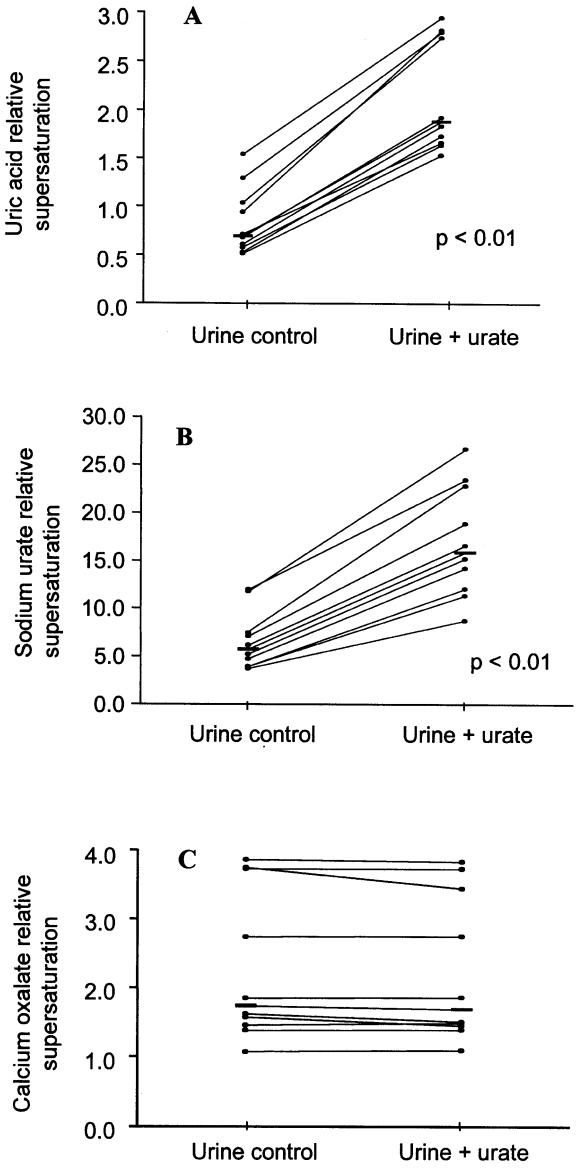

Figure 4. The Degrees of Relative Supersaturations of the Type B Control and Test Urines

(A) The degrees of relative supersaturations with uric acid of the type B control urines and in portions of the same urines spiked with dissolved urate. Bars indicate medians. This shows that the addition of the urate significantly increased the median relative supersaturation of the urines with respect to uric acid from a control value of 0.69485 to 1.8794 ($p \leq 0.01$).

(B) The degrees of relative supersaturations with sodium urate of the type B control urines and in portions of the same urine samples enriched with dissolved urate. Bars indicate medians. This shows that the addition of the urate significantly increased the median relative supersaturation of the urines with respect to sodium urate from a control value of 5.7052 to 15.8355 ($p \leq 0.01$)

(C) The degrees of relative supersaturations with calcium oxalate of the type B control urines and in portions of the same urine samples supplemented with dissolved urate. Bars indicate medians. This shows that the addition of the urate did not significantly alter the median relative supersaturation of the urines with respect to calcium oxalate from a control value of 1.73055 to 1.67575 (non-significant).

taking steps to reduce their calcium and oxalate excretion.

It is remarkable that only one previous study has examined the effect of dissolved urate on CaOx crystal formation in human urine [34]. These authors reported that the urate did not influence the formation of CaOx crystals in concentrated urine at pH 5.3. In that study, the concentration of dissolved urate in a number of urines was reduced by passing the samples through nylon coils onto which the enzyme uricase was immobilized. CaOx crystallization was then induced in the urines and in control samples of the same urines, which had not been subjected to enzyme digestion, by addition of oxalate and evaporation to a final osmolality of 1200 mOsmol/Kg. There are two main reasons why a promotory effect of dissolved urate would not have been detected in the studies of Hallson et al. [34]. First, evaporation of the urine samples to high osmolalities would have caused an inevitable increase in the concentration of low and high molecular weight components, some of which are well-documented inhibitors of CaOx crystallization [29]. Therefore, it is highly likely that such an increase in the concentration of urinary components could have potentially overwhelmed any promotory effect of the urate. Second, salting out of CaOx by dissolved urate occurs only in the pH range 5.7–6.5 [15]. At low pH, as used by Hallson et al. [34], CaOx is more soluble than UA [15]; hence, there will be no salting out of the former by the latter.

Taken together, results of the present study demonstrate that the urate's effects result directly from its ability to salt out CaOx from solution. This mechanism provides a rational, scientific explanation for the occurrence of CaOx urolithiasis in patients who tend to excrete high levels of urate, and for the apparent beneficial effect of allopurinol. As far as treatment is concerned, at present, nothing can be done to alter the excretion of macromolecular weight inhibitors, and we are left with the possibility of lowering urate, calcium, and oxalate excretion or finding some other agent which may attenuate urate's effects. For instance, pyrophosphate, magnesium, and citrate have been reported to inhibit crystal growth in undiluted human urine, and magnesium has been shown to raise the metastable limit of urine samples [35]. The real potential benefit of such agents is that, unlike high molecular weight compounds, their concentration can be increased therapeutically. A detailed study of the effects of these low molecular weight inhibitors, especially citrate and magnesium, on the urate-induced promotion of CaOx crystallization is therefore warranted. Also, as shown by Kallistratos et al. [15], pH has a profound effect on the solubility of CaOx and urate. In the present study, experiments were carried out at the original native pH values of the samples. Urinary pH can be easily controlled. Therefore, to determine whether alterations of urinary pH can lower the extent of salting out, it will be worthwhile repeating these experiments at different pH values as was originally reported by Kallistratos and coworkers [15].

Significance

The present manuscript constitutes the first presented evidence that the promotory effect of hyperuricosuria,

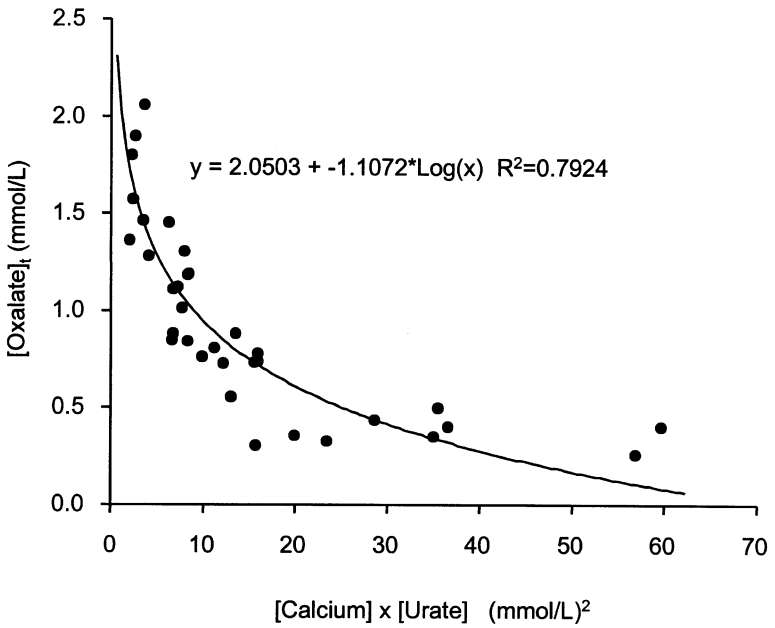

Figure 5. Relationship between the Urinary Concentrations of Calcium, Urate, and Total Oxalate That Induced Precipitation of Calcium Oxalate

The total concentrations of oxalate were obtained for all the samples by adding the endogenous concentration to the increase in concentration that was required to induce detectable spontaneous CaOx precipitation. The figure shows that the amount of oxalate required to elicit spontaneous calcium oxalate crystal formation decreases with increases in the product of the prevailing concentrations of calcium and urate.

simulated by the addition of dissolved sodium urate to human urine, on CaOx crystallization results from its ability to salt out CaOx from solution. Such a mechanism would explain the empirical observations linking hyperuricosuria to CaOx stone formation and more importantly, the success of allopurinol to reduce recurrence of CaOx stones in hyperuricosuric patients, without the need to invoke occurrence of urate crystals in urine, the presence of which occurs only rarely. From the treatment perspective, of particular interest is the relationship between the urinary concentrations of urate, calcium, and oxalate at which CaOx precipitation occurs. The data indicates that the urate's effect depends upon the prevailing concentrations of calcium and oxalate. This demonstrates for the first time that in addition to lowering urinary urate in hyperuricosuric or, for that matter, in normouricosuric CaOx stone-formers, steps should be taken to reduce their calcium and oxalate excretion. Relatively few samples were studied in this investigation. Results of further studies with a larger number of samples may allow the possible definition of an index relating urinary calcium, oxalate, and urate concentrations, thus enabling the identification of patients likely to benefit from allopurinol treatment and those in whom a reduction in calcium and oxalate excretion would also be beneficial. The manuscript also outlines anti-salting out strategies for future research for the prevention and/or treatment of CaOx calculi.

Experimental Procedures

Materials

Monosodium urate (NaU), sodium hydroxide (NaOH), and sodium chloride (NaCl) were purchased from Sigma Chemical Company, St. Louis, MO. Sodium oxalate and hydrochloric acid (HCl) were from BDH Chemicals Ltd. Poole, England. Multistix test strips were from Miles Laboratories, Mulgrave, Victoria, Australia. Millipore filters (0.22 μm ; GVWP 142 50) were from Millipore Corporation, Bedford, MA. Coulter Counter (Model TA II fitted with a Population Count Accessory) was from Coulter Electronics Ltd, Coldharbour Lane, Harpenden, Herts, United Kingdom. All other chemicals and materials used in this study were of the highest purity available.

Collection and Preparation of Urine Samples

Although the use of whole urine would have been ideal, centrifuged and 0.22 μm filtered urine was used in this study. This was because urine invariably contains particulate material [36] that precludes the use of a Coulter Counter to determine volume and number of crystalline CaOx deposited. It is remarkable that centrifugation and 0.22 μm filtration of urine is known to remove Tamm-Horsfall glycoprotein and some human serum albumin [36], neither of which has any significant effect on CaOx crystal growth in undiluted human urine [37]. With these reservations, the study was carried out using centrifuged and 0.22 μm filtered urine.

Twenty-four hour urine specimens were collected without preservative from 48 healthy men (mean age 38 years) who had no history of kidney stone disease. Absence of blood from the specimens was confirmed using Multistix test strips. The samples were refrigerated during the collection period and during storage before use and then centrifuged using a JA-14 fixed-angle rotor at 8000 × g for 15 min at 20°C in a Beckman J2-21 M/E centrifuge (Beckman Instruments, Palo Alto, CA). The supernatants were filtered through 0.22 μm Millipore filters.

Determination of the Salting Constant, *k*, in Undiluted Human Urine In Vitro

Since solubility product of CaOx is a measure of solubility of the salt, its values obtained in control (dissolved urate not added) and test (dissolved urate added) portions of the same urines were used in the Setschenow equation for determination of the salting constant.

Ten type B [25] centrifuged and 0.22 μm filtered urines were used in this experiment. This is because, unlike type A, they do not precipitate CaOx upon the addition of dissolved urate alone: this enables accurate determination of their total oxalate concentrations at which precipitation of CaOx occurs. All the samples were titrated against filtered, saturated solution of NaU and the maximum increase in the urate concentrations that could be achieved without causing the spontaneous precipitation of NaU was determined [25]. All the samples were then divided. A filtered (0.22 μm) saturated solution of NaU dissolved in 1mol/liter of NaOH was added to the test portion to increase its urate concentration to 1.3 mmol/liter less than the amount determined above. In practical terms, this meant that the urate concentration was increased by an average of 3-4

mmol/liter. The remaining portion of the sample was used as the control, and to this was added an identical volume of 1mol/liter NaOH. The pH of all the samples was adjusted to their original native value, and the osmolalities of the control and experimental samples were matched exactly by the addition of solid NaCl. Portions of all the samples, control and test, were retained for determination of their relative supersaturations, and the remaining lots were titrated against sodium oxalate solution to determine their empirical metastable limits with respect to CaOx crystallization.

The total oxalate concentrations at which precipitation of CaOx occurred were obtained for all the samples by adding the endogenous concentration to the increase in concentration, which was required to induce detectable spontaneous CaOx precipitation at the metastable limit. From these were calculated the products of the urinary concentrations of calcium and the total oxalate for the control (s_o) and experimental portions (s) of each sample. These values and the molar increases in urinary concentration of urate achieved in the experimental samples (c_s) were then applied to the Setschenow equation to calculate the salting constant, k, for the urines.

Determination of Empirical Metastable Limit with Respect to CaOx Crystallization and Visualization of the Precipitated Material

The experimental system used for the determination of empirical metastable limit of urine samples with respect to CaOx precipitation has been described in detail elsewhere [28]. Briefly, the empirical metastable limit is defined as the minimum amount of oxalate required to induce spontaneous, detectable CaOx crystallization. It is determined by titrating the urine sample with sodium oxalate solution, followed by incubation at 37°C for 30 min in a shaking water bath. The number of crystals >2.0 μm in each sample is then determined using a Coulter Counter. Once the metastable limit has been exceeded, the crystal number initially rises linearly with increasing oxalate concentration. The point at which this line intercepts the abscissa is taken as the minimum amount of oxalate necessary to induce detectable CaOx crystal formation and is defined as the empirical metastable limit of the urine.

To visualize the precipitated material, at the end of the titration, 1.0 ml aliquots of the samples loaded with sodium oxalate solution 30 μmol above their metastable limits were 0.22 μm filtered and the membranes were processed for scanning electron microscopy.

Determination of Relationship between Dissolved Urate Added and the Type and Amount CaOx Precipitated

Four centrifuged and 0.22 μm filtered urine samples, two each of type A and B [25], were used in this experiment. After retaining a portion of each urine for various biochemical measurements, the remaining sample was divided. While the first part was titrated against filtered, saturated solution of NaU as described previously [25], the second part was further divided into several 100 ml portions. These were supplemented with increasing volumes of filtered, saturated solution of NaU such that the maximum increase in urate concentration was 1.3 mmol/liter less than the amount determined above. The samples were incubated in a shaking water bath at 37°C for 90 min and checked for the presence of crystalline particles under the light microscope. All portions of type A urines spontaneously precipitated crystalline material, and its volume was determined using a Coulter Counter. Portions of the type B urines, on the other hand, did not spontaneously precipitate any particles, and their metastable limits with respect to CaOx crystallization were determined.

At the end of the experiment, 1.0 ml aliquots of the samples were 0.22 μm filtered and the membranes were processed for scanning electron microscopy. Also, small portions of the test samples were retained for determination of their urate concentrations.

Scanning Electron Microscopy

The filtration membranes containing crystalline material were dried overnight at 37°C. They were mounted on aluminium stubs, sputtered with gold for 180 s (SEM Autocoating Unit E5200, Polaron Equipment Ltd, Watford, UK), and examined using an ETEC Auto Scan Electron Microscope (Siemens AG, Karlsruhe, Germany) at an operating voltage of 20 kV.

Determination of Relative Supersaturations of the Control Urines and in Portions of the Same Urines Supplemented with Dissolved Urate

The relative supersaturations of the control and test urines, used for the determination of the salting constant, were calculated with respect to UA, NaU, and CaOx using the EQUIL computer program [30]. This required analysis of urines for pH, calcium and magnesium (atomic absorption), phosphate [38], urate (Technicon method SF4-0013FH4), chloride (Beckman Astra chloride chemistry module), sodium and potassium (Beckman Astra sodium and potassium chemistry module), oxalate [39], sulfate [40], ammonia [41], pyrophosphate [42], and citrate [43].

Determination of Relationship between the Urinary Concentrations of Calcium, Oxalate and Urate Which Induced Precipitation of CaOx

Thirty-four centrifuged and 0.22 μm filtered urine samples of type B, for the reason mentioned above, were used in this experiment. While an aliquot of each urine was retained for various biochemical measurements, the remaining sample was titrated against sodium oxalate solution to measure its empirical metastable limit with respect to CaOx crystallization. As mentioned above, this enabled the calculation of the total oxalate concentration of each sample at which precipitation of CaOx occurred. Various combinations of calcium, oxalate, and urate that induced precipitation of CaOx were explored for their significant relationship.

Statistical Methods

Each experiment was performed in triplicate. For the sake of clarity, data were plotted as mean values; nonetheless, statistical comparisons were performed using the Wilcoxson signed rank sum test at a 0.05 level of significance.

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