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Crystal growth of calcium oxalate in urine of stone-formers and normal controls

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Summary. In this study, the relative crystal growth rate (Vcr) of calcium oxalate (Caox) and a number of other parameters were determined in 17-h daily (d) and 7-h nocturnal fractions (n) of whole urine from 20 recurrent Caox stone formers (SF) and 29 age-matched male normal controls (NC). Vcr, which was determined by the gel crystallization method (GCM), showed the largest difference between SF and NC among all parameters under investigation. Mean values (± SD) obtained for Vcr were: 0.73 ± 0.58 (SF-d)/0.21 ± 0.22 (NC-d; $P < 0.001$) and 0.63 ± 0.58 (SF-n)/0.24 ± 0.25 (NC-n; $P < 0.01$). Significantly higher concentrations of Ca and lower concentrations of thermodynamic and kinetic effectors of Caox crystal growth were responsible for the higher crystal growth rates observed in SF as compared with NC, i.e., they should be partially causative in Caox urolithiasis. However, other properties of urine or the urinary tract (potentially, crystal agglomeration and adhesion) must be accounted for in the genesis of Caox stones.

Key words: Calcium oxalate – Crystal growth – Human urine – Urolithiasis

Many investigations have been carried out to demonstrate metabolic abnormalities or properties of urine that might be responsible for the formation of calcium oxalate (Caox) stones [1, 12, 14, 15, 18–20, 23–25]. Among other hypotheses, the supposition has been raised that Caox urolithiasis might result from a deficiency of crystal growth inhibitors in the urine of stone-formers as compared with normal controls.

During recent years, we have developed a photometric microtechnique for the efficient determination of relative crystal growth rates of Caox hydrates (Vcr) that may be applied to whole urine samples (gel crystallization method, GCM) [2–4,8]. The parameter Vcr reflects the total influence of all of its thermodynamic and kinetic effectors in urine except oxalate, thus enabling an estimate of the contribution of inhibition on the crystal growth rate of Caox.

The aim of this study was to evaluate the role of crystal growth effectors in the genesis of Caox urolithiasis by the determination of Vcr (Caox) in defined urinary fractions from well-matched groups of stone-formers and controls and its interpretation through other parameters.

Subjects and methods

Design of the study and sample handling

The following volunteers were included in the study: 20 men aged 40–50 years (mean, 46 ± 2 years) who were normocalcaemic, recurrent calcium oxalate stone-formers (> 2 stones) showing normal renal function (creatinine clearance, > 60 ml/min) and 29 age-matched (mean, 44 ± 3 years) healthy men as controls. All volunteers maintained their usual diet and activities.

The collection of urinary fractions was carried out on an outpatient basis in springtime within a period of 45 days so as to avoid seasonal variations of the parameters of interest [21]. A 17-h daily urine specimen (from 6 a.m. to 11 p.m.) and a 7-h overnight fraction (from 11 p.m. to 6 a.m.) were collected from each subject and a venous blood sample was drawn from fasting volunteers. The next day, a 2-h urinary fraction was also collected from fasting subjects. During the collection period, urinary fractions were kept cool at 6°–10°C. Samples (20 ml) of all urinary fractions were frozen without the addition of preservatives and then stored at −70°C–−80°C for further laboratory investigations, which were completed within a period of 2 months after collection.

Determination of parameters

Immediately after thawing of the samples at room temperature, pH values were measured electrometrically. Relative crystal growth rates of Caox (Vcr) were determined after centrifugation in undiluted samples of urine using the automated GCM [2–4, 8]. The measuring device consisted of an automated microphotometric system for transmitted light that was equipped with a rapid-scanning stage adapted to 96-well microtiter plates, an MPC 64, electronic control unit and an on-line HP9816S computer (Zeiss, Oberkochen, FRG; Hewlett-Packard). We used the dark-field mode of measurement and a gel matrix comprising 0.5% (w/w) agar-agar, 2 mmol/l sodium oxalate, and approximately 0.1 mmol/l Caox seed crystals. The solution used as standard in crystal growth experiments was

Table 1. Parameters of crystal growth of Caox in 17-h daily and 7-h nocturnal urinary fractions from 20 recurrent Caox stone-formers and 29 matched normal controls

Parameter	Fraction	Stone-formers (SF)	Controls (NC)	Significance		
				SF/NC	SFd/n	NCd/n
Vcr	d	0.73 ± 0.58	0.21 ± 0.22	***	NS	NS
	n	0.63 ± 0.58	0.24 ± 0.25	*		
Vcr (pH 5)	d	0.91 ± 0.59	0.33 ± 0.31	***	NS	NS
	n	0.77 ± 0.64	0.31 ± 0.3	*		
Vcr(pol)	d	0.82 ± 0.72	0.2 ± 0.23	***	NS	NS
	n	0.75 ± 0.74	0.25 ± 0.21	**		
Vcr × Oxn × V (ml)	d	680 ± 529	156 ± 174	***	*	*
	n	125 ± 127	67 ± 74	*		

Vcr, Relative crystal growth rate of Caox determined using the GCM in urine samples of original pH corresponding to the description in *Subjects and methods;* Vcr (pH 5), Vcr determined in urine samples adjusted to pH 5; Vcr (pol), Vcr determined using the measuring mode of polarized light; Vcr × Oxn × V, product of Vcr, normalized total concentration of oxalate and excretion volume; d, daily fractions; n, nocturnal fractions; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, corresponding to appropriate statistical tests

composed as follows: 131 mmol/l sodium, 40 mmol/l potassium, 25 mmol/l ammonium, 4 mmol/l calcium, 3 mmol/l magnesium, 20 mmol/l phosphate, 15 mmol/l sulfate, 2 mmol/l citrate, 149 mmol/l chloride and 250 mmol/l urea, pH 6. In addition to measurements at original pH, the crystal growth rate of Caox was determined in all urinary samples after adjustment to pH 5 [Vcr (pH 5)] so as to exclude artefacts due to potential precipitation of calcium phosphate at higher pH values. Furthermore, Vcr was determined in all native urinary samples using polarized light as the mode of measurement [Vcr (pol)].

Total concentrations of Ca, Mg, Na, K, phosphate, and sulfate were determined by ICP-coupled atomic emission spectroscopy (simultaneous spectrometer JY 32P; Instruments S.A.) [5]. Citrate, isocitrate, creatinine, urate, oxalate and ammonium were analyzed by enzymatic microphotometric analysis in 96-well microplates (TECAN 505 automated pipetting station; MR600, microplate reader Dynatech; kits commercially available from Boehringer, Mannheim, FRG) [9].

To avoid systematic differences between parameters in the specimens under regard due to potential analytical errors, measurements were carried out using only an alternating arrangement of samples from both groups within a series. Saturation ratios (activity product/solubility product) of calcium oxalate, uric acid and brushite were estimated using a computer program for the calculation of complex chemical equilibria written in HP-BASIC [6]. The following solubility products (valid for $I = 0$; given in mol^2/l^2) were used: Ksp (Caox) $= 3.63 \times 10^{-9}$, Ksp (brushite) $= 2.49 \times 10^{-7}$, Ksp (uric acid) $= 9.86 \times 10^{-10}$.

The probability for the significance of differences in parameters between daily and nocturnal urinary fractions was assessed by appropriate tests for paired data. Principally, distributions of corresponding data were evaluated using the Shapiro-Wilk normality test. In correspondence with the distributions assessed (normal, symmetrical, others), the paired *t*-test, the Wilcoxon rank-sum test, or the sign test was chosen. For unpaired data (SF vs NC), distribution was evaluated using the Fischer test. In correspondence with the kind of distribution and the homogeneity of the variances under regard, the two-sample *t*-test, an expanded *t*-test, or the median test was applied. Calculations were carried out using the Statistics Library HP98820A (Hewlett-Packard).

Results

Table 1 shows the relative crystal growth rates of Caox hydrates measured in native urine samples at original pH (Vcr) and after adjustment to pH 5 [Vcr(pH 5)]. The parameter Vcr(pol) was measured in the same samples at original pH; however, polarized light was used as the mode of microscopic measurement, predominantly indicating the growth of doubly-refracting whewellite. Because of the excess of soluble oxalate provided by the gel phase (2 mmol/l), urinary oxalate has no significant effect on the parameters measured by the GCM. However, its potential influence on the real crystal growth of Caox in urine can reasonably be taken into account using the product of Vcr × Oxn × V (Oxn, normalized concentration of total oxalate; V, corresponding urinary volume).

As may be seen from Table 1, all listed parameters of Caox crystal growth exhibited significant differences in their means between SF and NC. In general, these differences were more pronounced in daily urinary fractions than in overnight specimens. The ratio Vcr(SF)/Vcr(NC) was 3.5 for daily fractions and 2.6 for nocturnal fractions. The corresponding ratios for Vcr × Oxn × V were 4.4 (d) and 1.9 (n).

In contrast, the saturation ratios for Caox [S (Caox)] in SF and NC were of much lower discriminating power between these groups (Table 2). Among the daily urine specimens, S(Caox) in SF was only 1.6 times that in NC. However, no significant difference could be found for S(Caox) in corresponding overnight samples or with respect to the crystal phases of brushite and uric acid. In Fig. 1, individual measurements of Vcr in the two urinary fractions from SF and NC are illustrated. It can be seen that in spite of the significant differences in mean values, individual data showed an appreciably large range of overlap.

To interpret the crystal growth parameters shown in Table 1, we determined a series of urinary parameters for the specimens under regard. In Table 3 the excretion

Table 2. Saturation ratios (activity product/solubility product) for Caox monohydrate, brushite and uric acid calculated from complex chemical equilibrium distributions in 17-h daily and 7-h noctural urinary fractions from 20 recurrent Caox stone-formers and 29 matched normal controls

Parameter	Fraction	Stone-formers (SF)	Controls (NC)	Significance		
				SF/NC	SFd/n	NCd/n
S[Caox]	d	7.31 ± 3.55	4.72 ± 2.25	**	NS	NS
	n	6.04 ± 3.49	5.4 ± 3.43	NS		
S[brush]	d	1.98 ± 1.21	1.51 ± 1.16	NS	NS	NS
	n	1.47 ± 1.58	1.35 ± 1.15	NS		
S[UA]	d	2.03 ± 1.39	2.52 ± 2.21	NS	NS	NS
	n	2.66 ± 1.02	2.99 ± 1.22	NS		

S [Caox], saturation ratio for Caox monohydrate; S [brush], saturation ratio for brushite; S [UA], saturation ratio for uric acid; d, daily fractions; n, nocturnal fractions; NS, not significant * $P<0.05$; ** $P<0.01$; *** $P<0.001$, corresponding to appropriate statistical tests

Table 3. Excretion volume, pH and total concentrations of urinary constituents measured in 17-h daily and 7-h nocturnal urinary fractions from 20 recurrent Caox stone formers and 29 matched normal controls

Parameter	Fraction	Stone-formers (SF)	Controls (NC)	Significance		
				SF/NC	SFd/n	NCd/n
Volume; V (ml)	d	1,178 ± 443	896 ± 412	*	***	***
	n	363 ± 352	341 ± 171	NS		
pH	d	6.04 ± 0.51	6.06 ± 0.56	NS	*	NS
	n	5.72 ± 0.56	5.68 ± 0.47	NS		
Ca(T)	d	4.81 ± 2.17	3.37 ± 1.66	**	*	NS
	n	4.31 ± 2.63	3.55 ± 1.73	NS		
Mg(T)	d	3.29 ± 1.39	3.66 ± 1.65	NS	NS	**
	n	3.47 ± 1.86	4.65 ± 2.04	*		
Na(T)	d	174 ± 54	209 ± 44	*	NS	NS
	n	158 ± 71	203 ± 62	*		
K(T)	d	46.4 ± 17.1	61.7 ± 19.3	**	**	***
	n	36.7 ± 21.9	47.7 ± 15.8	*		
NH_4(T)	d	27 ± 14	33 ± 17	NS	NS	NS
	n	28 ± 14	39 ± 19	*		
Ox(T)	d	0.32 ± 0.13	0.34 ± 0.13	NS	*	NS
	n	0.27 ± 0.1	0.37 ± 0.18	NS		
Cit(T)	d	2.33 ± 0.96	2.52 ± 1.05	NS	NS	NS
	n	2.01 ± 1.02	2.4 ± 1.19	NS		
Isocit(T)	d	0.34 ± 0.1	0.44 ± 0.15	*	NS	NS
	n	0.31 ± 0.13	0.48 ± 0.2	**		
SO_4(T)	d	20.3 ± 6	23.1 ± 7.61	NS	NS	**
	n	20.5 ± 8.07	27.1 ± 8.9	*		
PO_4(T)	d	22.1 ± 7.35	25 ± 10.9	NS	NS	***
	n	22.2 ± 8.59	35.6 ± 13.7	***		
Cl(T)	d	168 ± 53.5	209 ± 49.2	**	NS	**
	n	148 ± 84.1	180 ± 56.4	NS		
Urate(T)	d	3.36 ± 0.91	3.95 ± 1.37	NS	NS	NS
	n	3.01 ± 1.56	3.56 ± 1.03	NS		
Crea(T)	d	11.3 ± 3.4	13.3 ± 4.27	NS	NS	NS
	n	11.3 ± 4.77	14.4 ± 4.88	*		

Ca, Mg, Na, K, NH_4, Ox, Cit, Isocit, SO_4, PO_4, CL, Urate and Crea(T) represent total concentrations of calcium, magnesium, sodium, potassium, ammonium, oxalate, citrate, isocitrate, sulfate, phosphate, chloride, urate and creatinine (given in mmol/l). d, Daily fractions; n, nocturnal fractions; NS, not significant, * $P<0.05$; ** $P<0.01$; *** $P<0.001$, corresponding to appropriate statistical tests

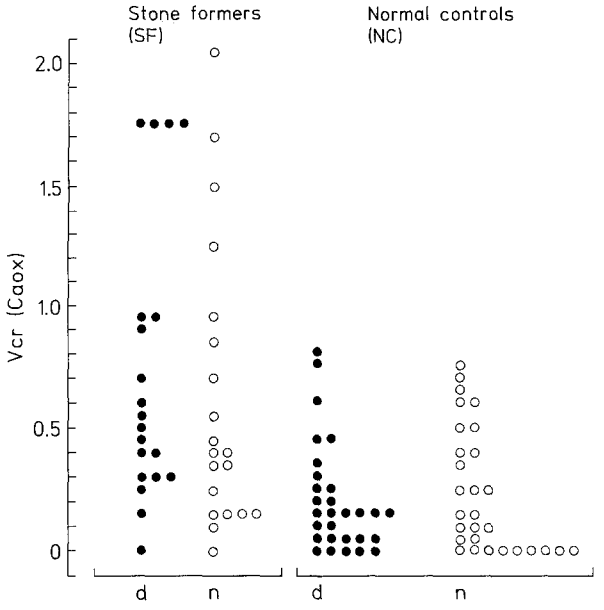

Fig. 1. Individually measured values for relative crystal growth rates of Caox (*Vcr*) in two urinary fractions from each of 20 recurrent Caox stone-formers and 29 matched normal controls. *d*, 17-h daily fractions; *n*, 7-h nocturnal fractions

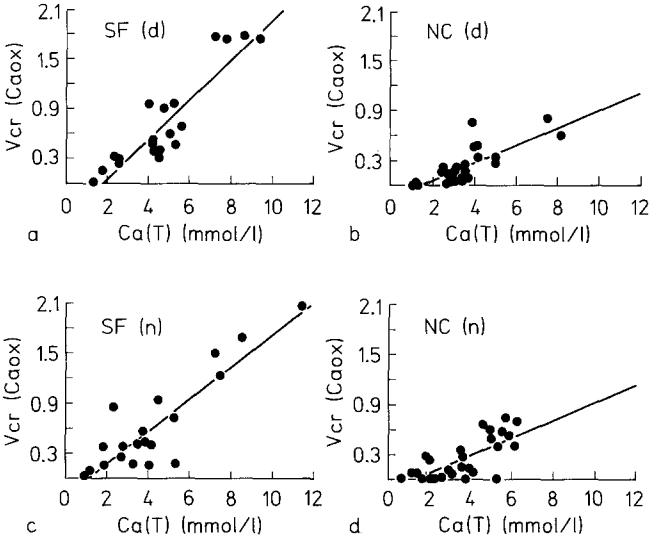

Fig. 2a–d. Correlation between the crystal growth rate of Caox (*Vcr*) and corresponding total concentrations of calcium (Ca(T)) in daily (*d*) and nocturnal urinary fractions (*n*) from 20 recurrent Caox stone-formers (*SF*) and 29 matched controls (*NC*)

volumes, pH values and total concentrations of urinary constituents are listed. Most of these parameters have been proven to be effectors of Caox crystal growth [4]. Concentrations were directly related to the growth parameters listed in Table 1. It may be seen from Table 3 that apart from excretion volumes, total calcium concentration was the only parameter that was higher in SF than in NC. All parameters known to decrease crystal growth by

Table 4. Excretion of urinary constituents in 17-h daily and 7-h nocturnal urinary fractions from 20 male recurrent Caox stone formers and 29 matched normal controls

Parameter	Fraction	Stone-formers (SF)	Controls (NC)	Significance SF/NC
Ca(T)xV	d	5.17 ± 2.03	2.79 ± 1.41	***
	n	1.26 ± 0.88	1.18 ± 0.74	NS
Mg(T)xV	d	3.58 ± 1.42	3.01 ± 1.34	NS
	n	0.91 ± 0.49	1.5 ± 0.82	NS
Na(T)xV	d	194 ±63	181 ±75	NS
	n	45 ±30	69 ±42	**
K(T)xV	d	51.3 ±18.4	50.6 ±18.9	NS
	n	8.5 ± 3.4	15.4 ± 8.4	**
NH_4(T)xV	d	28 ±11	25 ±10	NS
	n	7.6 ± 4.5	12.2 ± 6.3	*
Ox(T)xV	d	0.35 ± 0.13	0.28 ± 0.12	*
	n	0.07 ± 0.04	0.11 ± 0.04	**
Cit(T)xV	d	2.53 ± 0.94	2.06 ± 0.93	*
	n	0.52 ± 0.29	0.77 ± 0.5	NS
Isocit(T)xV	d	0.37 ± 0.11	0.36 ± 0.12	NS
	n	0.09 ± 0.05	0.15 ± 0.07	**
SO_4(T)xV	d	22.2 ± 5.6	18.6 ± 6.1	*
	n	5.7 ± 3.1	8.8 ± 4.5	NS
PO_4(T)xV	d	24.2 ± 7.3	19.6 ± 6.4	*
	n	6.3 ± 3.5	11.1 ± 4.6	***
Cl(T)xV	d	186 ±57	180 ±75	NS
	n	40 ±27	62 ±40	**
Urate(T)xV	d	3.67 ± 0.81	3.21 ± 1.09	NS
	n	0.77 ± 0.37	1.15 ± 0.55	NS
Crea(T)xV	d	12.2 ± 2.5	10.8 ± 3.4	NS
	n	2.85 ± 1.20	4.44 ± 1.66	**

Ca, Mg, Na, K, NH_4, Ox, Cit, Isocit, SO_4, PO_4, Cl, Urate and Crea(T)xV represent total excretions of calcium, magnesium, sodium, potassium, ammonium, oxalate, citrate, isocitrate, sulfate, phosphate, chloride, urate and creatinine, given in mmol/17 h and mmol/7 h. d, Daily fractions; n, nocturnal fractions; NS, not significant * $P<0.05$; ** $P<0.01$; *** $P<0.001$, corresponding to appropriate statistical tests

thermodynamic and kinetic mechanisms were higher in NC than in SF.

In Table 4, the total output of the urinary constituents measured in the different specimens are summarized. Because of their minor dependence on excretion volume, these parameters were related more to metabolic differences between the subjects under consideration than concentrations. Obviously, in the daily urinary fractions, the difference between SF and NC was more pronounced with respect to calcium (as compared with concentrations) and was either abolished or reversed for most of the other parameters. However, the output of calcium in nocturnal fractions was nearly the same in SF and NC, whereas, as a rule, the excretion of substances known to decrease Vcr was higher in NC than in SF.

Figure 2 demonstrates the correlation between the crystal growth of Caox (Vcr) and corresponding total

calcium concentrations in the two fractions (d and n) of SF and NC. The clearly slighter slopes of the regression lines Vcr = f [Ca(T)] in the NC groups corresponded to higher concentrations of the urinary constituents that depress the crystal growth of Caox.

The quantification of pH, calcium, magnesium, sodium, potassium, phosphate and creatinine in sera of both groups (data not shown) revealed no difference in any of these parameters between SF and NC.

Discussion

This study was aimed at gaining insight into the role of the crystal growth of Caox and its effectors in Caox stone formation. Therefore, an efficient micromethod that enables the determination of the relative crystal growth rates (Vcr) of Caox in whole urine (gel crystallization method, GCM) [2–4, 8] was applied to daily and nocturnal fractions of urine from well-matched groups of recurrent Caox stone-formers and healthy controls.

The measuring parameter Vcr obtained using the GCM is governed by all of the thermodynamic (determining crystal growth via supersaturation) and kinetic factors (affecting crystal growth at constant supersaturation) of urine except its oxalate content. The influence of oxalate on Vcr could be neglected because in the samples under regard, >90% of Ox(T) had been precipitated and the remaining small amount was overwhelmed by the excess of oxalate in the gel phase (2 mmol/l) during measurement.

It could be demonstrated (Table 1) that the mean crystal growth parameters measured at the original pH value in whole urine samples [Vcr and Vcr(pol)] as well as in those adjusted to pH 5 [Vcr (pH 5)] were significantly different in both groups. However, differences assessed for 17-h daily fractions [SF(d)−NC(d)] were more pronounced than those found for 7-h overnight collections [SF(n)−NC(n)]. The increase in Vcr with decreasing pH corresponds to the kinetic effect of pH on Caox crystal growth via the protonation of phosphate and citrate, as has been shown from measurements in artificial urine [10]. The influence of urinary oxalate and excretion volume on the Caox stone-forming potential in whole urine is taken into account using the product of Vcr × Oxn × V. It shows the same pattern as the parameters mentioned above in specimens from stone-formers vs healthy controls, thus indicating the dominating role of effects on crystal growth that are reflected by the measuring parameters Vcr, Vcr(pH 5) and Vcr(pol) (Table 1).

To account for the different Vcr values obtained in the urinary fractions studied, a series of other parameters were determined (Tables 2–4). As may be derived from Table 3, the higher crystal growth rates observed in urine specimens from SF as compared with NC were caused by both a higher concentration of urinary calcium and lower concentrations of the effectors that are known to decrease Vcr by thermodynamic and kinetic mechanisms (Mg, Na, K, NH_4, citrate, isocitrate, SO_4, PO_4) [7, 10]. This was found in daily fractions as well as in overnight collections and was confirmed by the slighter slopes of the regression lines plotting Vcr against Ca(T) for the fractions NC(d);NC(n) as compared with SF(d),SF(n) (Fig. 2).

However, in daily fractions, significantly higher excretion volumes were registered for the SF group, which seems to be a general finding in most studies on such subjects [15, 19, 23, 25]. In accordance with Tiselius [25], we think that "patients who are collecting urine tend to drink more than they usually do". As our patients had been advised to maintain normal fluid intake, their somewhat higher urinary volumes seem to be part of the well-known "stone clinic effect".

Therefore, concentration was converted to total output, thus excluding the dependence of parameters on excretion volume (Table 4). The significance of differences between the concentrations of crystal growth effectors found in daily fractions (Table 3) was abolished or even reversed when this parameter was converted into total excretion per collection time. In contrast, the difference in total calcium between SF and NC was enhanced when excretion rather than concentration was measured. These results suggest that the higher crystal growth rates found for daily fractions from stone-formers as compared with normals result from higher calcium output rather than from reduced excretion of crystal growth inhibitors. The higher output of Ca found in stone-forming subjects is in agreement with findings in a number of previous studies [1, 15, 19, 23].

However, lower crystal growth rates measured in the overnight fractions from NC as compared with SF may have been attributable to a higher output of Vcr-decreasing effectors (Mg, Na, K, NH_4, citrate, isocitrate, SO_4, PO_4) rather than to a lower output of calcium. It should be emphasized that lower excretion volumes in SF, which should be voided under normal conditions, would enhance the differences in crystal growth parameters between SF and NC.

The significantly higher crystal growth rates in the SF group demonstrate that the parameter Vcr is clearly indicative of the risk for Caox stone formation, in this respect being unambiguosly superior to calculated saturation ratios [S (Caox); Table 2]. The latter fact is attributable to the sensitivity of Vcr to both thermodynamic and kinetic effects, whereas S(Caox) is a purely thermodynamic measure that is not indicative of growth inhibition. However, considerable overlapping of individual Vcr values measured in the urine specimens from SF and NC may be observed (Fig. 1). Although the mean or median values in the corresponding groups differed significantly, some fractions from SF exhibited low crystal growth of Caox and some specimens from normal controls showed relatively high Vcr values.

In a number of studies evaluating Caox stone-formers on basis of so-called crystal formation risks, however these might be defined, none of the investigators could demonstrate measured or calculated parameters from urine fractions that completely distinguished groups of stone-formers from matched controls [1, 12, 14, 15, 18–20, 23–25]. These studies involved the determination of so-called inhibitory activities [20, 23], metastable limits of Caox precipitation [12, 14, 23], estimation of supersaturation [15, 19, 25] and other parameters [18, 24, 25].

Therefore, from the present study as well as in the literature, the conclusion may be drawn that urinary factors relevant to the nucleation and growth of Caox crystals may be only partially responsible for the genesis of Caox stones. The decrease in effectors of crystal growth noted in the present study may contribute to enhanced crystal growth in stone-formers' urine; however, it cannot be the only causative factor for stone formation. Urinary properties such as the promotion or inhibition of agglomeration [17] and/or the adhesion of crystals on surfaces [16] might also play a role in the process of stone formation. It could be that the coincidence of any of these factors with a sufficiently high crystal growth rate might simply be the cause of urolithiasis. The findings of Rose et al. [22] on the potential role of Tamm-Horsfall mucoprotein (THMP) as a promoter of Caox crystal agglomeration could recently be confirmed by us by means of a new flow model of crystallization. In the latter study [13], THMP could be shown to account for significant agglomeration and adhesion of Caox crystals from an in vitro specimen on a gel surface. Further experiments are necessary to establish the pathophysiological meaning of these results.

Apart from confirming the causative value of crystal growth in Caox stone formation, we found the gel crystallization method (GCM) to be a highly efficient diagnostic microprocedure for determining the course of the parameter Vcr in individuals suffering from Caox urolithiasis. Therefore, the GCM is especially suitable in clinical routine for following the efficacy of therapeutic measures in stone-forming patients [11] as well as their compliance with therapy for stone prophylaxis.

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