Urol Res (2000) 28:167–177

ORIGINAL PAPER

P. O. Schwille · A. Schmiedl · U. Herrmann
M. Manoharan · J. Fan · V. Sharma · D. Gottlieb

Ascorbic acid in idiopathic recurrent calcium urolithiasis in humans – does it have an abettor role in oxalate, and calcium oxalate crystallization?

Received: 24 September 1999 / Accepted: 16 December 1999

Abstract The role of ascorbic acid (ASC) in the pathophysiology of renal calcium stones is not clear. We evaluated ASC in blood and urine of fasting male patients with idiopathic calcium urolithiasis (ICU) and healthy volunteers. Using smaller subgroups, we also evaluated their response to exogenous ASC [either intravenous or oral ASC (5 mg/kg bodyweight)] administered together with an oxalate-free test meal. The influence of ASC on calcium oxalate crystallization, the morphology of crystals at urinary pH 5, 6 and 7, and the effect of increasing duration of urine incubation on urinary oxalate at these pHs, without and with addition of ASC, were studied too. In normo- and hypercalciuric ICU, blood and urinary ASC from fasting patients remained unchanged, but the slope of the regression line of urinary ASC versus urinary oxalate was steeper than in the controls; the plasma ASC half-life did not differ between controls, normo- and hypercalciuric ICU; the ASC-supplemented meal caused an increase in the integrated plasma oxalate in the normocalciuric subgroup versus controls. In normo- and hypercalciuric ICU urinary oxalate, the oxalate/glycolate ratio, and calcium oxalate supersaturation were increased, but urinary glycolate was unchanged. In the controls, oral ASC did not affect calcium oxalate crystallization, while in ICU, ASC inhibited crystal growth. In control urine calcium oxalate dihydrate and calcium oxalate monohydrate develops, while in ICU urine only the former crystal type develops. In vitro oxalate neoformation from ASC did not occur. It was concluded that (1) under normal conditions an abettor role of ASC for renal stones is not recognizable, (2) in ICU, urinary oxalate excess unrelated to degradation of exogenous ASC is exhibited, and that this is most likely unrelated to an initial increase in oxalate biosynthesis, and (3) ASC appears to modulate directly calcium oxalate crystallization in ICU, although the true mode of action is still not known.

Key words Ascorbic acid · Load studies · Plasma and urinary oxalate · Urinary oxalate/glycolate · Calcium oxalate crystallization · ASC stability in urine

P. O. Schwille (✉)
University Hospital – Department of Surgery,
Maximiliansplatz 2, 91023 Erlangen, Germany

A. Schmiedl · U. Herrmann · M. Manoharan · J. Fan · V. Sharma
Mineral Metabolism and Endocrine Research Laboratory,
Departments of Surgery and Urology,
University of Erlangen, Germany

D. Gottlieb
Guest scientist from the Weizmann Institute,
Department of Biochemistry, Rehovot, Israel

Introduction

Ascorbic acid (ASC) plays a key role in the biology of humans and many animal species. For instance, apart from its involvement in the prevention of scurvy, ASC contributes substantially to the status of oxidants as well as antioxidants [8]. Ascorbic acid influences urinary oxalate via its role as an oxalate precursor [45] and its instability on storage [1, 15]. In Western civilizations, which are characterized by a sedentary lifestyle and overeating, including vitamins, resulting in obesity and pathological calcifications in arteries and kidneys, there is a need for an improved knowledge of the body's ASC status and its response to exogenous ASC.

Most kidney stones are composed of calcium oxalate, or to a lesser extent of calcium phosphate, but some are mixtures of the two. Among the factors leading to so-called idiopathic calcium urolithiasis (ICU), a disorder with high recurrence rates that puts an enormous financial strain on health care institutions, ASC-stimulated oxaluria is under discussion as a risk factor capable of initiating stone formation; the issue is, however, still controversial [1, 21, 45]. In one study, long-term and regular intake of varying amounts of ASC, i.e., up to 2 g and more per day, was not found to be associated with a higher risk for renal stones [9]; others found hyperox-

aluria when ASC intake exceeded 2 g per day [42, 43]. An ASC-related drive toward stone formation might therefore simply arise from excessive ASC intake [13, 17]; an alternative mechanism might be an inappropriate response to a fixed amount of exogenous ASC. Although, on a population basis, dietary habits in terms of quantity and quality of food do not differ between ICU patients and non-stone-forming healthy individuals [11, 38], there are reports of intrinsic abnormalities of metabolism in stone patients, for example, after intake of a standard meal, with or without addition of ASC [6, 7, 33, 38, 39]. Such findings support the view that ICU patients are maladapted to some dietary constituents. More systematic investigations into the ASC status in ICU are not available. For example, the response to ASC loading, in particular the sequence of metabolic events under these conditions in the gastrointestinal tract, possibly leading to altered composition of urine, is insufficiently known. Many investigators feel that our knowledge of the pathophysiology of ICU would greatly benefit from a better insight into the former. In particular, it is not known whether ASC taken in amounts between the recommended daily intake (approx. 70 mg) and 1 g – widely practiced as ASC supplement in food – renders the urine of a non-stone forming healthy individual prone to form stones (so-called abettor role); if this is so, it would be desirable to identify in which organ (intestine, liver, kidney) or environment (blood, urine) the abnormality arises.

Against this background the present work was conceived. The main objectives were to investigate (1) ASC in plasma and urine of fasting patients, (2) the kinetics of the disappearance from the plasma of systemically administered ASC, (3) the concomitant response of plasma and urinary ASC and oxalate, and (4) the ratio of urinary oxalate to urinary glycolate before and after the intake of an ASC-supplemented meal. Additional aims were to study calcium oxalate crystallization in postprandial urine collected after oral ASC challenge, and urinary oxalate stability after direct addition of ASC to urine. Our investigations show that ASC is not an abettor of renal stone formation in otherwise healthy individuals; in contrast, ICU is associated with ASC-independent abnormal urinary oxalate, and ASC-dependent alteration of calcium oxalate crystallization.

Materials and methods

Participants

Adult renal stone patients were diagnosed as ICU, on the basis of medical history, stone analysis, and a plain X-ray film of the kidney–ureter–bladder region documenting the formation of radiopaque stones. Systemic metabolic disorders such as overt diabetes, primary hyperparathyroidism, and others frequently associated with stones were absent. Overall, renal function was normal (serum creatinine < 1.4 mg/dl). The degree of calciuria [normocalciuria (NC); absorptive, and the combination of absorptive and fasting, hypercalciuria (synonymous idiopathic hypercalciuria; IHC)] was assessed from the calcium/creatinine ratio in urine of fasting patients (0.12 mg/mg) and post-calcium load (> 0.27 mg/mg), as appropriate, using our standardized laboratory program [38, 39]. The majority of patients were stone-free when presenting for the investigations described below. Stone analysis revealed either pure calcium oxalate (approx. 60%), or admixtures of calcium phosphate. Healthy subjects served as controls. All participants ate their usual free home diet, used no vitamin supplementation in the two antecedent weeks, and fasted overnight for 12 h before attending the laboratory.

Study protocols

Several trials were carried out to determine whether ICU and healthy subjects differ with respect to the levels of endogenous ASC, and to study the effects of exogenously administered ASC, as well as associated parameters of relevance to crystal and stone formation.

Trial one: ascorbic acid status in plasma and urine of fasting patients

A total of 69 males (30 healthy controls; 20 NC, 19 IHC patients) participated. The age range for both groups was 19–65 (mean 42) years. The body mass index [kg/(m)^2] was slightly higher in ICU than in controls.

Trial two: response to exogenous ascorbic acid in small subgroups

Since trial one showed ASC in fasting blood and urine to be largely unsuspicious, it was decided to administer ASC either intravenously or orally, but in both instances together with a meal with a known ASC content. This approach did not alter meal-mediated postprandial metabolism, but allowed us to investigate whether (1) ASC disappearance from the blood differs during the postprandial period in ICU and controls, (2) the widely practiced ASC supplementation of food leads to anomalous urine composition, especially with regard to oxalate. Since it has been reported that in ICU there is preferential intestinal uptake of supplemental ASC, ultimately leading to hyperoxaluria and renal stones [6], we monitored plasma and urinary ASC, oxalate, glycolate, and citrate, to obtain crude information on whether or not these substances differ between ICU and controls. With respect to the intravenous ASC load, it was felt that variations in oxalate, should they occur, can be interpreted on the basis of simultaneous measurement of plasma ASC and oxalate, urinary oxalate and glycolate: i.e., if oxalate did accumulate in plasma, then, could it be due to neoformation from ASC or enhanced oxalate biosynthesis as crudely reflected by urinary glycolate?

With both types of load (intravenous, oral) the extra ASC administered was 5 mg per kg bodyweight, a dosage matching the maximal intestinal ASC absorption as observed after administration of 500–600 mg, and achieving peak blood levels of 20–30 mg/l at about 3 h post-load [29].

The meal, a commercial liquid formula diet, contained 11 mg ASC but no oxalate, and it carried an acid load (120 milliequivalent titratable protons after prior ashing in mineral acid); details on its content of other nutrients and energy supply have been described elsewhere [39]. After prior collection of urine from 2-h fasting patients, the meal was taken in 300 ml demineralized water, and urine was collected over 3 h thereafter. Before and during the loads, samples of venous forearm blood were drawn into chilled heparinized tubes (15 for intravenous ASC; seven for oral ASC). Six controls (three males, three females, mean age 40 years), six NC (four males, two females, mean age 39), and six IHC patients (five males, one female, mean age 39) were recruited for the intravenous ASC load, another six controls (four males, two females; mean age 28), seven NC (males; mean age 35), and seven IHC patients (males; mean age 37, of which five met the criteria of absorptive hypercalciuria) for the oral ASC load. Plasma was obtained immediately. One aliquot (300 µl) was mixed with 100 µl of 10% (w/v) metaphosphoric acid (to prevent ASC degradation) and used for the determination of total ASC; another aliquot (0.5 ml) was immediately shock-frozen in liquid nitrogen for analysis of oxalate

and citrate; all samples, freshly voided and then shock-frozen urine included, were stored at −80 °C.

Trial three: ascorbic acid effects on calcium oxalate crystallization

Details of the crystallization test procedure have been reported [10, 34], and also a brief description has been given [40]. The test permits the light-microscopic determination of calcium oxalate nucleation (in terms of the metastable limit of calcium oxalate solubility, so-called tolerable oxalate concentration), calcium oxalate crystal growth and agglomeration time.

The focus of the trial was on the effects of ASC supplementation of the meal (see trial 2) versus the effects of the meal alone. A total of 33 males participated [15 controls (meal alone, $n = 8$; meal + ASC, $n = 7$); 18 ICU patients (meal alone, $n = 9$; meal + ASC, $n = 9$)]. The two subgroups (meal alone, meal with ASC supplementation) were matched for age and body mass index. For ICU the metabolic activity of stone disease (for definition see reference 40) was kept comparable. In addition, factors capable of promoting or inhibiting crystallization and stone formation were considered, such as urinary pH and volume – the latter because it influences crystallization inhibitors in non-linear fashion [12] – albumin, non-albumin protein, calcium, oxalate, phosphate, magnesium, citrate, calcium/citrate ratio, and calcium oxalate supersaturation.

In addition, the freshly voided urine of one male control subject and one male ICU patient, both showing a similar ASC concentration, was studied after ASC was added to give a total concentration of 4 mmol/l; the pH was stabilized (by adding microliter amounts of 6 M HCl or NaOH) at 5.0, 6.0 and 7.0, respectively; thereafter urine was processed for calcium oxalate crystallization as described elsewhere [10, 34], except that the tube with unequivocal calcium oxalate precipitation was incubated for 120 (instead of 90) min. The urine was suctioned through a 0.22 μm filter (Type GS, Millipore, Bedford, Mass. USA), the filter rinsed with bidistilled water, air-dried, and kept dry for the study of crystal type and composition by scanning electron microscopy (SEM), and energy-dispersive X-ray analysis (EDX) of elements. SEM was performed in the usual manner on identical segments of each filter, using carbon cover of the sample.

Trial four: Stability of ascorbic acid

In another series of the urines from the two individuals studied for calcium oxalate crystallization (see trial 3), crystallization was not induced; instead, the urine was incubated at 37 °C over 60, 90, or 120 min, at pH 5, 6, or 7, without and with addition of ASC in an amount to give 4 mM; thereafter oxalate was measured.

Chemical analyses

The ASC taken orally, the ASC added to urine in vitro, and all other chemicals used were of analytical grade (Fluka, Buchs; Switzerland). Ascorbic acid administered intravenously was obtained from Merck, Darmstadt, Germany. Standard equipment was used for SEM (Jeol, JSM 840, operated at 25 kV) and EDX (EDAX, type SW 1900) for analysis of crystals. Ascorbic acid in urine and plasma was determined by high performance liquid chromatography [24]; oxalate and glycolate in urine, oxalate in plasma ultrafiltrate, were all determined by high performance ion chromatography [25, 40], total protein in urine by the Lowry method [22], and urinary albumin by immuno-nephelometry. All other analyses used routine or other well-documented methodologies [10, 34, 38, 39].

Calculations, statistics

Urinary ASC clearance was calculated using the standard formula, the post-load cumulative response of plasma ASC, oxalate and citrate by summing differences (post-test meal load value minus baseline), relative calcium oxalate supersaturation in urine (with calcium oxalate solubility in water set as 0) from the activity products [28], net gastrointestinal alkali absorption by the method of Oh [30], correlation coefficients by bivariate regression analysis. The ASC clearance half-life from plasma was extrapolated from the curve with the best fit. Data were given as mean values and standard error, if not otherwise indicated; the significance ($P \leq 0.05$) of differences between groups was tested using the *t*- or U-test for unpaired and paired observations, as appropriate. The software STATISTICA (Stat-Soft, Tulsa; USA) was used.

Results

Ascorbic acid under fasting conditions

Table 1 shows that in male controls and male ICU patients fasting plasma ASC varied between 2.9 and 17.9 mg/l (controls, ICU); in recent reports the order of magnitude of ASC in plasma of healthy normals was from 5 to 30 mg/l [4, 18, 19, 20, 25]. In another report somewhat higher plasma ASC was found to be characteristic for females [31]. On plotting the individual ASC values, about 20–30% of the patients in the IHC subgroup of ICU were below the lower limit in the controls (data not shown).

For fasting individuals, the urinary ASC excretion rate and the mean ASC clearance were higher in NC than in IHC, but overall (NC + IHC) ASC in stone patients did not statistically differ from controls (Table 1). Urinary oxalate excretion in fasting individuals (Table 1) varied between 1 and < 7 mg per 2 h (for

Table 1 ASC concentration in fasting plasma, ASC and oxalate excretion rate in urine from 2-h fasting patients, and ASC clearance in male patients with idiopathic calcium urolithiasis (*ICU*), classified by calciuria (*NC*, *IHC*), and in healthy males (*Controls*). Data are mean values (range). $^{*}P < 0.05$, $^{**}0.05 < P < 0.10$, versus IHC

	Controls		ICU					
	Mean	Range	NC		IHC		NC + IHC	
			Mean	Range	Mean	Range	Mean	Range
Number	30		20		19		39	
ASC in plasma (mg/l)	10.9	(6.6–16.7)	12.1	(6.1–16.6)	10.3	(2.9–17.9)	11.2	(2.9–17.9)
ASC in urine (mg)	0.67	(0.07–16.1)	1.5*	(0.2–18)	0.67	(0.07–18)	1.0	(0.07–18)
ASC clearance (ml/min)	0.69	(0.05–8.0)	0.97**	(0.2–12.8)	0.63	(0.1–10.2)	0.75	(0.1–12.8)
Oxalate in urine (mg)	1.8	(1.3–2.4)	1.9*	(1.3–6.3)	2.1	(1.3–4.7)	2.0	(1.3–6.3)

controls and ICU). Urinary ASC and oxalate in urine from fasting patients correlated directly (controls, $r = 0.58$, $P < 0.01$; NC, $r = 0.71$, $P < 0.001$; IHC, $r = 0.42$, $P < 0.07$). On pooling of NC and IHC the slope of the regression line differed significantly from that of controls ($P < 0.05$). Thus, at any given level of urinary ASC in fasting individuals, the accompanying urinary oxalate is higher in males with ICU and unclassified for calciuria than in male controls. Further insight into the possible origin of the oxalate excess in urine of ICU was therefore deemed necessary and was expected from studies on the response to exogenous ASC (see below).

Ascorbic acid loading tests

Intravenous ascorbic acid

Figure 1 shows that when ASC administration was preceded by the test meal – both loads together achieving a total ASC supply of 300–410 mg – this combined challenge was followed by uniform ASC clearance from the plasma. Neither NC nor IHC patients differed substantially from the controls; the fitted curves obeyed first order kinetics ($y = a \cdot x^b$, $r = 0.99$, $P < 0.01$; Fig. 1). The mean peak values observed at 6 min post-injection varied between 60 and 75 mg/l (complete data and statistics are available on request). During the early post-load phase, the mean plasma ASC was higher in NC and IHC. This phenomenon may be attributed to the predominance of males among the NC and IHC participants, contrasting with the reportedly higher values of females [31]. Furthermore, as the transmembrane exchange of both ASC and dehydroascorbic acid in leukocytes occurs within a few minutes and is considered a superior indicator of the overall ASC status [19, 46], the largely unaltered ASC clearance from plasma (Fig. 1) corroborates the assumption that in middle-aged ICU as a whole (NC + IHC), as studied here, ASC storage within blood cells is undisturbed.

The early post-load plasma oxalate levels in the three groups were slightly higher than baseline, but the post-load cumulative response differed only insignificantly [NC −0.02 (SE 0.27); IHC −0.18 (0.29); controls −0.23 (0.15), all μmol/l; $P = 0.22$ (NC vs. controls), and $P = 0.62$ (IHC vs. controls)]. Fasting and post-ASC load urinary excretion of ASC, oxalate and glycolate were also statistically indistinguishable. The mean post-load excretion for ASC oxalate and glycolate is given in Table 2. For further details on urinary oxalate and glycolate see below.

Oral ascorbic acid

Figure 2, upper half, illustrates the effect of the ASC-supplemented oxalate-free formula meal. In plasma, the ASC peak values of approximately 20 mg/l were developed at timepoint 180 min post-load. There were no statistical differences between the groups at any of the timepoints studied, and the cumulative response of ASC was unchanged. Other investigators have observed similar plasma ASC peaks 3 h following oral intake of 0.5 g

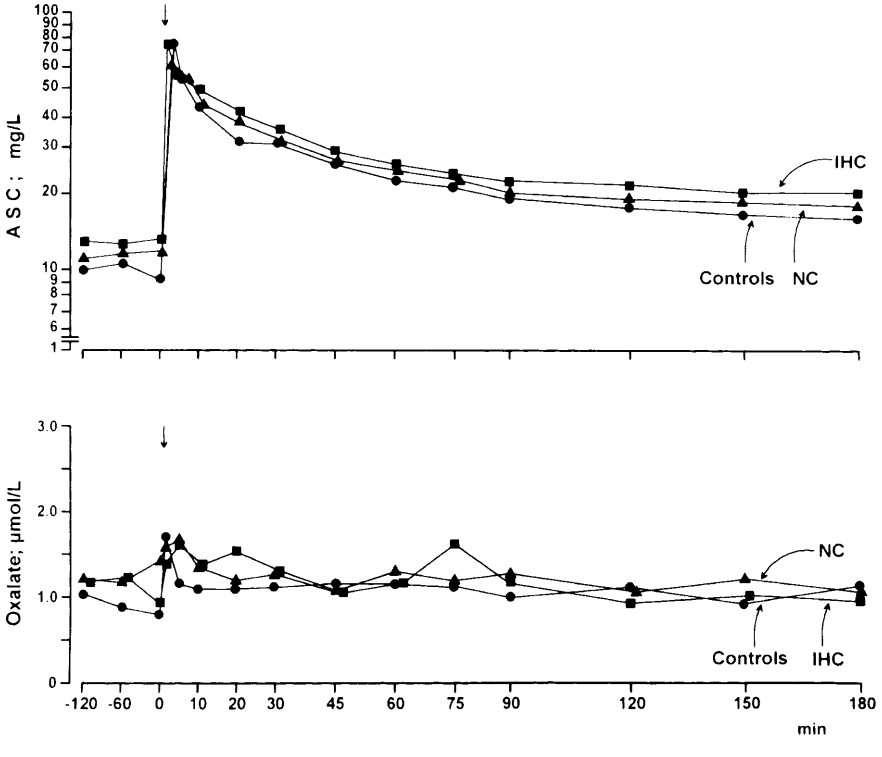

Fig. 1 Effect of a test meal taken at timepoint zero (↓) and followed by intravenous injection of ascorbic acid (ASC) (5 mg per kg bodyweight) on the mean response of plasma ASC and oxalate in healthy controls (●) and ICU patients with either normocalciuria (▲) or absorptive hypercalciuria (■). The number of participants per group was six. Note the logarithmic Y-scale for ASC

ASC, thereafter the values declined [29]. It is therefore unlikely that, in the present work, had observation time been prolonged, even higher ASC peaks would have emerged beyond 180 min. Oxalate concentration in plasma of controls and IHC varied only minimally.

Table 2 The mean post-load excretion for ascorbic acid, oxalate and glycolate. For each value the SE is given in brackets

	NC	IHC	Controls
ASC (mg)	250 (24)	250 (13)	220 (15)
Oxalate (μmol)	62 (6)	64 (4)	48 (8)
Glycolate (μmol)	81 (15)	116 (18)	89 (18)

However, in NC the mean baseline value was lower than in the former two groups, and in the post-load period of NC there was a trend toward higher oxalate, resulting in a significantly higher cumulative oxalate response than in controls. Plasma citrate was in the range 2.0–2.5 mg/dl throughout – i.e., initially, during, and at the end of the post-load period; statistical differences were not recognizable (data not shown).

Figure 2, lower half, illustrates the situation in the 3 h postprandial urine. In NC and IHC there was a lower median ASC but a higher median oxalate excretion than in controls; in IHC, oxalate was significantly increased, while in NC it approached the level of significance versus controls. Citrate was unchanged (me-

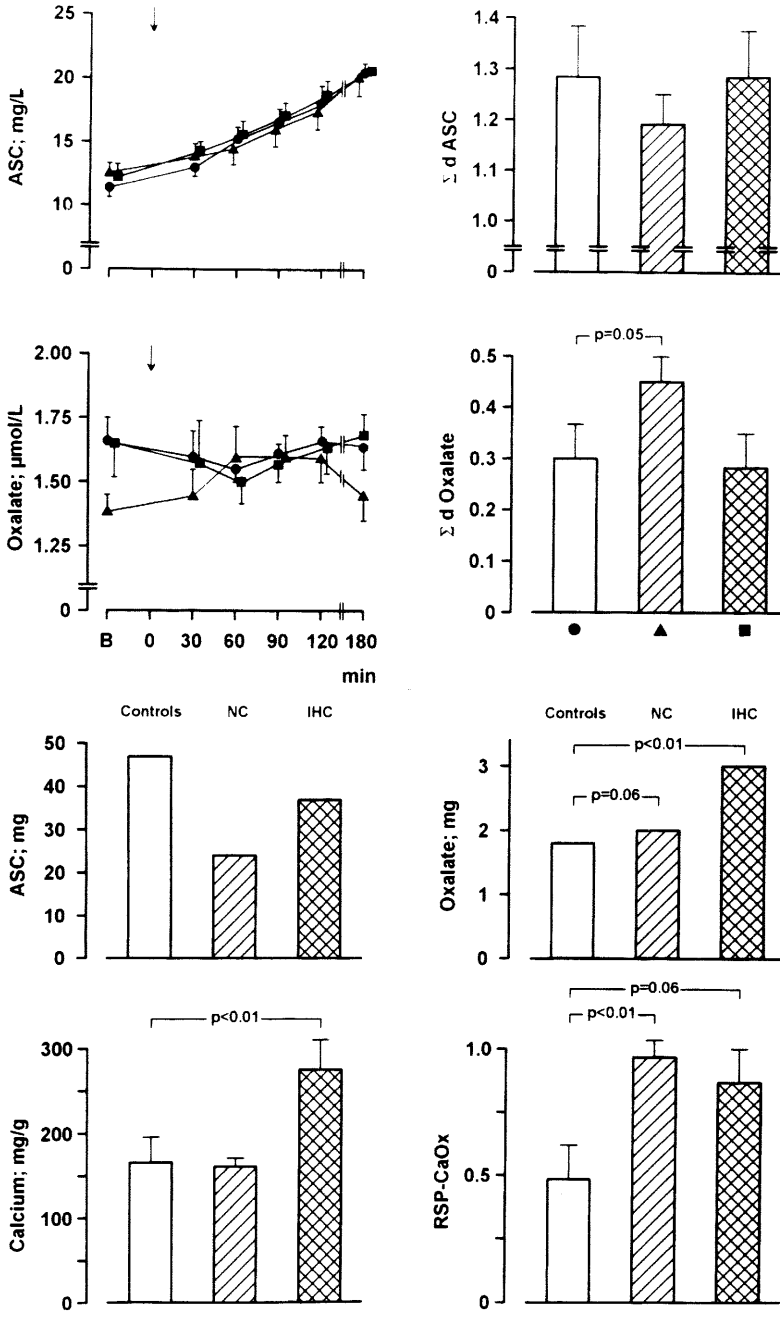

Fig. 2 Effect of ASC supplementation (5 mg per kg bodyweight) of a test meal taken at timepoint zero (↓) on the response of ASC in plasma and urine of controls (●, $n = 6$), and ICU patients presenting with either normocalciuria (▲, NC; $n = 6$) or idiopathic hypercalciuria (■, IHC; $n = 7$). *Upper half*: *Left* profile of ASC and oxalate in plasma (*B* mean of values at timepoints −120 and −60 min). *Right*: plasma integrated ASC and oxalate, given as sum of differences ($\sum$ d) from B, after prior $\log_{10}$ transformation. Mean values and standard error. *Lower half*: excretion rate in 3-h urine. ASC and oxalate are given as medians; calcium (factorized for the accompanying creatinine) and the relative supersaturation product (*RSP*) of CaOx are given as means and standard error

dian values were 57, 57, 52 mg, in controls, NC, and IHC, respectively). Calciuria was normal in NC, but increased in IHC. In ICU as a whole (NC + IHC) the average calcium oxalate supersaturation was higher than in the controls, but the accompanying urine volume was lower (data not shown) and therefore should explain at least in part that calcium oxalate metastability was at the upper limit, by definition constituting a risk factor for stones. The decline of urinary pH was comparable in the three groups (mean values were 5.4 – 5.7), precluding the possibility that in NC and IHC the kidneys are unable to excrete the protons derived from the acid meal load, irrespective of whether these stem from the meal itself or the metabolic degradation of supplementary ASC.

Like ASC and citrate in postprandial urine (see above), their excretion varied widely in urine of fasting individuals (data not shown), while oxalate was more homogeneous. For the three substances, the median (range) of postprandial changes from the fasting state (calculated on a per hour basis) are given in Table 3. The accompanying median postprandial change from the fasting state of net gastrointestinal alkali absorption in NC, IHC and controls was 1.7, −2.4, and 2.5 milliequivalent, respectively. All these data (ASC, citrate, oxalate, alkali absorption) were statistically indistinguishable; it is worth noting that although the citrate response shown by stone patients appeared low, their ASC response was not higher than that of the controls.

Figure 3 shows that the molar ratio of urinary oxalate to urinary glycolate differed significantly [0.43 (SE 0.03), 0.54 (0.05), 0.34 (0.06), in NC, IHC and controls, respectively], irrespective of whether the urine was taken from a fasting individual or it was ASC-richer postprandial urine. However, the underlying urinary glycolate was statistically indistinguishable [mean values, in μmol, 62 (NC), 65 (IHC), 81 (controls)]. Elevated urinary glycolate was postulated to be characteristic of those situations where hyperoxaluria resulted from oxalate overproduction [27, 44]; this made it unlikely that in the present work where urinary glycolate remained unchanged, oxalate biosynthesis was increased.

Ascorbic acid effects on calcium oxalate crystallization

Table 4 shows the data obtained from the thirty-three participants studied. Since the urinary calcium oxalate supersaturation in postprandial urine of NC and IHC was similarly high (Fig. 2), categorization of ICU into these subgroups was abandoned. Overall ICU as a whole and controls were almost ideally matched for age and body mass index; the metabolic activity was kept comparable in ICU receiving either the meal and supplementary ASC or the meal alone. Irrespective of whether additional ASC was taken with the meal, calcium oxalate crystallization of controls was virtually unchanged. This holds true for tolerable oxalate, crystal diameter, and agglomeration time. If there was any change at all, it was a slight tendency toward smaller crystals. In contrast, in ICU patients not taking supplementary ASC, not only was the long known average higher calcium excretion noted but also a smaller urinary volume, and a higher urinary concentration of calcium, oxalate, magnesium, phosphorus, albumin and non-albumin protein. In addition, there was a higher calcium/citrate ratio and the tolerable oxalate concentration was low versus controls. In ICU patients taking the ASC-supplemented meal, tolerable oxalate concentration was lower than in those not taking ASC; despite this, the crystal diameter was significantly reduced and the changes in agglomeration time and crystal growth rate were statistically insignificant. The borderline stimulation of calcium oxalate nucleation and inhibition of calcium oxalate crystal growth, obviously ASC-mediated, were not accompanied by differences in pH, calcium oxalate supersaturation or concentration of citrate and protein(s). Assessment of crystal number, although feasible with the crystallization test in use [10, 34], was unfortunately not undertaken.

In urine of the control and the ICU individual, in which the concentrations of ASC, citrate, oxalate, calcium, and the calcium oxalate supersaturation were close to the respective group means shown in Table 4, and in which crystallization was induced at pH 5, 6 and 7, with or without addition of ASC (4 mmol/l), SEM revealed the following: at pH 5 and 6 calcium oxalate monohydrate was the dominant crystal phase in urine of the control individual. At pH 7 calcium oxalate dihydrate was exclusively present in this urine. Conversely, when the stone patient's urine was at pH 5, calcium oxalate dihydrate was present, and this crystal type persisted up to pH 7. Once ASC was added to the control urine at the pre-set pH, number, size and agglomeration of crystals were not affected, whereas the same procedure in the patient's urine resulted in increased crystal number, but crystal size and agglomeration appeared unchanged. Scanning electron microscopy and EDX, subsequently applied to the calcium oxalate crystals, failed to detect any calcium phosphate phase and a phosphorus peak, respectively, but EDX showed the two calcium-specific energy peaks (k_α, k_β) that are regularly seen when analyzing CaOx crystals using this technique (data not shown).

Table 3 The median (range) of postprandial changes from the fasting state (calculated on a per hour basis)

	NC	IHC	Controls
ASC (mg)	22 (2; 58)	33 (–2; 70)	41 (5; 63)
Oxalate	−0.5 (−2; 0),	−0.5 (−2; 1),	−0.5 (−1; 0)
Citrate (mg)	−15 (−111; 48),	−12 (−61; 36),	3 (78; 50)

Ascorbic acid stability and urinary oxalate

Neoformation of oxalate in urine, from precursors also present in urine, in particular ASC, has been reported

[1]. Theoretically, therefore, the bulk of calcium oxalate crystal mass formed in fresh urine, especially of stone patients, could stem from newly formed oxalate or alternatively from oxalate present initially in the urine. This question was more specifically addressed, using aliquots of urine from the control individual and the stone patient mentioned in trial 3 (see above, last section). Figure 4 shows that in both individuals oxalate tends to increase with increase in pH of the urine. However, oxalate remains uninfluenced by the addition

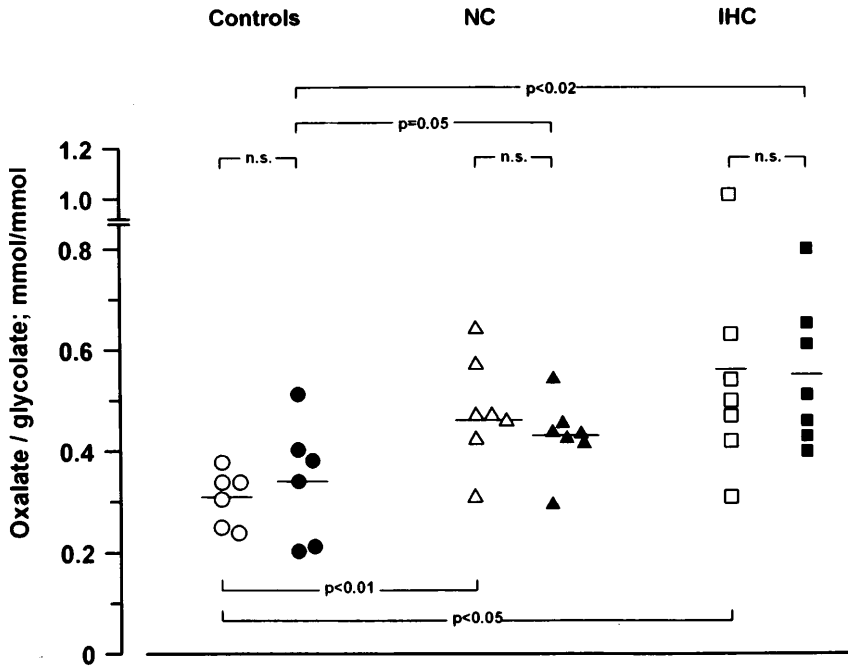

Fig. 3 Molar urinary ratio oxalate/glycolate: mean value; *open symbols* fasting urine, *solid symbols* post-ASC load urine

Table 4 Data from the participants of trial three; includes general features, urinary volume and other parameters, and CaOx crystallization, all determined in postprandial urine collected after ingestion of a meal with ASC supplementation (Meal + ASC) and without (Meal alone). Mean values (SE). (* total protein minus albumin, *N* at nucleation, *30* at 30 min post-nucleation, Δ value at 30 min post-nucleation minus value at nucleation)

	Controls			ICU patients		
	Meal alone $n = 8$	Meal + ASC $n = 7$	*P*-value	Meal alone $n = 9$	Meal + ASC $n = 9$	*P*-value
General features						
Age (years)	33 (2)	32 (2)	0.77	37 (4)	34 (3)	0.48
Body mass index [kg/(m)^2]	23.6 (1.2)	23.8 (1.3)	0.91	26.1 (1.7)	23.5 (0.7)	0.17
Metabolic activity (score)				22 (2)	20 (3)	0.76
Variables in urine						
Volume (ml)	347 (54)	430 (75)	0.38	237 (42)	237 (39)	1.0
pH	5.74 (0.19)	5.37 (0.13)	0.14	5.74 (0.14)	5.68 (0.21)	0.81
Oxalate (mmol/l)	0.08 (0.02)	0.09 (0.02)	0.87	0.16 (0.04)	0.17 (0.03)	0.91
Calcium (mmol/l)	2.73 (0.58)	2.52 (0.99)	0.67	5.37 (1.09)	5.12 (0.66)	0.85
Magnesium (mmol/l)	2.46 (0.41)	2.41 (0.75)	0.60	4.10 (0.52)	3.45 (0.62)	0.44
Citrate (mmol/l)	1.98 (0.74)	2.73 (1.72)	0.60	1.93 (0.44)	1.79 (0.39)	0.82
RSP-CaOx	0.29 (0.14)	0.26 (0.15)	0.90	0.71 (0.09)	0.72 (0.11)	0.94
Calcium/citrate (mmol/mmol)	1.76 (0.36)	1.44 (0.21)	0.83	3.28 (0.52)	3.57 (0.50)	0.70
Phosphorus (mmol/l)	9.3 (2.3)	9.6 (2.1)	0.42	12 (2.5)	14.1 (2.8)	0.55
Albumin (mg/l)	2.0 (0.7)	4.9 (3.8)	1.0	5.5 (3.5)	2.3 (0.6)	0.54
Non-albumin protein* (mg/l)	13 (2)	14 (6)	0.83	20 (5)	16 (2)	0.81
CaOx crystallization						
Tolerable oxalate (mmol/l)	0.59 (0.08)	0.59 (0.09)	0.96	0.49 (0.03)	0.40 (0.03)	0.06
Crystal diameter(N) (μm)	3.2 (0.2)	3.2 (0.2)	0.98	3.8 (0.2)	2.8 (0.2)	0.008
Crystal diameter(30) (μm)	6.2 (0.7)	6.0 (0.5)	0.84	9.2 (0.7)	6.4 (0.5)	0.004
Δ Crystal diameter (μm)	3.0 (0.5)	2.9 (0.5)	0.81	5.4 (0.7)	3.5 (0.3)	0.028
Agglomeration time (min)	34 (4)	32 (2)	0.66	33 (4)	30 (0)	0.44
Crystal growth rate (μm/min)	0.10 (0.03)	0.09 (0.02)	0.72	0.18 (0.04)	0.12 (0.01)	0.14

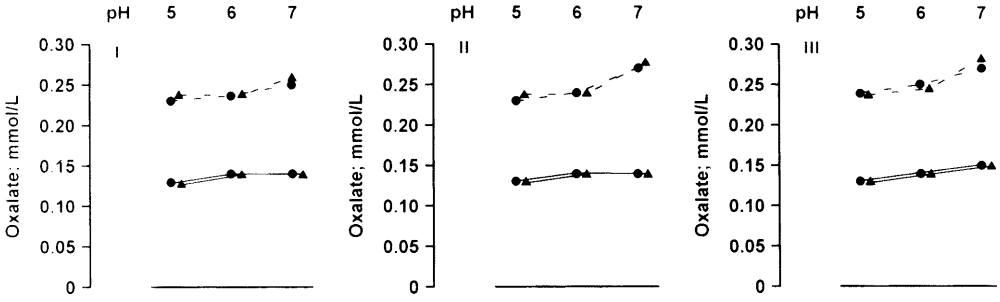

Fig. 4 Effects of urine with a pH of 5, 6 or 7, prolongation of incubation time (I, II, III: 60, 90, 120 min), and added excess of ASC on oxalate concentration in uncrystallized urine: non-stone-forming urine [*lines*, without (●) and with (▲) 4 mmol/l ASC]; stone-forming urine [*broken lines*, without (●) and with (▲) 4 mmol/l ASC]

of ASC, and also by the duration of incubation, mimicking the duration (120 min) of the crystallization test in use.

Discussion

Ascorbic acid and the risk for ICU

Work done by others shows that the stone risk in men is not increased by ASC supplementation of food over the long-term [9]. Collectively, our data are in agreement with this observation and another made more recently [2]. Also, the data on plasma and urinary ASC presented here do not permit the conclusion that in males with ICU the metabolism of ASC is malregulated, either at the level of the intestine, or the kidney. Nor is there any evidence of an increase in the conversion of exogenously (intravenous, oral) administered ASC into oxalate, either in the blood or in the urine at a pH between 5 and 7. Conversely, ICU patients may be afflicted by an etiologically as yet unidentified intrinsic disorder that is responsible for a permanent ASC-independent excess of urinary and, to some extent, of plasma oxalate. The former, together with higher-than-normal urinary calcium, may lead to the well-known pre-existent higher degree of calcium oxalate supersaturation in urine of ICU patients. In previous work of ours on ICU, an excess of oxalate in postprandial urine produced after a similar oxalate- and glycolate-free test meal as in the present study was reported [36]. This also demonstrated that the accompanying intestinal absorption of ^{14}C-oxalate was low and therefore cannot have accounted for the higher oxaluria [36]. The high urinary oxalate/glycolate ratio may therefore be a key to the further understanding of the pathophysiology of this disorder (see also below).

Ascorbic acid in plasma and urine

Because of the pilot character of the present study the number of participants, from whom data were included in Table 1, was too small to be broken down into categories taking different amounts of ASC with their usual diet – smokers and non-smokers [23], younger [26] and older individuals. This shortcoming also made it impossible to decide whether plasma ASC, when in the lower range, was also related to such factors as ASC in cells or tissues, that possibly influence ASC in plasma from fasting individuals. For example, in younger male ICU patients, with a mean age of less than 30 years, the ASC content of red blood cells was found to be lower than in controls of a similar age, while in plasma no difference in levels of ASC could be detected [26]. Therefore in ICU, ASC apparently is controlled by additional factors. The urinary elimination of ASC has been judged useless in the evaluation of the overall ASC status [18]. This view is supported by the present data in Table 1, showing that the ASC excretion rate in urine of fasting controls and ICU varies enormously (note that some of the extremes in Table 1 differ by a factor >200). Overall [controls, stone patients (NC + IHC)], ASC reabsorption by renal tubuli during fasting appears high; this observation contrasts with another, postulating that in normals of either sex there is a low renal ASC threshold as assessed from intravenous ASC loading, and that the low threshold prevents plasma ASC from rising above about 15 mg/l [19]. Our data appear to show that, if renal handling of ASC is altered among males with ICU, the finding may be restricted to the NC subgroup in this. ASC excretion rate and the mean ASC clearance were significantly higher than in IHC (Table 1).

Ascorbic acid, oxalate, glycolate

The eating of an ASC-supplemented meal by ICU patients has no effect on calciuria, and rarely on the provision of alkali from the intestinal tract, but it does reveal abnormal oxalate. The true cause of the excess oxalate in postprandial plasma (NC), and both urine from fasting individuals and postprandial urine (NC, IHC), is unknown. Hyperoxaluria of intestinal origin is characterized by a high urinary oxalate/glycolate ratio, due mainly to high oxalate rather than low glycolate [3, 27, 44]. In our work the significant increase in the oxalate/glycolate ratio in NC and IHC versus controls, observed in both fasting and postprandial urine (Fig. 3) – the latter urine having been collected after ingestion of the oxalate- and glycolate-free but ASC-rich meal – is a strong argument against intestinal oxalate

hyperabsorption. This also supports the theory that in the presence of normal glycolate hepatic oxalate biosynthesis is not increased. It is postulated that the post-load rise in plasma oxalate in NC, accompanied by only marginally higher urinary oxalate (Fig. 2), indicates oxalate self-regulation by the kidney; in analogy, the situation in IHC might reflect that normal plasma oxalate (Fig. 2) but high urinary oxalate are sequelae of altered renal oxalate transport (secretion, reabsorption). Although renally mediated disordered oxalate homeostasis in ICU has not been systematically considered so far, its existence would mean that oxalate may not be simply a waste product of metabolism but instead serves some physiological function. This interpretation of plasma and urinary oxalate extends, but is at variance with that of Cowley et al. [6, 7]. Using a similar protocol for ASC load, these authors ascribed the urinary oxalate excess in ICU to intestinal hyperabsorption of ASC, some of which is then degraded to oxalate.

Ascorbic acid and calcium oxalate crystallization

In animal experiments where there was feeding of ASC over the long-term, increased renal tissue calcifications were observed, but the underlying mechanisms remained unclear [41]. Others, collecting carefully protected urine to prevent falsely high oxalate from the instability of ASC, failed to show a significant increase in urinary oxalate and indices of calcium oxalate supersaturation of urine [2]. Also in the present work, with examination of patients under rigidly controlled conditions during the fasting and post-test meal load on the day in the laboratory, definitive ASC stimulation of urinary calcium oxalate supersaturation and calcium oxalate crystallization in postprandial urine of controls and stone patients could not be observed (Table 4). In our calcium oxalate crystallization test, crystal diameter (synonym crystal growth) and crystal agglomeration time are inversely related [10, 35]. Therefore, the observation that after ASC loading in ICU crystal growth is diminished in the presence of unchanged crystal agglomeration time, is interpreted to mean that this parameter is unrelated to crystal diameter, but rather to crystal number. We reported that in urine from fasting individuals and postprandial urine of ICU there is a trend toward enhanced spontaneous crystalluria, i.e., a greater number of calcium phosphate, not of calcium oxalate crystals [16, 34]. Furthermore, within the hydration shell of crystals, a number of inorganic and organic anions can substitute each other [5]. These findings suggest that some phosphorus may be detectable in calcium oxalate crystals, particularly since phosphorus is regularly found when calcium oxalate crystals generated by the procedure described here are dissolved and wet-chemically analyzed (P. O. Schwille, manuscript in preparation). However, in the present work, using SEM and EDX examination of one ICU patient and one control individual, we cannot infer that phosphorus plays a role. In addition, ASC appears unable to modulate calcium oxalate crystallization in a way triggering the formation of calcium-containing stones (via enhanced crystal growth and shortening of agglomeration time). Conversely, calcium oxalate phase transformation (from monohydrate to dihydrate) was detected in urine of the control individual, provided the urine was less acidic and rich in ASC. Differences in urinary pH have been reported as underlying calcium oxalate monohydrate and calcium oxalate dihydrate as contained in calcium oxalate stones [32]. Therefore, it cannot be ruled out that increasingly deprotonated ASC is able to influence calcium oxalate crystallization directly, i.e., beyond the level of calcium oxalate supersaturation of urine. This could occur at the surface of calcium oxalate crystals or the level of the crystal hydration shell, thereby affecting crystal hydration and shape.

Ascorbic acid stability and oxalate

We showed previously that upon addition of metaphosphoric acid to plasma and shock-freezing of freshly voided urine ASC degradation to dehydro-ascorbic acid and oxalate can be prevented [25]. Until now it has been uncertain whether in undiluted urine, with the pH varying from <5 to 7, there is oxalate neoformation during the calcium oxalate crystallization test. Such a possibility can be ruled out (Fig. 4), although results from a larger series of study participants would be desirable. Since addition of ASC to urine, in amounts up to 1 g, the concentrations frequently seen upon ingestion of ASC did not lead to more oxalate, we cannot plausibly explain why the urinary oxalate/glycolate ratio is high in ICU vs controls (Fig. 3). Earlier observations showed that ASC is only one of numerous di- or tricarboxylic acids present in urine. All of these are to some degree prone to degradation to oxalate on incubation at 37 °C [1]. Such a mechanism might have contributed to the initially higher oxalate in the urine of the stone patient as depicted in Fig. 4, to the higher oxalate in the post-ASC load urine of NC and IHC stone patients (Fig. 2, lower half), and to the steeper slope of oxalate when regressed for ASC in urine of fasting ICU patients. In an unpublished series of experiments in our laboratory, comprising more than 200 male ICU patients not receiving ASC supplementation of the oxalate-free test meal, there is a similarly high oxalate/glycolate ratio both in fasting and postprandial urine, but roughly normal oxalate in fasting plasma. Therefore, our present observations, if confirmed by independent laboratories, would indicate that in ICU patients there may exist a pool of yet unidentified non-ASC organic acid(s) [1] or other mechanisms, capable of modulating oxalate in urine and, within limits, plasma.

Conclusions

Although ASC is essential in the human diet for prevention of scurvy, not all its biological effects are identified as yet. The question is whether ASC can act as a poison, prophylactic, or panacea [14]. While recent articles on ASC as a possible stimulant of the risk for calcium oxalate urinary tract stones in healthy men document that such a role is unlikely [2, 9], our present observation that ASC can directly interfere with calcium oxalate crystallization in whole urine of ICU patients indicates that future investigations in this area are worth doing; these should include the use of superior tools for examining the various stages of crystallization and long-term studies in non-stone-forming and stone-forming individuals.

Acknowledgements We are grateful to the numerous practicing urologists for giving us the opportunity to cooperate in the care for kidney stone patients. We also owe special thanks to our coworkers G. Hoffmann, B. Schreiber, K. Schwille for technical assistance, and I. Goldberg for secretarial work. This work was supported by the W. Sander Foundation for the Advancement of Medical Sciences, Munich, Germany, the University of Erlangen Hospital Research Fund, Erlangen, Germany, and the Deutsche Forschungsgemeinschaft, Bonn, Germany. The leading author (POS) dedicates this article to the late Professor Gerd Hegemann, former chair of the Department of Surgery of the University of Erlangen, in commemoration of his strong support during the development of a Division of Research at this academic hospital.

References

1. Asper R, Bigler M, Georgi U, Schmucki O (1987) In vitro-Oxalatbildung im Urin. Fortschr Urol Nephrol 25: 152
2. Auer BL, Auer D, Rodgers AL (1998) The effect of ascorbic acid ingestion on the biochemical and physico-chemical risk factors associated with calcium oxalate kidney stone formation. Clin Chem Lab Med 36: 143
3. Barratt TM, Kasidas GP, Murdoch I, Rose GA (1991) Urinary oxalate and glycolate excretion and plasma oxalate concentration. Arch Dis Childhood 66: 501
4. Benzie IFF, Strain JJ (1997) Acute post-ingestion changes in plasma ascorbic acid concentration: relationship to dose and to existing body stores. Nutr Res 17: 187
5. Brown CM, Purich DL (1992) Physical-chemical processes in kidney stone formation. In: Coe FL, Favus MJ (eds) Disorders of bone and mineral metabolism. Raven, New York, p 613
6. Chalmers AH, Cowley DM, Brown JM (1986) A possible etiological role for ascorbate in calculi formation. Clin Chem 32: 333
7. Cowley DM, McWhinney BC, Brown JM, Chalmers AH (1987) Chemical factors important to calcium nephrolithiasis: evidence for impaired hydroxycaboxylic acid absorption causing hyperoxaluria. Clin Chem 33: 243
8. Crawford RD (1995) Proposed role for a combination of citric acid and ascorbic acid in the production of dietary iron overload: a fundamental cause of disease. Biochem Mol Med 54: 1
9. Curhan GC, Willett WC, Rimm EB, Stampfer MJ (1996) A prospective study of the intake of vitamins C and B6, and the risk of kidney stones in men. J Urol 155: 1847
10. Fan J, Schwille PO, Manoharan M, Gottlieb D, Schmiedl A (1995) Calcium oxalate nucleation growth and aggregation in undiluted urine of healthy controls and patients with idiopathic recurrent calcium urolithiasis evaluated by a one-time small-volume test procedure – effects of alkali citrate. Clin Lab 41: 139
11. Fellström B, Danielson BG, Karlström B, Lithell H, Ljunghall S, Vessby B (1989) Dietary habits in renal stone patients compared with healthy subjects. Br J Urol 63: 575
12. Fleisch H (1980) Mechanisms of stone formation: role of promoters and inhibitors. Scand J Urol Nephrol Suppl 53: 53
13. Griffith HM, O'Shea B, Maguire M, Keogh B, Kevany JP (1986) A case-control study of dietary intake of renal stone patients. II. Urine biochemistry and stone analysis. Urol Res 14: 75
14. Halliwell B (1999) Vitamin C: poison, prophylactic or panacea? Trends Biochem Sci 24: 255
15. Halliwell B, Gutteridge JMC (1989) Free radicals in biology and medicine. Oxford University Press, Oxford
16. Herrmann U, Schwille PO (1992) Crystalluria in idiopathic recurrent calcium urolithiasis – dependence on stone composition. Urol Res 20: 157
17. Hughes C, Dutton S (1981) High intakes of ascorbic acid and urinary oxalate. J Hum Nutr 35: 274
18. Jacob RA, Skala JH, Omaye TS (1987) Biochemical indices of human vitamin C status. Am J Clin Nutr 46: 818
19. Lee W, Davis KA, Rettmer RL, Labbe RF (1988) Ascorbic acid status: biochemical and clinical considerations. Am J Clin Nutr 48: 286
20. Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, Graumlich JF, King J, Cantilena LR (1996) Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. Proc Natl Acad Sci USA 93: 3704
21. Liebman M, Chai W, Harvey E, Boenisch L (1997) Effect of supplemental ascorbate and orange juice on urinary oxalate. Nutr Res 17: 415
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265
23. Lykkesfeldt J, Loft S, Nielsen JB, Poulsen HE (1997) Ascorbic acid and dehydroascorbic acid as biomarkers of oxidative stress caused by smoking. Am J Clin Nutr 65: 959
24. Manoharan M, Schwille PO (1994) Measurement of ascorbic acid in human plasma and urine by high performance liquid chromatography. Results in healthy subjects and patients with idiopathic calcium urolithiasis. J Chromatogr B 654: 1334
25. Manoharan M, Schwille PO (1997) Measurement of oxalate in human plasma ultrafiltrate by ion chromatography. J Chromatogr B 700: 261
26. Manoharan M, Schwille PO, Schmiedl A (2000) Are plasma and red blood cell (RBC) levels of antioxidative vitamins and uric acid disordered in idiopathic calcium urolithiasis (ICU)? A prelminary report. In: Rodgers AL, Hibbert BE, Hess B, Khan SR, Preminger GM (eds) Urolithiasis 2000. University of Cape Town, Cape Town, p 547
27. Marangella M, Petrarulo M, Vitale C, Cossedu M, Linari F (1992) Plasma and urinary glycolate assays for differentiating the hyperoxaluria syndromes. J Urol 148: 986
28. Marshall RW, Robertson WG (1976) Nomograms for the estimation of the saturation of urine with calcium oxalate, calcium phosphate, magnesium ammonium phosphate, uric acid, sodium acid urate, ammonium acid urate, and cystine. Clin Chim Acta 72: 253
29. Melethil S, Subrahmanyam MB, Chang CJH, Mason WD (1987) Megadoses of vitamin C. A pharmacokinetic evaluation. Ann NY Acad Sci 498: 491
30. Oh MS (1989) A new method for estimating GI absorption of alkali. Kidney Int 36: 915
31. Oreopoulos DG, Lindeman, Vander-Jagt DJ, Tzamaloukas AH, Bhagavan HM, Garry PJ (1993) Renal excretion of ascorbic acid: effect of age and sex. J Am Coll Nutr 12: 537
32. Pierratos AE, Khalaff H, Cheng PT, Psihramis K, Jewett MAS (1994) Clinical and biochemical differences in patients with

pure calcium oxalate monohydrate and calcium oxalate dihydrate kidney stones. J Urol 139: 240
33. Rao PN, Gordon C, Davies D, Blacklock NJ (1982) Are stone-formers maladapted to refined carbohydrates? Br J Urol 54: 575
34. Schmiedl A, Schwille PO (1994) Tolerable oxalate in the urine: Principles, inprovement of measuring method, and assessment in idiopathic calcium urolithiasis. Clin Lab 40: 757
35. Schwille PO, Schmiedl A (1999) Effects of magnesium citrate and magnesium-alkali citrate on calcium oxalate and calcium phosphate crystallization in undiluted urine. An acute load-response study in renal calcium stone patients and healthy controls. In: Kidney Stones, Borghi L, Meschi T, Briganti A, Schianchi T, Novasini A (eds), Editoriale Bios, Cosenza, p 565
36. Schwille PO, Hanisch E, Scholz D (1984) Postprandial hyperoxaluria and intestinal oxalate absorption in idiopathic renal stone disease. J Urol 132: 650
37. Schwille PO, Manoharan M, Rümenapf G, Wölfel G, Berens H (1989) Oxalate measurement in the picomole range by ion chromatography: values in fasting blood and urine of controls and patients with idiopathic calcium urolithiasis. J Clin Chem Clin Biochem 27: 87
38. Schwille PO, Schmiedl A, Rümenapf G (1998) Disorders of Calcium Metabolism – Appraisal and Management. In: Whitfield H, Hendry B, Kirby R, Duckett J (eds) Textbook of Genito-Urinary Surgery. Blackwell Scientific Publications, Oxford, p 771
39. Schwille PO, Schmiedl A, Herrmann U, Wipplinger J (1997) Postprandial hyperinsulinemia, insulin resistance and inappropriately high phosphaturia are features of younger males with idiopathic calcium urolithiasis – attenuation by ascorbic acid supplementation of the test meal. Urol Res 25: 49
40. Schwille PO, Schmiedl A, Herrmann U, Fan J, Gottlieb D, Manoharan M, Wipplinger J (1999) Magnesium, citrate, magnesium citrate and magnesium-alkali citrate as modulators of calcium oxalate crystallization in urine: observations in patients with recurrent idiopathic calcium urolithiasis. Urol Res 27: 117
41. Singh PP, Kiran R, Pendse AK, Ghosh R, Surana SS (1993) Ascorbic acid is an abettor in calcium urolithiasis: an experimental study. Scanning Micros 7: 1041
42. Urivetzky M, Kessaris D, Smith AD (1992) Ascorbic acid overdosing: A risk factor for calcium oxalate nephrolithiasis. J Urol 147: 1215
43. Wandzilak TR, D'Andre SD, Davis PA, Williams HE (1994) Effect of high dose vitamin C on urinary oxalate levels. J Urol 151: 834
44. Wandzilak TR, Hagen LE, Hughes H, Sutton RAL, Smith LH, Williams HE (1991) Quantitation of glycolate in urine by ion-chromatography. Kidney Int. 39: 765
45. Williams HE, Wandzilak TR (1989) Oxalate synthesis, transport and the hyperoxaluric syndromes. J Urol 141: 742
46. Wolf G (1996) The mechanism of uptake of ascorbic acid into osteoblasts and leucocytes. Nutr Rev 54: 150