Chemical Factors Important to Calcium Nephrolithiasis: Evidence for Impaired Hydroxycarboxylic Acid Absorption Causing Hyperoxaluria

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An investigation of variables important to calcium stone formation in urine indicated significantly increased daily excretion of calcium and oxalate and decreased excretion of ascorbate and citrate by recurrent calcium stone formers. In addition, urine volume, sodium, mucopolysaccharide, and protein were also significantly increased. We compared the uptake of citrate and ascorbate from the gut into the blood in normal controls and stone formers. These studies indicated significantly depressed absorption of both these hydroxycarboxylic acids in recurrent calcium stone formers. We also found that concurrent administration of citrate inhibited ascorbate absorption and increased urinary oxalate excretion after an ascorbate load in normal subjects and stone formers. These findings suggest a mechanism that explains hyperoxaluria in stone patients on the basis of a malabsorption of citrate, ascorbate, and possibly other hydroxycarboxylic acids.

Additional Keyphrases: calculous disease · oxalate · citrate · ascorbate

In westernized societies, approximately 70–80% of all renal calculi contain calcium oxalate as a major component (1). The incidence of calcium nephrolithiasis in western societies is increased in warmer climates (2) and is particularly high in Queensland, Australia (16.8 hospital admissions per 10 000 population; Brown JM and Bain C, unpublished observation). This contrasts with the relative rarity of this disease in developing tropical countries in Asia (3).

In most cases, the etiology of these stones is poorly understood, although several factors are believed to contribute, either singly or in combination (4):

- increased urinary excretion of oxalate and, to a lesser degree, calcium (5, 6)
- decreased concentrations in urine of inhibitors of stone formation such as pyrophosphate, citrate, mucopolysaccharides, and glycoproteins (7, 8)
- precipitation of chemically unrelated substances in urine, such as urate, resulting in co-precipitation of calcium salts (9)
- abnormal urine pH, which affects the solubility of most stone constituents (2, 10)
- increased urine osmolality, which increases the concentration of all stone constituents (11)

We have measured most of these variables in recurrent calcium stone formers to determine which differ consistently from values for our normal population, and thereby gain clues to the etiology of these stones in patients with recurrent calcium nephrolithiasis. Our results indicate the presence of a defective carrier in recurrent calcium stone formers, resulting in decreased uptake of two hydroxycarboxylic acids from the gut. The abnormal excretion of other key urinary metabolites in stone formers is partly explained by this finding.

Materials and Methods

Specimen collection: Timed plasma specimens were obtained from EDTA-anticoagulated blood taken from an indwelling venous catheter. Twenty-four-hour urine specimens were collected into a container divided into three compartments. Different preservatives were used in each compartment: 0.1 mol of HCl, 0.1–0.2 mmol of sodium thimerosal, or 0.1–0.2 mmol of sodium thimerosal plus 7–8 mmol of EDTA. The last-mentioned preservative, which stabilizes ascorbate and inhibits its conversion to oxalate (12), was used for ascorbate and oxalate estimations. The acid preservative was used for calcium and occasional oxalate estimations. For all other estimates we used the thimerosal fraction—including urate assay after solubilization of this analyte by adding alkali to the thimerosal-containing urine.

Analyses: Citrate in urine and plasma was assayed by enzymic methods (13, 14), ascorbate by colorimetric methods (15, 16). Urinary oxalate was measured by an enzymic method (17) and later by a liquid-chromatographic method (18, 19). The specificity of both oxalate methods were confirmed by comparison with other reliable methods for urinary oxalate (19, 20). Calcium was measured by EGTA titration of the fluorescent calcium–calcium complex (Corn Medical, Essex, England) and glycolate and glyoxylate by enzymic and fluorimetric methods, respectively (21, 22). Urinary mucopolysaccharides were analyzed by precipitation with Alcian Blue (23), urinary protein by co-precipitation with Ponceau S (24), and urinary ammonia by an enzymic method (Boehringer Mannheim Ammonia Analysis Kit).

Patients: The patients, 18 women and 25 men, ranged in age from 20 to 63 years (mean, 44 years). The duration of their stone histories varied from 1 to 30 years, with a mean of 9.6 years. All had formed more than one stone and their therapy consisted of careful instructions to avoid foods of high oxalate or calcium content and to maintain a high fluid intake. At the time of the study, all were healthy, with normal renal function as evidenced by plasma creatinine and urea concentrations. None of the patients had hyperparathyroidism, enteric hyperoxaluria, vitamin D intoxication, or renal tubular acidosis. The calcium absorption of 38 of the 43 patients was studied in hospital, with these patients on self-selected diets. When challenged with 1000 mg of orally administered calcium, 13 were classified as hypercalcemic, the rest as normocalcemic. All patients were normocalcemic on an intake of 150 mg of calcium daily and all had had radiopaque stones demonstrable on abdominal roentgenograms. Stones were available from 14 patients, and these, analyzed by a wet chemical method (25), were found to be mixed calcium oxalate/phosphate calculi. All the patients used in the load studies were recurrent formers of calcium oxalate/phosphate stones, as confirmed by chemical analysis of the stones.

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Received September 9, 1986; accepted November 18, 1986.
The normal subjects consisted of healthy hospital personnel who were taking no medication. There were approximately equal numbers of men and women in the group, ranging from 22 to 55 years old (mean, 32 years).

**Plasma studies:** In these studies we performed two types of experiments. The first, termed the "citrate-load" study, was performed on six recurrent stone formers and five healthy subjects. In this study, the subjects took 2 g of potassium citrate and 0.4 g citric acid, dissolved in 10 mL of water (test adapted from reference 26). The plasma citrate concentration was measured 0, 10, 20, 30, 45, and 60 min later (Figure 1).

The second experiment, termed the "citrate--ascorbate load" study, was performed on two healthy men. This experiment was performed in three parts. On day 1, the subjects ingested 2 g of ascorbic acid; on day 2, 4 g of potassium citrate and 0.8 g of citric acid, taken orally in 20 mL of water; and on day 3, 2 g of ascorbate was given along with 4 g of potassium citrate and 0.8 g of citric acid in 20 mL of water. Plasma citrate and ascorbate concentrations were measured 0, 10, 20, 30, 45, 60, 120, 180, 240, and 300 min after the load on each day (Figure 2). Both participants took one 500-mg ascorbate tablet per day for five days before the experiment, to saturate the tissue ascorbate stores.

Before each of these experiments the participants fasted for 12 h (overnight) but were permitted to drink water before and during the experiment. In the citrate-load studies the results were expressed as the plasma citrate concentration at time \( t \) minus that at time 0. This procedure allowed for differences in the basal plasma concentrations of citrate between individuals. For the citrate--ascorbate load studies, results for ascorbate were expressed as described for the citrate load. In addition, the change in plasma ascorbate concentration (time \( t \) -- time 0) when ascorbate was given alone was subtracted from the equivalent change obtained with the combined citrate--ascorbate load (termed "ascorbate difference" in Figure 2A). This procedure gives an index of the extent of inhibition or enhancement of ascorbate uptake by citrate.

**Urine studies:** We collected basal 24-h urines from normal subjects and stone formers who were taking their usual diets at home (Table 1). The 2-g oral ascorbate load test was done as previously described (27) on four healthy males. This experiment was then repeated on another day in these normal individuals except that the 2 g of ascorbate was taken with 4 g of potassium citrate plus 0.8 g citric acid in 20 mL of water.

The experimental protocol was as follows: After a 12-h overnight fast, collect a baseline specimen of urine, from 06:00 to 08:00. Give the ascorbate or combined citrate--ascorbate load at 08:00 and collect four three-hourly urine specimens, followed by a 12-h (overnight) collection. In this experiment, participants were not allowed breakfast but could eat a self-selected lunch and dinner and drink water *ad libitum* (27).

A similar experiment was also done on four recurrent stone formers, except that a single 24-h collection was made after each load instead of the three-hourly collections.

**Statistics:** In correlating data we used the product moment correlation and paired \( t \)-test (28). In comparing urinary excretions of normals and stone formers we used the Wilcoxon nonparametric two-sample statistic (29).

**Results**

In Table 1 we compare the results for all variables measured in 24-h urines collected at home with the subjects on usual diets, from both recurrent calcium stone formers and a normal population. In our stone formers, the urinary calcium and oxalate were significantly above normal and excretion of citrate and ascorbate was decreased. These results agree with previously reported findings for such patients (27, 30--32).

![Fig. 1. Uptake of citrate into blood after an oral load](image)

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Citrate was given at time 0 and all plasma concentrations of citrate have been normalized by subtracting the concentration at time 0. The results are indicated as means (± 1 SD) for six recurrent calcium stone formers (Φ), five normals (Ο). The asterisk indicates significance (\( p < 0.01 \)).

![Fig. 2. Effect of citrate on ascorbate absorption in two normal persons](image)

*Fig. 2. Effect of citrate on ascorbate absorption in two normal persons*

Combined oral citrate--ascorbate and ascorbate loads were given at time 0. The plasma ascorbate was normalized for the time-0 result. In A, the results of the combined ascorbate/citrate load are indicated as filled symbols (Φ, Α), whereas open symbols (Ο, Δ) are used for the ascorbate load given alone. The square symbols (Ο, Δ) indicate ascorbate excretion when citrate was taken. In A, the "Ascorbate Difference" was obtained by subtracting the normalized concentration of plasma ascorbate after an ascorbate load from an equivalent time course concentration obtained when the combined oral citrate--ascorbate load was taken.
The excretion of sodium by stone formers was also significantly increased (p <0.01). For the normal subjects, there was a significant correlation between urinary sodium and calcium excretion (r = 0.61; p <0.01; n = 28), confirming previous results (33). However, this correlation was poor in the case of the recurrent calcium stone formers (r = 0.07; p not significant; n = 43), suggesting that factors other than sodium are more important in modulating calcium excretion in these patients.

The volume of urine was significantly greater for stone formers, presumably reflecting compliance with medical advice to drink more fluid. This finding suggests that dehydration per se may not be a major factor leading to the recurrent stone formation. The slightly but significantly higher values for protein and mucopolysaccharide excretion probably reflect damage to the renal pelvis caused by the presence of calculi in some of the patients.

In a previous study we demonstrated impaired ascorbate absorption from the gut of patients with recurrent calcium nephrolithiasis, leading to decreased urinary ascorbate excretion (27). Both citrate and ascorbate are hydroxy-carboxylic acids, so we performed the oral loading test with citrate to see whether we could find evidence of depressed citrate absorption in these patients. Figure 1 depicts our results. The mean value for citrate in the stone formers was significantly lower as compared with normals 10 min after the oral intake, and the mean concentrations of citrate in plasma at the other times were also lower than those seen in normals. Others’ (30) failure to show depressed citrate absorption may be because they did not allow for baseline citrate concentrations in plasma. These authors, however, did find an abnormal function of the citrate carrier in the kidney.

To investigate whether citrate and ascorbate compete in vivo for transport by a common carrier in the gut, we performed a combined ascorbate–citrate load test in two normal subjects. We compared the results with two control days where the same doses of citrate and ascorbate were given singly. Figure 2 shows the results of the combined loadings in two normal subjects; they indicate that citrate inhibits absorption of ascorbate from the gut for as long as 3–4 h after the load was taken. In this combined load experiment, the plasma citrate concentration peaked about 1.5 to 2 h after the load, then gradually decreased. The uptake of citrate into plasma in this experiment was unaffected by ascorbate (data not shown).

To investigate whether the citrate-induced ascorbate malabsorption was clinically significant in terms of oxalogenesis, we measured the urinary oxalate excretion in four normal subjects and four stone formers, all of whom took oral citrate plus ascorbate. We further compared this oxalate excretion with that when ascorbate was given alone. The results (Figure 3) show that citrate administration with
ascorbate significantly enhanced oxalate output: the hourly oxalate excretions in urine were all significantly greater than the baseline collection (−2 h to time 0; p < 0.025). In comparison, when oral ascorbate was given alone there was no significant difference between the baseline and other hourly urinary oxalate excretions. In the normal persons, the 24-h urinary oxalate excretions after the combined load were 0.50, 0.54, 0.60, and 0.62 mmol/day and the values were significantly (p = 0.025) greater as compared with 0.25, 0.30, 0.36, and 0.44 mmol/day, respectively, for the 2 g of ascorbate given alone (reference range: 0.50 mmol/day; 20, 21). The response of the four recurrent stone formers to the combined load did not differ significantly from that of the normal subjects (0.53, 0.62, 0.77, and 0.88 mmol of oxalate per day).

The urinary ascorbate excretion by the normal persons receiving the combined citrate–ascorbate load was about 30% depressed in the first 3-h collection (0–3 h; p = 0.1; Figure 3), but was significantly increased in the next collection period (3 to 6 h; p = 0.05). However, the amount of ascorbate excreted over the 24-h period by the normal subjects was similar whether the ascorbate was given alone or in combination with citrate, 20.0 to 25.1% of the ascorbate intake being excreted as ascorbate (27). The stone formers, as compared with normals, excreted significantly less (p = 0.05) of the ascorbate intake as ascorbate (3.7–20.0%). The oral citrate load, given alone, did not affect urinary ascorbate (Figure 2) or oxalate excretion (data not shown).

**Discussion**

Of the factors important to stone formation that we studied here, only a few differed significantly between stone formers and normals in 24-h urine specimens they collected while on their usual diets at home. Perhaps the most clinically significant of these were the decreased citrate and increased calcium and oxalate excretions. Citrate inhibits stone formation by complexing calcium (7, 8). Oxalate and calcium form insoluble calcium oxalate crystals in urine, thereby increasing the possibility of stone formation (5, 6). The hypoexcretion of ascorbate is also important, because it is due to malabsorption and leads to increased oxalate formation from ascorbate in the gut—and thus hyperoxaluria in recurrent calcium stone formers (27).

The decreased urinary excretion of citrate and ascorbate by recurrent calcium-stone formers (Figure 1; 27) suggests the possibility of an inherent defect in a common carrier which absorbs hydroxyoxycarboxylic acids from the gut. To test whether these two organic anions compete with one another for absorption by a common carrier in the gut, the combined ascorbate–citrate load test was performed. This test, in two healthy people, showed that citrate depressed ascorbate absorption from the gut into the blood (Figure 2), with the profile of citrate uptake corresponding with the time of maximal inhibition of ascorbate excretion at about 90 to 120 min. These results suggest that there may be a common carrier for these structurally related anions (34). The enhanced ascorbate uptake 4–5 h after the load may indicate the increase in availability of the carrier for ascorbate after the citrate had cleared from the gut (see Figure 2). This rebound with ascorbate was also seen in the case of urine collected 3-6 h after the combined load (Figure 3). The inability of ascorbate to inhibit citrate uptake in the experiment (data not shown) is consistent with a greater affinity of the carrier for citrate, and this would be consistent with the more avid uptake of citrate from the gut (Figure 1) as compared with ascorbate (Figure 2B).

That citrate inhibits the uptake of ascorbate has significant bearing on the etiology of calcium-stone disease, because in stone formers the malabsorption of ascorbate will be potentiated by the presence of citrate. Other structurally related dietary hydroxyoxycarboxylic acids such as malate and tartrate may also compete for a defective carrier. These interactions in turn should increase the amount of ascorbate available for conversion to oxalate in the gut and may subsequently result in hyperoxaluria. Indeed, findings supporting this hypothesis were obtained for normal persons and stone formers who took the combined citrate–ascorbate loads, the oxalate excretion in both groups being significantly increased over the normal reference interval.

In addition to this mechanism, the increased citrate concentration in the gut lumen may significantly influence oxalate and calcium absorption and may explain the poor correlation found between urinary sodium and calcium excretion in stone formers. For example, the excess citrate in the gut may result in more calcium being bound to this anion, because citrate forms a strong complex with calcium (10). This would have two major effects. Firstly it would increase the absorption of oxalate from the gut by binding calcium and thereby increase the formation of soluble oxalate salts, which are more readily absorbed; calcium oxalate is insoluble and poorly absorbed from the gut (35, 36). Secondly the calcium citrate complex is more readily absorbed from the gut than are calcium complexes with other anions (Cowley DM, Brown JM, McWhinney BC, Chalmers AH, unpublished observation; 37), and this would contribute to the hypercalciuria seen in stone patients. To sum up, the basic mechanism responsible for recurrent calcium stone diseases may be malabsorption of hydroxycarboxylic anions. Figure 4 depicts the above mechanisms that we believe lead to hypercalciuria and hyperoxaluria in stone patients. We do not know whether the defect in hydroxycarboxylic acid uptake in our patients is inherited or acquired secondary to some insult to the gut. Certainly none of our patients had any symptoms of gastrointestinal disease, but 38% of them gave a positive family history of renal calculi, an incidence comparable to that found in other studies (38, 39).

In relation to the treatment of patients with recurrent calcium stone disease, foods with a high citrate–ascorbate content, such as citrus fruits, probably are contraindicated, because this combination produced hyperoxaluria in all the subjects we studied. Although the citrate dose in our loading

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**Fig. 4.** Model comparing handling of ascorbate, calcium citrate, and hydroxycarboxylic acids in the gut of recurrent calcium-stone formers and normals.
experiments was high, it was approximately equivalent to the citrate content of 500 g of oranges, as purchased, and (or) 50 mL of lemon juice (40). Obviously, dietary ascorbate is essential, but we recommend a sensible daily intake of <200 mg/day. In agreement with our present and previous findings, high-dose ascorbate intake (4 g/day) in calcium stone patients can re-activate stone formation in these patients (41). Treatment of recurrent calcium-stone formers with high-dose citrate supplements (4) is still recommended, if these supplements are not given simultaneously with a diet high in calcium or ascorbate.

The combination of impaired hydroxybutyryllyc acid carrier, together with western dietary preferences and the ready availability of foodstuffs rich in ascorbate and citrate in warmer climates, may explain the high incidence of calcium nephrolithiasis in certain western communities as compared to the developing world. Our current studies are aimed at investigating the effects of other organic anions on ascorbate absorption and ascorbate-induced oxalogenesis in stone formers and normals. In addition, we will be studying the effect of these anions on calcium absorption and excretion.

We thank the John P. Kelly Mater Research Foundation for a grant supporting this project.

References