Hypercalciuria is the most common metabolic abnormality found in patients with nephrolithiasis (1–5). Hypercalciuria raises urine supersaturation with respect to the solid phases of calcium hydrogen phosphate (CaHPO₄; brushite) and calcium oxalate (CaOx), enhancing the probability of nucleation and growth of crystals into clinically significant stones (4).

To establish an animal model of hypercalciuria, we successively inbred 59 generations of the most hypercalciuric progeny of hypercalciuric Sprague-Dawley rats, each of which now excretes eight to 10 times as much calcium as controls. These rats uniformly form calcium phosphate (apatite) kidney stones and have been termed genetic hypercalciuric stone–forming (GHS) rats. The addition of a common amino acid and oxalate precursor, hydroxyproline, to the diet of the GHS rats leads to formation of calcium oxalate (CaOx) kidney stones. Hydroxyproline-supplemented GHS rats were used to test the hypothesis that the thiazide diuretic chlorthalidone would decrease urine calcium excretion, supersaturation, and perhaps stone formation. All GHS rats received a fixed amount of a standard 1.2% calcium diet with 5% trans-4-hydroxy-L-proline (hydroxyproline) so that the rats would exclusively form CaOx stones. Half of the rats had chlorthalidone (Thz; 4 to 5 mg/kg per d) added to their diets. Urine was collected weekly, and at the conclusion of the study, the kidneys, ureters, and bladders were radiographed for the presence of stones. Compared with control, the addition of Thz led to a significant reduction of urine calcium and phosphorus excretion, whereas urine oxalate excretion increased. Supersaturation with respect to the calcium hydrogen phosphate fell, whereas supersaturation with respect to CaOx was unchanged. Rats that were fed Thz had fewer stones. As calcium phosphate seems to be the preferred initial solid phase in patients with CaOx kidney stones, the reduction in supersaturation with respect to the calcium phosphate solid phase may be the mechanism by which thiazides reduce CaOx stone formation.

Thiazide diuretic agents and analogous molecules such as chlorthalidone (Thz) reduce urine calcium excretion in normal people (22,23), patients with idiopathic hypercalciuria (24,25), hypoparathyroid people (26,27), and rats (28,29). Thiazide diuretics are commonly used to treat CaOx stone disease (1–5).

Eight studies have examined the effect of thiazide diuretics on preventing recurrent stone disease (30). A meta-analysis revealed that in all six studies in which treatment lasted for >2 yr, there was a significant reduction in stone recurrence rate; however, there was no reduction in stone recurrence rate in the two studies in which the duration of follow-up was <2 yr (30). Recently, Evan and co-workers (31,32) demonstrated that in humans with CaOx nephrolithiasis, the initial solid phase is formed around the thin limb of Henle’s loop and is composed of apatite. The apatite solid phase enlarges past the vasa recta and erodes through the urothelium, where it provides a nucleating site for CaOx, allowing for CaOx crystal growth into a clinically significant kidney stone.

Unlike in humans, the diet of a rat can be controlled precisely and urine collected quantitatively during studies that last...
weeks to months. We used hydroxyproline-supplemented GHS rats to ask whether chlorthalidone would reduce urine supersaturation with respect to a solid phase and, if so, whether it would alter supersaturation with respect to CaHPO₄ or CaOx and reduce stone formation. We found that chlorthalidone reduced urine calcium and phosphorus excretion, whereas oxalate excretion increased significantly. Supersaturation with respect to CaHPO₄ fell, whereas supersaturation with respect to CaOx was unchanged. Rats that were fed chlorthalidone had fewer stones than the control rats. As a calcium phosphate solid phase seems to be the initial mineral in hypercalciuric patients with CaOx kidney stones, the reduction in supersaturation with respect to the CaHPO₄ may be the mechanism by which thiazides reduce CaOx stone formation.

Materials and Methods
Establishment of Hypercalciuric Rats
The GHS rats were derived from Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) as described previously (6–20,33,34).

Study Protocol
Twenty-four 59th generation male GHS rats, initially weighing on average 211 g, were placed in metabolic cages for 18 wk. Each rat was initially provided with 13 g/d food, an amount that we have previously shown is completely consumed by a rat of this size (35), and deionized distilled water ad libitum. At 12 wk, the amount of food was increased to 15 g/d to account for the increased dietary needs of the now larger rats (6,20). Any rat that ate <12 g/d food until week 12 or ate <14 g/d food from week 12 until the conclusion of the study or drank <15 ml of water on any day would have been excluded from the remainder of the study; however, all rats met these prospective criteria throughout the study.

Twelve rats were placed in each of two groups. The control (Ctl) group was fed the standard diet, which consisted of 1.2% calcium and 0.65% phosphorus with 5% (by weight) trans-4-hydroxy-L-proline (OHP; ICN Biomedicals, Aurora, OH) added. The Thz group was fed similarly (standard diet plus 5% OHP) with chlorthalidone (1 mg/15 g food; Sigma, St. Louis, MO). This dose was calculated to provide approximately 4 to 5 mg/kg body wt per 24 h, a dose that we have previously shown to be effective in reducing calcium excretion in rats (28). Male rats, as opposed to the female rats that were used in a number of previous studies (7–10,12,13,15), were used because of their greater baseline oxalate excretion when fed this standard calcium diet (6,16,20). Previously, we have shown that GHS rats develop calcium phosphate (apatite) stones when fed this 1.2% calcium diet (8,10,12,15,20). The addition of 5% OHP to this diet results in the formation of CaHPO₄ stones (6,20). Every 2 wk, two successive 24-h urine collections were obtained. The first 24-h urine sample was collected in thymol and was used for measurement of pH, uric acid, and chloride. The second 24-h urine sample was collected in concentrated HCl and was used for all other measurements. Both samples were refrigerated at 4°C until measurement. All biochemical measurements were completed within 2 wk.

At the conclusion of the experiment (18 wk), each rat was killed. The kidneys, ureters, and bladder were dissected en block, and radiographic analysis was performed on 10 of the 12 rats in each group, which were chosen at random. The presence of stones was determined in a blinded manner. The other two rats in each group were reserved for pathologic studies, which were not done because of technical difficulties.

Chemical Determinations
Calcium was measured by reaction with arsenazo III and then determined photometrically at 650 nm (36). Creatinine was determined by a modification of the Jaffe method by formation of a creatinine-picomate complex (37). Inorganic phosphorus was measured by reaction with ammonium molybdate to form a colored phosphomolybdate complex (38). Uric acid was measured after oxidation by uricase to produce allantoin and hydrogen peroxide (39). Magnesium was determined by combination with calmagite (40). Ammonia was determined by coupled enzyme system using glutamate dehydrogenase and NADPH (41). Sodium was determined by a selective electrode (42), and potassium was determined using a valinomycin membrane attached to a potassium electrode (43). Chloride was measured by colorimetry using a silver/silver chloride electrode (44). Oxalic acid was measured using oxalate oxidase, which oxidizes oxalate to hydrogen peroxide and carbon dioxide. The hydrogen peroxide then reacts with 3-methyl-2-benzothiazoline hydrozone and 3-(dimethyl)benzoic acid to form an indamine dye that is monitored photometrically (45). Citric acid was determined using citrate lyase, which catalyzes the conversion citrate to oxaloacetic acid, which then is converted to malic acid, in the presence of malate dehydrogenase. The malic acid oxidizes NADH to NAD⁺, which is monitored photometrically (46). pH was measured by an ion-selective electrode. Sulfate was measured by turbidity after barium precipitation (47). We have used these methods previously (6–20,33,34).

Urine Supersaturation
The CaOx ion activity product was calculated using the computer program EQUIL developed by Finlayson and associates (48–50). The computer program calculates free ion concentrations using the concentrations of measured ligands and known stability constants. Ion activity coefficients are calculated from ionic strength using the Davies modification of the Debye-Huckel solution to the Poisson-Boltzman equation. The program simultaneously solves for all known binding interactions among the measured substances. Oxalate, phosphorus, and calcium ion activities were used to calculate the free-ion activity products. The free ions in solution are considered to be in equilibrium with the dissolved CaOx governed by a stability constant (K) of 2.746 × 10⁻⁷ M⁻¹ and with the dissolved CaHPO₄ (brushite) governed by a K of 0.685 × 10⁻¹¹ M⁻¹. The value of CaOx in a solution at equilibrium with a solid phase of CaOx, the solubility of CaOx, is 6.16 × 10⁻⁸ M/L. The value of brushite in a solution at equilibrium with a solid phase of brushite, the solubility of brushite, is 3.981 × 10⁻⁷ M/L. The relative supersaturation for CaOx is calculated as the ratio of the free-ion activity product of calcium and oxalate in the individual urine to the solubility of CaOx. The relative supersaturation for brushite is calculated as the ratio of the free-ion activity product of calcium and phosphate in the individual urine to the solubility of calcium phosphate. Ratios of 1 connote a sample at equilibrium, above 1 supersaturation, and below 1 undersaturation. We used this computer program previously and found excellent correspondence between calculated and experimentally measured saturation in urine and blood (7–10,12,15–17) and in bone culture medium (51–53).

Statistical Analyses
All values are expressed as mean ± SEM. Tests of significance were calculated by t tests and linear regression, as appropriate, using conventional computer programs (BMDP; University of California, Los Angeles, CA). P < 0.05 was considered significant.
Results

Urine Ion Excretion, Volume, and pH

Every 2 wk, two successive 24-h urine collections were obtained. The individual urine collections for the 24 rats divided into two groups were analyzed separately and then were averaged over the first 6 wk (weeks 1 to 6), the second 6 wk (weeks 7 to 12), and the final 6 wk (weeks 13 to 18).

With respect to urine calcium, when compared with Ctl, during all three time periods, Thz induced a significant decrease in urine calcium excretion (Figure 1, top). With respect to urine oxalate, when compared with Ctl, during all three time periods, Thz induced a significant increase in oxalate excretion (Figure 1, middle). With respect to urine phosphorus, when compared with Ctl, during the first and third time periods, Thz induced a significant decrease in urine phosphorus excretion; however, during the second time period, there was no difference in urine phosphorus between the two groups (Figure 1, bottom).

With respect to urine pH, when compared with Ctl, during...
the second and third time periods, Thz induced a significant decrease in urine pH; however, during the first time period, there was no difference in urine pH between the two groups (Figure 2, top). With respect to urine citrate, when compared with Ctl, during the second and third time periods, Thz induced a significant increase in urine citrate; however, during the first time period, there was no difference in urine citrate between the two groups (Figure 2, middle). With respect to urine ammonium, when compared with Ctl, during all three time periods, Thz induced a significant increase in urine ammonium excretion (Figure 2, bottom).

With respect to urine volume, during all time periods, there was no difference in urine volume between the Ctl groups (Figure 3, top). With respect to animal weight, when compared with Ctl during all three time periods, Thz induced a significant decrease in animal weight (Figure 3, middle). With respect to urine creatinine, when compared with Ctl, during all three time periods, Thz induced a significant decrease in urine creatinine (Figure 3, bottom).

Supersaturation

When compared with Ctl, during all three time periods, Thz induced a significant decrease in urine supersaturation with respect to the CaHPO4 solid phase (Figure 4, top). When compared with Ctl, during all three time periods, Thz did not alter urine supersaturation with respect to the CaOx solid phase (Figure 4, bottom).

Supersaturation in Relation to Ion Excretion

Urine supersaturation with respect to the CaHPO4 solid phase was correlated directly and significantly with urine calcium excretion in both the Ctl rats and the rats that were fed Thz (Figure 5, top). The regressions were significantly different; at a given level of calcium, the Thz-fed rats had a lower supersaturation with respect to the CaHPO4 solid phase than the Ctl rats. Urine supersaturation with respect to the CaHPO4 solid phase was correlated inversely and significantly with urine oxalate excretion in both the Ctl rats and the rats that were fed Thz (Figure 5, middle). The regressions were significantly different; at a given level of oxalate, the Thz-fed rats had a lower supersaturation with respect to the CaHPO4 solid phase than the Ctl rats. Urine supersaturation with respect to the CaHPO4 solid phase was correlated directly and significantly with urine phosphorus excretion in both the Ctl rats and the rats that were fed Thz (Figure 5, bottom). The regressions were significantly different; at a given level of phosphorus, the Thz-fed rats had a lower supersaturation with respect to the CaHPO4 solid phase than the Ctl rats.
solid phase was correlated directly and significantly with urine phosphorus excretion in both the Ctrl rats and the rats that were fed Thz (Figure 5, bottom). The regressions were significantly different; at a given level of phosphorus, the Thz-fed rats had a lower supersaturation with respect to the CaHPO₄ solid phase than the Ctrl rats.

Urine supersaturation with respect to the CaOx solid phase was correlated inversely and significantly with urine calcium excretion in the Ctrl rats but not in the rats that were fed Thz (Figure 6, top). Urine supersaturation with respect to the CaOx solid phase was correlated directly and significantly with urine oxalate excretion in both the Ctrl rats and the rats that were fed Thz (Figure 6, middle). There was no difference in the regressions between the two groups of rats. Urine supersaturation with respect to the CaOx solid phase was correlated inversely and significantly with urine phosphorus excretion in the Ctrl rats but not in the rats that were fed Thz (Figure 6, bottom).

Stone Formation
The Ctrl rats had visible stones in 18 of 20 kidneys, whereas the rats that were fed Thz had visible stones in 12 of 20 kidneys ($P < 0.01$).

Discussion
In this study, we have shown that administration of the thiazide diuretic Thz to GHS rats that were fed the oxalate precursor, hydroxyproline, reduces urine calcium excretion, as expected, but also leads to an increase in urine oxalate excretion and a fall in urine phosphorus excretion. The net result of these changes in urine ion excretion is a reduction in supersaturation with respect to the CaOx solid phase with no change in supersaturation with respect to the CaOx solid phase. Thz reduced stone formation.

In kidney biopsies of hypercalciuric humans with CaOx nephrolithiasis, Evan et al. (31,32) found that the initial site of crystallization seemed to be around the thin limb of Henle’s loop. It is interesting that the crystal phase consisted of a calcium phosphate solid phase (apatite) and not CaOx. The crystallization extended toward the collecting duct before eroding into the urothelium, where it provided a heterogeneous nucleating site for CaOx stone formation. These crystals, on the face of the papilla, have been termed Randall’s plaques (54,55). Fracture of this crystal from the urothelium can result in a clinically significant kidney stone. The reduction in stone formation in the GHS rats in this study may be secondary to reduction in an initial calcium phosphate solid phase. That a CaHPO₄ solid phase has not, as yet, been detected in GHS rats that were fed the standard diet with 5% additional hydroxyproline does not mean that it was not present at some time during the initial stone formation.

The decrease in urine calcium excretion in the GHS rats that were fed Thz is almost certainly due to increased renal tubular calcium reabsorption (22,56). We have previously shown in rats (28) and humans (57) that the decrease in urine calcium excretion is allowed to persist as a result of the Thz-induced reduction in intestinal calcium absorption. The increase in urine oxalate may be due to a reduction in calcium excretion, which was associated with a reduction in the number of stones formed. The chlorthalidone-induced reduction in urine calcium excretion allowed less substrate for CaOx stone formation, less oxalate incorporation into stones, and an increase in urine oxalate excretion. In the rats that were fed Thz, there was 35.1 mg/rat more oxalate excreted over the course of the study compared with the control rats, which is readily accounted for by a reduction in stone formation by Thz, of only 58.2 mg of crystal. Previously, we showed that the GHS rats that were fed 5% hydroxyproline formed only CaOx stones, yet their urine oxalate excretion did not increase (20). The failure of the urine oxalate to increase was ascribed to consumption of the oxalate by the CaOx stones.

The decrease in urine phosphorus excretion after feeding the GHS rats Thz may be explained by our previous study in...
normal rats (28). We showed previously that when rats are fed Thz, there is a decrease in intestinal calcium absorption, which allows the continued reduction in urine calcium excretion (28). The reduction in calcium absorption would lead to an increase in calcium in the intestinal lumen, which would be available to bind intestinal phosphate and reduce the available phosphate for absorption and subsequent excretion. In patients with renal insufficiency and renal failure, calcium has been shown to complex with intestinal phosphorus, leading to a decrease in intestinal phosphorus absorption (58). We have also shown that the administration of Thz to humans leads to a reduction in intestinal calcium absorption (57).

We have previously shown that the GHS rats that are fed a standard diet form calcium phosphate stones (8,10,12,15,20), which also seems to be the favored initial ion complex in humans with CaOx stones (31,32). The addition of the common amino acid and oxalate precursor hydroxyproline leads to formation of CaOx kidney stones (6,20). In this study, Thz induced a reduction only of supersaturation with respect to CaHPO₄; there was no change in the supersaturation with respect to CaOx. In GHS rats that were not given additional hydroxyproline, we showed previously that reduction of supersaturation with respect to the CaHPO₄ solid phase leads to a reduction in stone formation (10).

Thz is known to induce a metabolic alkalosis in rats and humans (59). During metabolic alkalosis, urine pH falls, as confirmed in this study, as a result of enhanced proton secretion into the lumen, leading to increased net acid excretion (59). The increase in ammonium excretion, found in this study, may be due to a reduction in urinary phosphorus and thus titratable acidity, requiring more of the daily endogenous acid load to be excreted by an increase in ammonium.

The effect of thiazide diuretics on urinary oxalate excretion in humans has not been explored in great detail. Three randomized, prospective trials of thiazide diuretics as treatment for calcium nephrolithiasis measured urine oxalate excretion before and during thiazide therapy (60–62). Borghi et al. (60) found that indapamide reduced urine oxalate excretion in years 2 and 3 of therapy compared with baseline; no decrement in oxalate excretion was seen in the control group. Ettinger et al. (61) reported reduction of oxalate in patients who were treated with Thz, but a greater reduction was seen in patients who were given 25 mg/d than in those who received 50 mg/d. Again, there was no change in oxalate excretion in the control group. Scholze et al. (62) reported no decrease in urine oxalate in patients who received hydrochlorothiazide after 1 yr of therapy but did find a reduction in oxalate excretion in the placebo group. Martins et al. (63) performed a prospective crossover trial comparing the biochemical effects of hydrochlorothiazide and indapamide. Neither drug caused a change in oxalate excretion during 3 mo of therapy. In a retrospective study, Ahlstrand et al. (64) found no change in urine oxalate after 1 yr of therapy with bendroflumethiazide. Yendt et al. (65) found no decrease during the first year of treatment with hydrochlorothiazide, but a significant reduction occurred in patients who were treated for >1 yr. Parks and Coe (66) found that urine oxalate increased after initiation of therapy to prevent stone recurrence but the increase did not differ regardless of whether the patients were treated with thiazides. The reduction in oxalate absorption seen in many human studies has been assumed to be due to thiazide’s decreasing intestinal calcium absorption, leaving more dietary calcium in the gut lumen to bind oxalate and prevent its reabsorption. An alternative hypothesis was proposed by Hatch and Vaziri (67), who found that thiazide diuretics reduced the net absorptive flux of oxalate across rabbit colon in vitro. As indicated above, the increase in urine oxalate excretion in the GHS rats that were given hydroxyproline may be due to a reduction in urine calcium excretion, resulting in less available urinary calcium for CaOx stone formation. Another possible explanation for the discrepant results between rats and humans is the origin of the urine oxalate. In the rats that are given hydroxyproline, oxalate is produced endogenously from metabolic precursors, whereas in humans, the diet is a significant source of oxalate. In humans, dietary oxalate is usually approximately 100 mg/d, and dietary oxalate accounts for 40 to 50% of urine oxalate excreted (68). If little oxalate is normally absorbed from the intestine in rats, then reducing calcium absorption in the intestine should have little effect on oxalate absorption and subsequent excretion.

Thus, we found that Thz reduced urinary calcium and phosphorus excretion, whereas urinary oxalate excretion increased significantly. Supersaturation with respect to CaHPO₄ fell, whereas supersaturation with respect to CaOx was unchanged. Rats that were fed Thz had fewer stones than the Ctl rats. As a calcium phosphate solid phase seems to be the initial mineral in patients with CaOx kidney stones, the reduction in supersaturation with respect to CaHPO₄ may be the mechanism by which thiazides reduce CaOx stone formation in humans. Further studies in humans who are treated with Thz will be necessary to test this hypothesis.

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