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Increased dietary oxalate does not increase urinary calcium oxalate saturation in hypercalciuric rats

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Background. Human calcium oxalate (CaOx) nephrolithiasis may occur if urine is supersaturated with respect to the solid-phase CaOx. In these patients, dietary oxalate is often restricted to reduce its absorption and subsequent excretion in an effort to lower supersaturation and to decrease stone formation. However, dietary oxalate also binds intestinal calcium, which lowers calcium absorption and excretion. The effect of increasing dietary oxalate on urinary CaOx supersaturation is difficult to predict.

Methods. To determine the effect of dietary oxalate intake on urinary supersaturation with respect to CaOx and brushite ($CaHPO_4$), we fed 36th and 37th generation genetic hypercalciuric rats a normal Ca diet (1.2% Ca) alone or with sodium oxalate added at 0.5%, 1.0%, or 2.0% for a total of 18 weeks. We measured urinary ion excretion and calculated supersaturation with respect to the CaOx and $CaHPO_4$ solid phases and determined the type of stones formed.

Results. Increasing dietary oxalate from 0% to 2.0% significantly increased urinary oxalate and decreased urinary calcium excretion, the latter presumably due to increased dietary oxalate-binding intestinal calcium. Increasing dietary oxalate from 0% to 2.0% decreased CaOx supersaturation due to the decrease in urinary calcium offsetting the increase in urinary oxalate and the decreased $CaHPO_4$ supersaturation. Each rat in each group formed stones. Scanning electron microscopy revealed discrete stones and not nephrocalcinosis. X-ray and electron diffraction and x-ray microanalysis revealed that the stones were composed of calcium and phosphate; there were no CaOx stones.

Conclusion. Thus, increasing dietary oxalate led to a decrease in CaOx and $CaHPO_4$ supersaturation and did not alter the universal stone formation found in these rats, nor the type of stones formed. These results suggest the necessity for human studies aimed at determining the role, if any, of limiting oxalate intake to prevent recurrence of CaOx nephrolithiasis.

Key words: kidney stones, brushite, nephrolithiasis, hypercalciuria, CaOx, calcium reabsorption, bone resorption, GHS rats.

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Idiopathic hypercalciuria is common in patients with nephrolithiasis and raises urinary supersaturation with respect to calcium oxalate (CaOx), the most common solid phase found in renal stones, as well as brushite ($CaHPO_4$) [1, 2]. Idiopathic hypercalciuria appears to have a genetic origin, and its pathogenesis involves a complex interaction of increased intestinal calcium absorption, increased bone resorption, and decreased renal tubule calcium reabsorption [3, 4].

Through over 37 generations of successive inbreeding of the most hypercalciuric progeny of hypercalciuric Sprague-Dawley rats, we have established a strain of rats, each of which excrete abnormally large amounts of urinary calcium [5–15]. The principal mechanism for the excessive calcium excretion in these rats appears to be an increase in intestinal calcium absorption [13]. The increased intestinal calcium absorption appears to be mediated not by an increase in the serum level of $1,25(OH)_2D_3$, but by an increase in the number of intestinal vitamin D receptors [12]. When these hypercalciuric rats are fed a very low-calcium diet, their urine calcium excretion remains elevated compared with that of similarly treated control rats, indicating a defect in renal calcium reabsorption and/or an increase in bone resorption [11]. The bone from these hypercalciuric rats releases more calcium, compared with bone of control rats, when exposed to increasing amounts of $1,25(OH)_2D_3$ [8], and inhibition of bone resorption substantially decreases the hypercalciuria when these rats are fed a low-calcium diet [14]. In addition, a primary defect in renal calcium reabsorption is observed during carefully controlled clearance studies [6]. We have shown that both the bone and kidney of the hypercalciuric rats have an increased number of vitamin D receptors [8, 12, 15]. Thus, these hypercalciuric rats appear to have a systemic abnormality in calcium homeostasis. They absorb more intestinal calcium. They resorb more bone, and they fail to reabsorb filtered calcium adequately. As each of these hypercalciuric rats forms renal stones, we have termed the rats

genetic hypercalciuric stone-forming (GHS) rats [7, 9]. Calcium transport abnormalities similar to those documented in the GHS rats have been observed in patients with idiopathic hypercalciuria [16].

Therapy for patients with idiopathic hypercalciuria and CaOx nephrolithiasis may include restriction of dietary oxalate, even though their urinary oxalate excretion is often no greater than that found in non–stone-forming people [1, 16, 17]. Oxalate restriction is used to reduce intestinal oxalate available for absorption, which should lead to a decrease in urinary oxalate excretion [18–20]. However, dietary oxalate also binds intestinal calcium so that a reduction in oxalate will increase the available, unbound calcium for absorption [17, 21–25]. Because of the complex interaction between intestinal calcium and oxalate and the subsequent absorption of each of these ions, a reduction in dietary oxalate could potentially reduce, not change, or even increase urinary CaOx supersaturation.

The purpose of this study was to use the GHS rats to determine the effect of an increase in dietary oxalate on urinary calcium excretion and CaOx supersaturation. We found that increasing dietary oxalate led to a decrease in CaOx supersaturation and did not alter the universal stone formation found in these GHS rats, nor the type of stones formed. Thus, reducing dietary oxalate in patients with CaOx nephrolithiasis may not appear warranted if these results, observed in the GHS rat, can be applied to humans.

METHODS

Establishment of hypercalciuric rats

Initially, 20 male and 20 female adult Sprague-Dawley rats (Charles River Laboratories, Kingston, NY, USA) were screened for hypercalciuria. The rats were placed in individual metabolic cages and were allowed five days to adjust to the cage and diet. During this time, the animals were fed 13 g/day of a diet containing 1.2% calcium, 0.90% phosphorus, 0.24% magnesium, 0.40% sodium, and 0.43% potassium, as well as 2.2 IU vitamin D_3/g of food. Deionized distilled water was provided ad libitum. Two successive 24-hour urine collections in 0.25 ml 12 N HCl were then obtained on days 6 and 7 to measure urine calcium excretion. The three male and three female rats with the greatest calcium excretion were used to breed the next generation. A similar protocol was used to select the two to three most hypercalciuric males and the three to four most hypercalciuric females for inbreeding of subsequent generations [5, 6, 8–15].

Study protocol

Twenty 36th and twelve 37th generations of female GHS rats, for a total of 32 rats, each weighing 150 g, were kept in the metabolic cages and continued on the same diet for a total of 18 weeks. Rats were offered 15 g/day of the diet that we have previously shown is the amount of food consumed by a female rat of this weight [26–28]. Rats were fed either this diet alone or with sodium oxalate added as 0.5%, 1.0%, or 2.0% for a total of 18 weeks. Every two weeks, two successive 24-hour urine collections were obtained. The first 24-hour urine was collected in thymol and was used for all measurements except oxalate, and the second 24-hour urine was collected in concentrated HCl for measurement of oxalate. Both samples were frozen at −70°C, and biochemical measurements were determined within two weeks. At the conclusion of the experiment (18 weeks), each rat was killed, and the entire urinary tract was examined, by direct vision, for stones. The kidneys, ureters, and bladder were dissected in block and mounted on radiographic film. Any stone found was analyzed for crystal type and composition. Any rat that ate less than 12 g of food or drank less than 15 ml of water on any day of the study would have been excluded from the entire study; however, all rats met these prospective criteria throughout the study.

Chemical determinations

Calcium was measured by reaction with arsenazo III and then determined photometrically at 650 nm [29]. Creatinine was determined by a modification of the Jaffe method by formation of a creatinine-picrate complex [30]. Inorganic phosphorus was measured by reaction with ammonium molybdate to form a colored phosphomolybdate complex [31]. Uric acid was measured after oxidation by uricase to produce allantoin and hydrogen peroxide [32]. Magnesium was determined by combination with calmagite [33]. Ammonia was determined by coupled enzyme system using glutamate dehydrogenase and nicotinamide adenine dinucleotide phosphate [34]. Sodium was determined by a selective electrode [35] and potassium using a valinomycine membrane attached to a potassium electrode [36]. Chloride was measured by colorimetry using a silver/silver chloride electrode [37]. 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-benzothiazolinone hydrozone and 3-(dimethyl)benzoic acid to form an indamine dye [38]. Citric acid was determined using citrate lyase, which catalyzes the conversion citrate to oxaloacetic acid, which is then converted to malic acid, in the presence of malate dehydrogenase. The malic acid oxidizes NADH to NAD^+ [39]. pH was measured by an ion-selective electrode.

Urinary supersaturation

The CaOx ion activity product was calculated using the computer program EQUIL, developed by Finlayson and Brown et al [40–42]. The computer program calcu-

lates 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 an equilibrium with the dissolved CaOx governed by a stability constant (K) of 2.746×10^3 M^{-1} and with the dissolved $CaHPO_4$ governed by a K of 0.685×10^3 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^{-6} M per liter. The value of the $CaHPO_4$ in a solution at equilibrium with a solid phase of $CaHPO_4$, the solubility of $CaHPO_4$, is 3.981×10^{-7} M per liter. 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 $CaHPO_4$ 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 one connote a sample at equilibrium, above one supersaturation, and below one undersaturation.

The ability of this computer program to accurately predict the saturation of urine or other solution with respect to the solid phase is excellent [10, 11, 40, 43, 44]. With a series of 20 artificial solutions, the equilibrium calcium concentration and the extent of calcium precipitation were predicted with average errors of 5% ± 9% and 5 ± 8% (mean ± SD), respectively [40]. We have used this computer program previously and found excellent correspondence between calculated and experimentally measured saturations in urine and blood [5, 9–11] and in bone culture medium [43–45].

Transmission electron microscopy, x-ray microanalysis, and electron diffraction

Small pieces of the stones were placed in Beem capsules and were embedded in Spurr epoxy resin without any chemical fixation or dehydration. The blocks were cut on a Sorval MT6000 microtome with a diamond knife. Two hundred nm sections were placed on carbon-coated formvar grids for microanalysis and selected area electron diffraction. Microscopy was carried out on a Phillips EM430 equipped with an Oxford Instruments AN10000 microanalysis system.

Statistical analysis

All values are expressed as mean ± SE. Tests of significance were calculated by analysis of variance with the Bonferroni correction for multiple comparisons using conventional computer programs (BMDP; University of California, Los Angeles, CA, USA) on a digital computer. A $P < 0.05$ was considered significant.

RESULTS

Urinary ion excretion

Every two weeks, two successive 24-hour urine collections were obtained. The individual urine collections for the 32 rats divided equally into four groups were analyzed separately and were then averaged over the first six weeks, the second six weeks, and the final six weeks.

With respect to urinary oxalate, over the first six weeks, there was an increase in oxalate excretion with the addition of 1.0% and 2.0% dietary oxalate compared with control and 0.5% oxalate; rats receiving 2.0% oxalate excreted more oxalate than rats receiving 1.0% oxalate (Fig. 1A). During the second six weeks, there was an increase in oxalate excretion with 0.5%, 1.0%, and 2.0% oxalate compared with controls; rats receiving 1.0% and 2.0% oxalate excreted more oxalate than rats receiving 0.5% oxalate. During the final six weeks, there was an increase in oxalate excretion with 0.5%, 1.0%, and 2.0% oxalate compared with controls. Thus, compared with controls, at all time periods, urinary oxalate excretion was increased with 1.0% and 2.0% dietary oxalate and in the latter two time periods with 0.5%, 1.0%, and 2.0% dietary oxalate.

With respect to urinary calcium, over the first six weeks there was a decrease in calcium excretion with the addition of 2.0% oxalate compared with controls, 0.5% and 1.0% oxalate (Fig. 1B). Over the second six weeks, there was a decrease in urinary calcium with the addition of 0.5%, 1.0%, and 2.0% oxalate compared with controls. Over the third six weeks, there was a decrease in urinary calcium with 0.5%, 1.0%, and 2.0% oxalate compared with controls; 2.0% oxalate was lower than all other groups.

With respect to urinary phosphorus, over the first six weeks there was an increase in urinary phosphorus with 2.0% oxalate compared with controls, 0.5% and 1.0% oxalate (Fig. 1C). Over both the second and third six weeks, there was an increase in urinary phosphorus with 1.0% oxalate compared with controls and with 2.0% oxalate compared with controls, 0.5% and 1.0% oxalate.

With respect to urinary sodium, over the first six weeks there was an increase in sodium excretion with 1.0% and 2.0% oxalate compared with controls and with 2.0% oxalate compared with 0.5% and 1.0% oxalate (Fig. 2A). Over the second six weeks, there was an increase in sodium excretion with 0.5%, 1.0%, and 2.0% oxalate compared with controls, and with 2.0% oxalate compared with 0.5% and 1.0% oxalate. Over the third six weeks, there was an increase in sodium excretion with both 1.0% and 2.0% oxalate compared with controls and 0.5% oxalate.

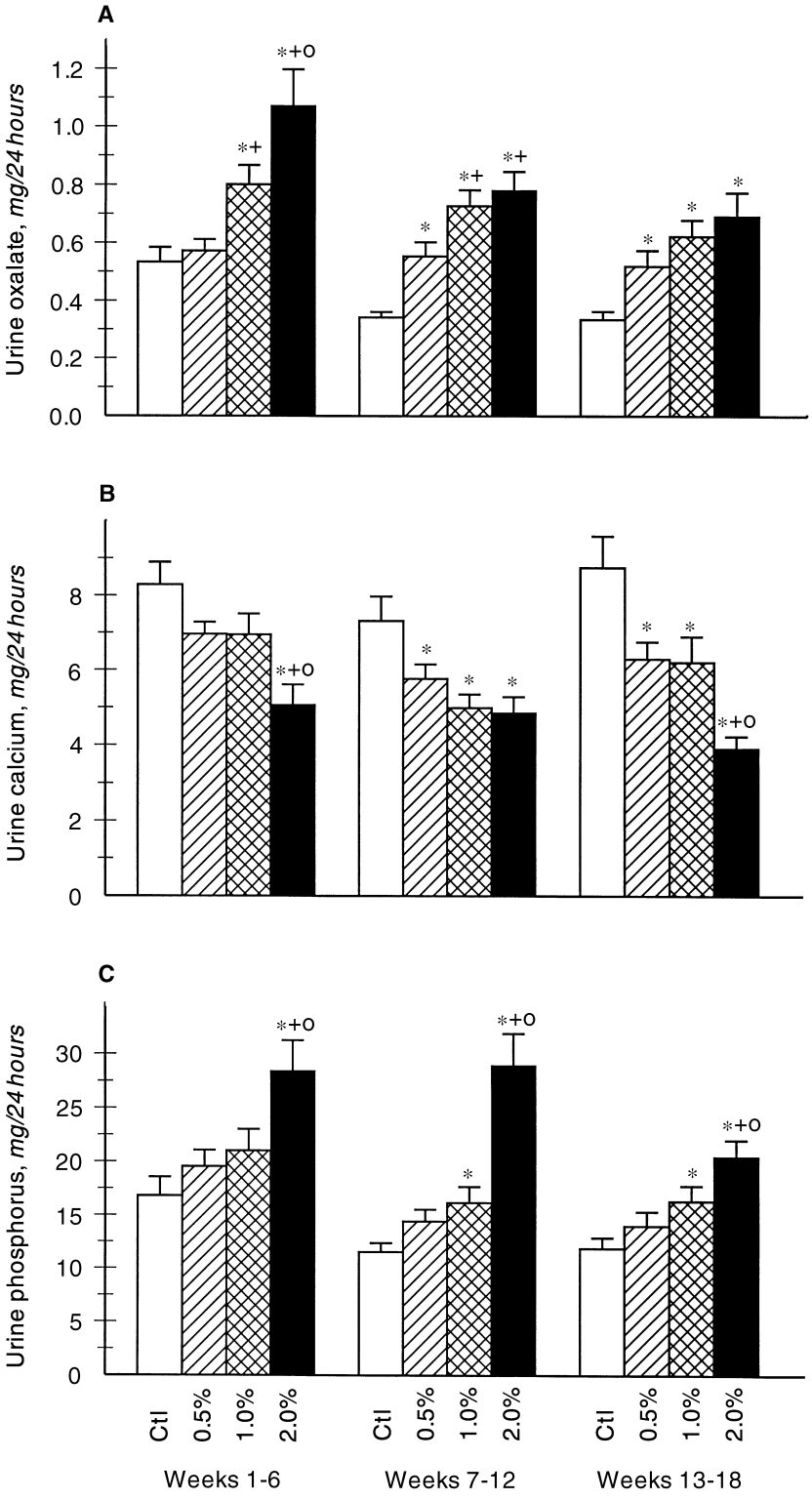

Fig. 1. Urine oxalate, calcium, and phosphorus excretion (mean ± SE) in GHS rats fed 15 g/day of a 1.2% Ca diet alone or with added sodium oxalate at 0.5%, 1.0%, or 2.0% for a total of 18 weeks. Every two weeks, individual 24-hour urine collections were obtained. The individual urine collections for each of the eight rats in the each of the four groups were analyzed separately and were then averaged over the first six weeks (weeks 1–6), the second six weeks (weeks 7–12), and the final six weeks (weeks 13–18). Abbreviations are: Ctl, GHS rats fed 15 g/day of the 1.2% Ca diet without added oxalate; 0.5%, GHS rats fed 15 g/day of the 1.2% Ca diet with 0.5% sodium oxalate; 1.0%, GHS rats fed 15 g/day of the 1.2% Ca diet with 1.0% sodium oxalate; 2.0%, GHS rats fed 15 g/day of the 1.2% Ca diet with 2.0% sodium oxalate; *, different from Ctl, $P < 0.05$; +, different from 0.5%, $P < 0.05$. °, different from 1.0%; $P < 0.05$.

With respect to urinary magnesium, over the first six weeks there was no change in magnesium excretion with the addition of oxalate (Fig. 2B). Over the second six weeks, there was an increase in magnesium excretion with 2.0% oxalate compared with 1.0% oxalate, and over the third six weeks, there was a decrease in magnesium excretion with 2.0% oxalate compared with controls.

With respect to urinary pH, over the first six weeks

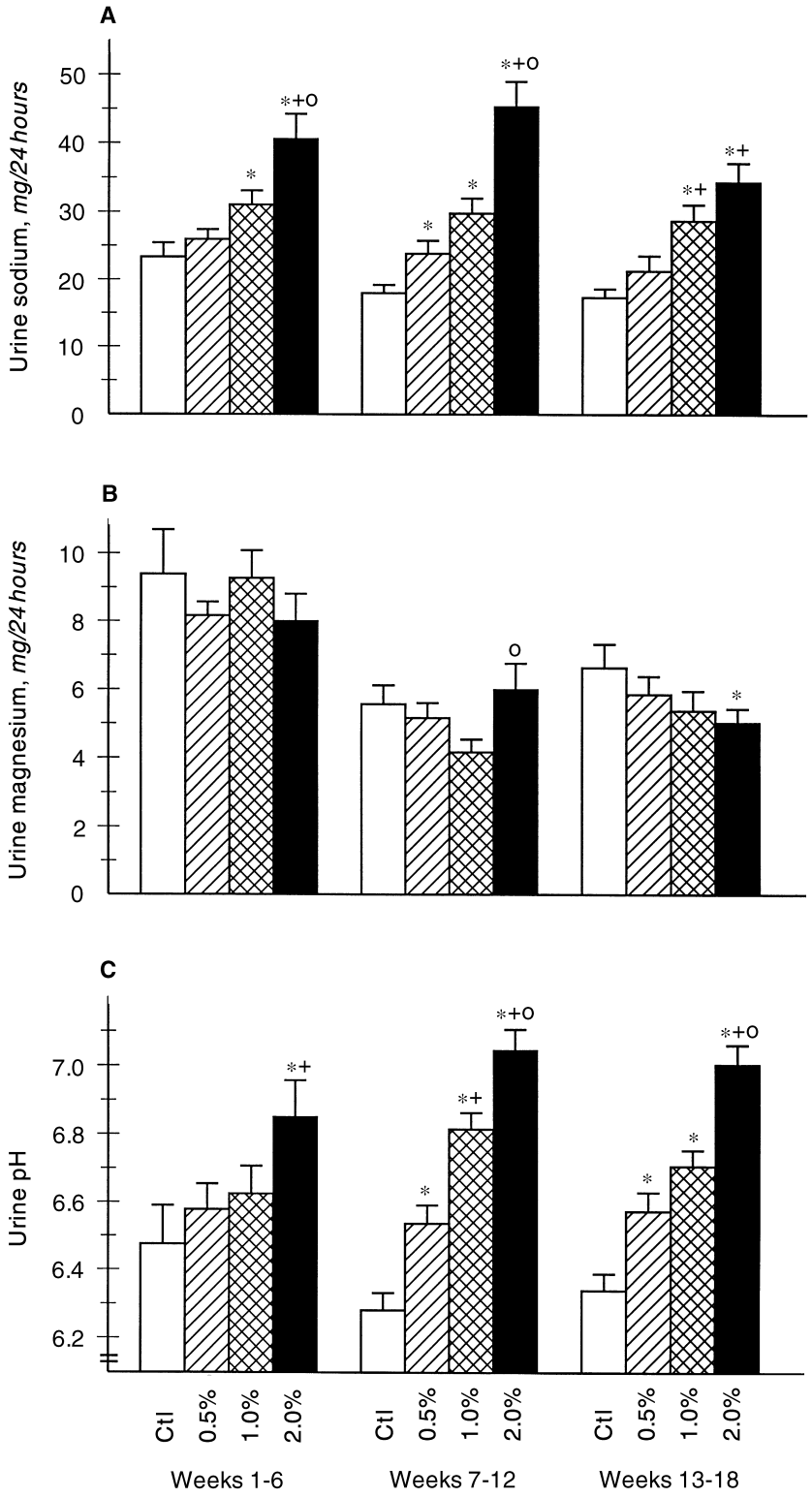

Fig. 2. Urine sodium and magnesium excretion and urine pH (mean ± SE) in GHS rats fed 15 g/day of a 1.2% Ca diet alone or with added sodium oxalate at 0.5%, 1.0%, or 2.0% for a total of 18 weeks. Abbreviations are the same as in Figure 1.

there was an increase in urinary pH with 2.0% oxalate compared with controls and 0.5% oxalate (Fig. 2B). Over the second six weeks, there was an increase in urinary pH with 0.5%, 1.0%, and 2.0% dietary oxalate compared with controls, an increase with 1.0% compared with 0.5% and with 2.0% compared with 0.5% and 1.0% oxalate. Over the final six weeks, there was an increase in urine pH with 0.5%, 1.0%, and 1.5% dietary oxalate compared

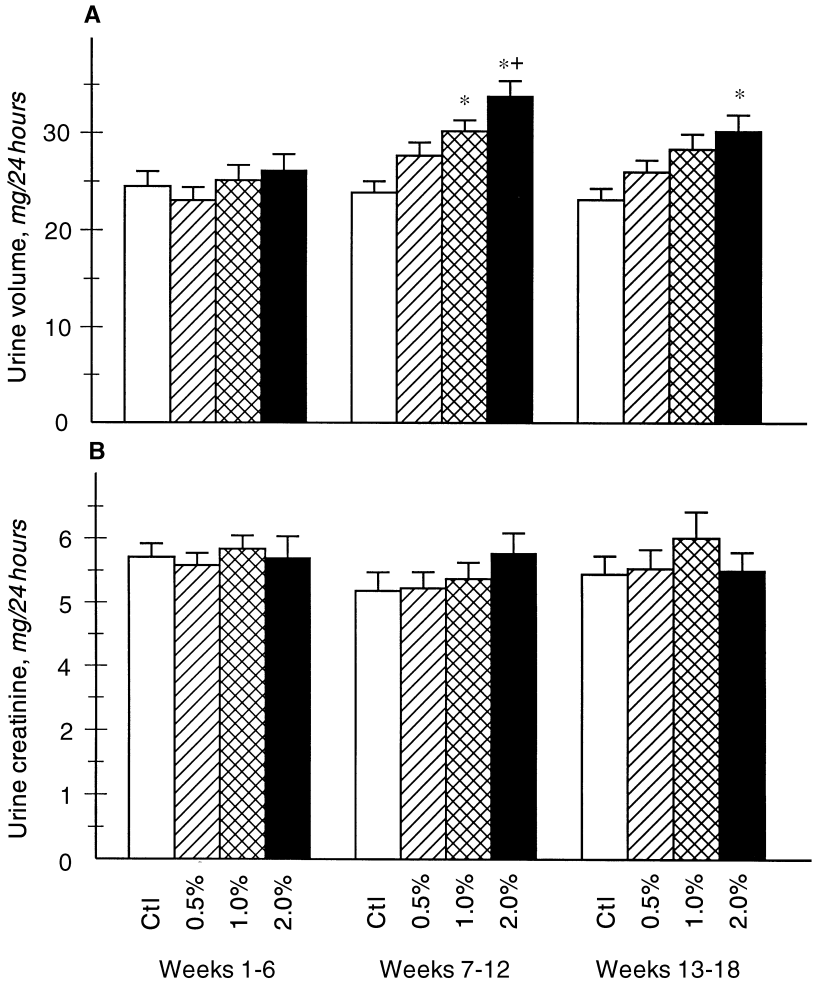

Fig. 3. Urine volume and creatine excretion (mean ± SE) in GHS rats fed 15 g/day of a 1.2% Ca diet alone or with added sodium oxalate at 0.5%, 1.0%, or 2.0% for a total of 18 weeks. Abbreviations are the same as in Figure 1.

with controls, and an increase with 2.0% dietary oxalate compared with 0.5% and 1.0% oxalate.

With respect to urinary volume, over the first six weeks there was no difference in urinary volume in any group (Fig. 3A). Over the second six weeks, there was an increase in urinary volume with 1.0% and 2.0% oxalate compared with controls and with 2.0% oxalate compared with 1.0% oxalate. Over the third six weeks, there was an increase in urinary volume with 2.0% oxalate compared with controls. With respect to urinary creatinine, there was no difference in urinary creatinine in any group during the entire study (Fig. 3B).

There was no difference in urinary potassium in any group during any time period of the study (data not shown). Over the second six-week period, urinary ammonium was increased in the group receiving 2% oxalate compared with the controls and the group receiving 0.5% oxalate; there was no difference in urinary ammonium between any other group at any other time period of the study (data not shown). Over the second six-week period, urinary citrate was increased in the group receiving 2% oxalate compared with the controls. There was no difference in urinary citrate between any other group at any other time period of the study (data not shown). During the second and third periods, urinary uric acid was lower in all of the groups receiving oxalate compared with controls. There was no difference in urinary uric acid between any other group at any other time period of the study (data not shown).

Urinary supersaturation

Using the individual ion concentrations, the urinary supersaturation with respect to CaOx and $CaHPO_4$ for each of the rats in the each of the groups was calculated separately and was then averaged over the first six weeks, the second six weeks, and the final six weeks.

With respect to CaOx supersaturation, over the first six weeks, there was no change in supersaturation with the addition of oxalate (Fig. 4A). Over the second six weeks, there was a decrease in CaOx supersaturation with 2.0% dietary oxalate compared with controls, 0.5% and 1.0% dietary oxalate. Over the final six weeks, there

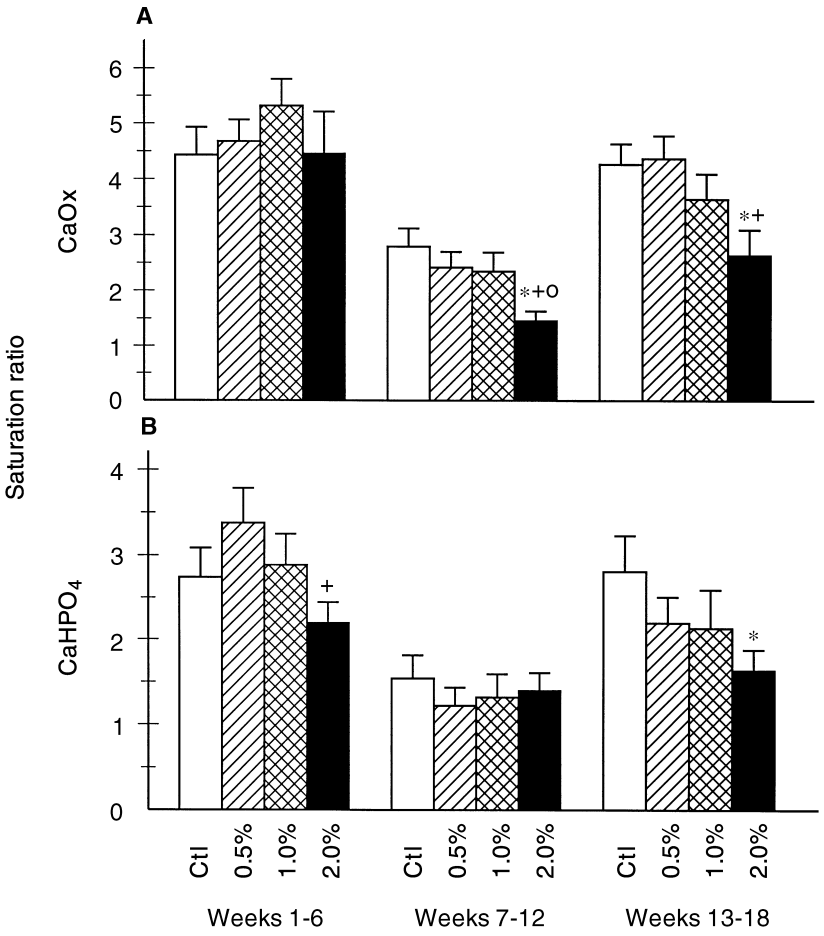

Fig. 4. Relative saturation ratio of calcium oxalate (CaOx) and brushite ($CaHPO_4$) in GHS rats fed 15 g/day of a 1.2% Ca diet alone or with added sodium oxalate at 0.5%, 1.0%, or 2.0% for a total of 18 weeks. Abbreviations are the same as in Figure 1.

was a decrease in CaOx supersaturation with 2.0% dietary oxalate compared with controls and 1.0% dietary oxalate.

With respect to $CaHPO_4$ supersaturation, over the first six weeks, there was a decrease in supersaturation with the addition of 2.0% dietary oxalate compared with 0.5% oxalate (Fig. 4B). Over the second six weeks, there was no change in supersaturation with the addition of oxalate. Over the final six weeks, there was a decrease in $CaHPO_4$ supersaturation with 2.0% dietary oxalate compared with controls.

Stone formation and identification

At the conclusion of the study, 18 weeks, all rats in all groups were found to have formed stones. We observed solitary stones, multiple stones, and ureteral stones (Fig. 5). The electron diffraction pattern of several representative calculi from each of the groups revealed the typical diffraction pattern of poorly crystalline apatite in all cases (Fig. 6). X-ray microanalysis of several representative calculi from each of the groups demonstrated only the presence of calcium and phosphorus (Fig. 7).

DISCUSSION

We used a well-characterized animal model of idiopathic hypercalciuria [5–15] to determine the effect of alterations in dietary oxalate on urinary ion excretion and supersaturation with respect to the key solid phases CaOx and calcium hydrogen phosphate ($CaHPO_4$). We found that increasing dietary oxalate led to an increase in urinary oxalate and phosphate excretion and a fall in calcium excretion. Increasing dietary oxalate did not increase urinary CaOx supersaturation; rather, it fell at the highest level of oxalate studied. Increasing dietary oxalate did not alter the universal occurrence of calcium phosphate stones observed in these rats.

Increasing dietary oxalate intake led to an increase in urine oxalate excretion. In rats, oxalate is absorbed by a nonsaturable, energy-independent mechanism in the duodenum, jejunum, ilium, and colon [46]. Oxalate is an end product of several metabolic pathways, including metabolism of glyoxylate and ascorbic acid [47, 48]. The quantity of urinary oxalate is the sum of intestinal absorption and endogenous production [16]. The increase in dietary oxalate intake would presumably not alter

Fig. 5. Electron micrograph of a representative rat stone embedded in Spurr epoxy resin without any chemical fixation or dehydration, showing agglomerates of needle-like crystals of apatite (magnification ×13,350).

endogenous oxalate production, but would increase the quantity of oxalate available for absorption and subsequent excretion.

As dietary oxalate was increased, there was a concomitant fall in urinary calcium. This may be secondary to increased dietary oxalate binding the constant amount of dietary calcium, resulting in an unabsorbable solid phase of CaOx [21–25]. As dietary oxalate was increased, there was an increase in urinary phosphorus. As is well known from clinical nephrology, intestinal calcium binds with phosphate to prevent its absorption [49, 50]. The binding of intestinal calcium with the increasing amounts of oxalate would decrease calcium available for phosphate binding. Thus, as dietary oxalate was increased, more phosphate should be available for intestinal absorption, resulting in increased urinary phosphorus excretion. Alternatively, urinary phosphorus excretion could have increased due to the increased urinary sodium excretion, or perhaps, the decrease in absorbed calcium might have increased the serum level of parathyroid hormone, resulting in enhanced phosphaturia [51].

With increasing dietary oxalate, provided as sodium oxalate, there was an expected increase in urinary sodium [52]. Increasing urine sodium would increase urine calcium excretion [53–55]; however, with increasing dietary oxalate, urine calcium excretion actually fell, indicating that the effect of oxalate to bind intestinal calcium was greater than the effect of sodium to increase urinary calcium excretion. If the oxalate were administered without sodium, it is likely that there would have been a greater fall in urinary calcium, resulting in even lower CaOx supersaturation. With increasing dietary oxalate, urine pH rose. Perhaps the increase in urinary phosphorus bound tubular hydrogen ions, leading to an increase

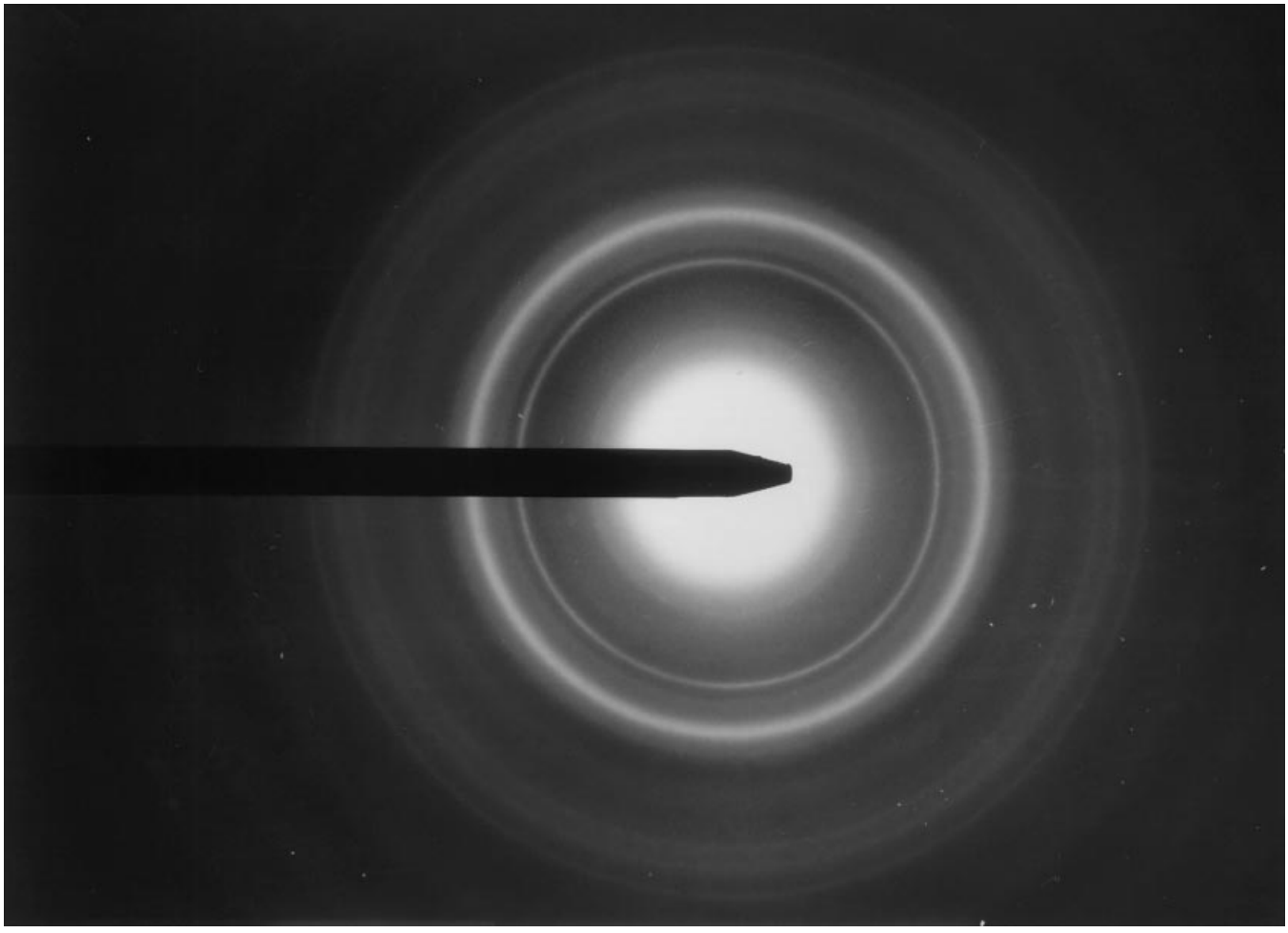

Fig. 6. Electron diffraction pattern of the rat kidney stone from Figure 5 showing the characteristic D spacings of apatite.

in titratable acidity so that the protons were excreted at a higher urinary pH. Although the presumed mechanisms for the alteration in urinary ions appear to result from well-accepted metabolic interactions, further studies will be necessary to confirm the precise relationships that alter the absorption and subsequent excretion of individual ions and ionic complexes when dietary oxalate is altered.

With increasing dietary oxalate, the increase in urinary oxalate was offset by the decrease in urinary calcium so that there was no increase in CaOx supersaturation. At the highest oxalate intake, the increase in urinary volume, in concert with the changes in ion excretion, led to an actual fall in CaOx supersaturation during the second and third time periods. Similarly, the increase in urinary phosphate induced by the increase in dietary oxalate was offset by the decrease in urinary calcium so that there was no increased supersaturation with respect to $CaHPO_4$.

The GHS rats uniformly make a poorly crystalline apatite stone, a solid phase composed only of calcium and phosphate without detectable oxalate [9]. Additional dietary oxalate did not influence the type of stones formed, which is not surprising as there was no increase in supersaturation with respect to the solid phase of CaOx and no change in supersaturation with respect to the solid phase of $CaHPO_4$. However, the supersaturation with respect to the CaOx solid phase is far greater than the supersaturation with respect to the $CaHPO_4$ solid phase, which suggests that the GHS rats should form a CaOx solid phase. However, we have previously shown that in the GHS rats, increasing urinary CaOx supersaturation led to an increase in the formation product, the saturation at which the solid phase will spontaneously precipitate, for this solid phase [5]. We have confirmed these results in humans [56]. Thus, examination of supersaturation is necessary, but not sufficient, to predict the formation of a solid phase.

Humans with idiopathic hypercalciuria who form CaOx stones are often told to limit oxalate intake in an effort to decrease oxalate excretion and decrease CaOx stone formation [1, 16, 17]. Although there may by intu-

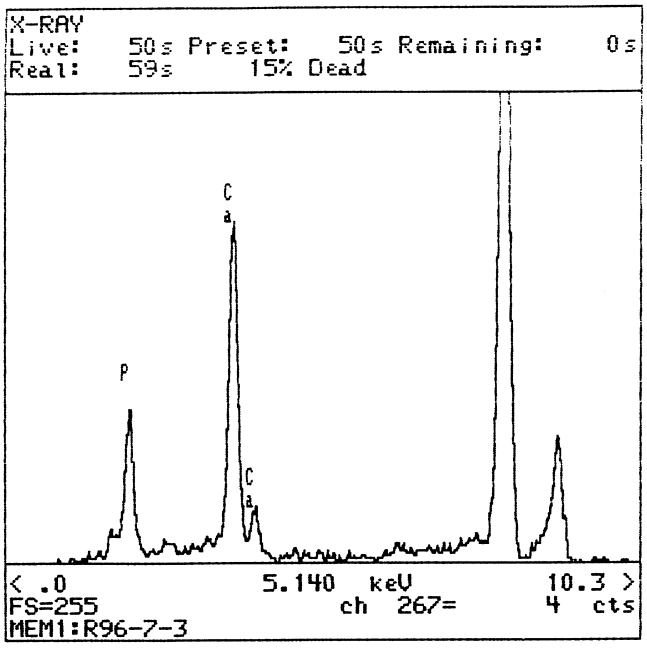

Fig. 7. X-ray microanalysis of a selected area from the above micrograph showing that only calcium and phosphorus are present in the calcified area. The peak at higher energy comes from the copper grid.

itive logic to this approach, there are no experimental studies that support this therapy. The GHS rat appears to be a reasonable model of human idiopathic hypercalciuria [5–15]. The results from this study, in which increasing dietary oxalate led to an actual decrease in CaOx supersaturation in the GHS rats, suggest that limiting dietary oxalate in hypercalciuric humans may be of no therapeutic benefit in preventing CaOx stone formation. Further studies in which humans with idiopathic hypercalciuria are fed graded amounts of oxalate, their supersaturation and formation products determined, and their rate of stone formation documented, are necessary to confirm the results of this short-term animal study in humans.

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