Sulfate homeostasis, NaSi-1 cotransporter, and SAT-1 exchanger expression in chronic renal failure in rats

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Background. It is known that hypersulfatemia, like hyperphosphatemia, occurs in chronic renal failure (CRF). The aim of this study was to assess the effects of CRF on sulfate homeostasis and on sodium sulfate cotransport (NaSi-1) and sulfate/oxalate-bicarbonate exchanger (Sat-1) expression in the kidney. In addition, sulfate homeostasis was compared with phosphate homeostasis.

Methods. Experimental studies were performed in adult male rats at three and six weeks after 80% subtotal nephrectomy (Nx) or sham-operation (S) ($N = 9$ per group). Transporter protein and mRNA expressions were measured by Western blot and RNase protection assay (RPA), respectively. Results were quantitated by densitometric scanning (Western) and electronic autoradiography (RPA), and were expressed in densitometric units (DUs; Western) and cpm (RPA).

Results. Creatinine clearance was lower in Nx-3 compared with S-3 rats (0.23 vs. 0.51 mL/min/100 g body weight, $P < 0.001$) and was further impaired in Nx-6 rats (0.15 vs. 0.48, $P < 0.001$). Sulfatemia was significantly higher in Nx-3 rats (1.08 vs. 0.84 mmol/L, $P < 0.05$) and further increased in Nx-6 rats (1.42 vs. 0.90 mmol/L, $P < 0.01$). Fractional sulfate excretion (FE_{SO4}) was increased by twofold in Nx-3 and Nx-6 rats compared with corresponding S rats. Phosphatemia did not differ between Nx-3 rats and controls, but was increased in Nx-6 rats ($P < 0.01$). Total amounts of both NaSi-1 and Sat-1 proteins were significantly decreased in both Nx-3 and Nx-6 rats when compared with controls. However, NaSi-1 protein and mRNA densities did not significantly change in Nx-3 rats, but were significantly increased in Nx-6 rats when compared with controls (4.8 vs. 3.7 DU/µg protein, $P < 0.05$, and 7.1 vs. 2.8 cpm/µg RNA, $P < 0.01$, respectively, for protein and mRNA). In contrast to NaSi-1, Sat-1 protein density was significantly decreased both in Nx-3 (2.9 vs. 3.6 DU/µg protein, $P < 0.05$) and Nx-6 rats (2.4 vs. 3.4 DU/µg protein, $P < 0.05$), and Sat-1 mRNA density significantly decreased in Nx-6 rats (10.7 vs. 14.7 cpm/µg RNA, $P < 0.05$). Na-PO$_4$ cotransporter (NaPi-2) protein total abundance and density were decreased at three and six weeks in Nx rats.

Conclusions. These results demonstrate that both NaSi-1 and Sat-1 total protein abundances are decreased in CRF, which may contribute to the increase in fractional sulfate excretion. Strikingly, NaSi-1 density was not decreased in CRF three weeks after Nx, and furthermore, increased six weeks after Nx, in contrast to NaPi-2 density, which was decreased at both times. The significance of this difference remains to be determined, but may explain why hypersulfatemia occurs earlier than hyperphosphatemia in CRF.

It is well known that hypersulfatemia, like hyperphosphatemia, occurs in chronic renal failure (CRF) [1–5]. Plasma sulfate concentrations can increase eightfold in patients with severe reductions in glomerular filtration rate (GFR) and may exceed 2.5 mmol/L [2, 6]. In contrast to phosphate, very little is known about the cellular and molecular mechanisms of sulfate regulation in the clinical setting of renal impairment.

The kidney plays a crucial role in maintaining sulfate homeostasis. Sulfate is freely filtered at the glomerulus and reabsorbed in the proximal tubule. Only 10 to 15% of the filtered load is excreted in urine. The driving force for sulfate tubular transport from lumen within the tubular epithelial cell is dependent on the transmembrane sodium gradient [2, 7]. Once sulfate has been accumulated, its exit across the peritubular face of the cell is mediated by anion exchange. Two sulfate transporters, a sodium-sulfate (Na-SO$_4$) cotransporter (NaSi-1) and a sulfate/oxalate-bicarbonate anion exchanger (Sat-1), have been identified [8–10] and implicated in this physiological process. NaSi-1 and Sat-1 proteins have been localized to the apical and basolateral membrane of the proximal tubule, respectively [11, 12]. In addition to the proximal tubule, NaSi-1 is expressed in the ileum [13], while Sat-1 is also expressed in the liver [9]. These two transporters are thought to be responsible for inorganic sulfate entry and exit from the proximal tubular cells.

The renal NaSi-1 cotransporter has been shown to be regulated in various physiological and pathophysiological alterations of sulfate homeostasis. Regulation of the NaSi-1 mRNA and protein by dietary sulfate intake has been demonstrated [14, 15]. The decreased serum sulfate concentrations observed in vitamin D deficiency [16], hypothyroidism [17], chronic hypokalemia [18], and ibuprofen administration [19] have been attributed to a down-regulation of the NaSi-1 cotransporter mRNA and protein. Therefore, all of these studies confirm that the NaSi-1 cotransporter plays a key role in sulfate homeostasis by modulating the renal reabsorption of the anion. Moreover, Sat-1 could also contribute to the maintenance of sulfate homeostasis, although no data are available on the regulation of this transporter in health and disease.

The clinical sequelae of hypersulfatemia in CRF are unknown. Sulfate is not thought of as a "uremic toxin," although this remains the subject of controversy [2]. However, chronic hypersulfatemia, like hyperphosphatemia, could contribute to the abnormalities of calcium metabolism observed in CRF and thereby to the pathogenesis of renal osteodystrophy. Indeed, an increase in calcium excretion has been observed when extracellular sulfate concentration increases [5, 6, 20, 21]. Hypersulfatemia, by leading to complex formation with calcium, can also induce hypocalcemia, thereby aggravating secondary hyperparathyroidism [6]. Maintenance of sulfate homeostasis may therefore be clinically important in CRF. However, to date there are no studies on the mechanisms involved in the development of hypersulfatemia and, in particular, on the regulation of NaSi-1 and Sat-1 in CRF.

The aim of this study was to assess the effects of CRF in rats on (*1*) serum sulfate concentrations, (*2*) the renal clearance of sulfate, and (*3*) the expression of the two sulfate transporters, NaSi-1 and Sat-1. In addition, we also compared sulfate with phosphate homeostasis and the expression of sulfate transporters with that of the sodium-phosphate (Na-PO$_4$) cotransporter (NaPi-2).

METHODS

Animals and diet

Male Sprague-Dawley rats (Iffa Credo, Saint Aubin les Elbeuf, France) weighing 130 to 150 g were used and were subjected to renal ablation as described [22–24]. Approximately 80% of total kidney mass was removed using a two-stage procedure to induce CRF. First, the two poles of the left kidney were consecutively excised and weighed. The amount of excised parenchyma was 60% of the left kidney mass, based on the mean kidney weight (0.47 ± 0.02 g/100 g body weight) of five control rats raised under the same conditions. Bleeding was prevented by finger pressure at sites of excision and coating cut surfaces with collagen powder (Pangen, Fournier,

France). At the second stage four days later, the right kidney was removed. Control rats underwent laparotomy and kidney decapsulation in parallel with uremic rats. All animals received a standard diet containing 20% casein, 0.57% calcium, 0.23% sulfate, 0.46% phosphate, and vitamin D$_3$ 100 IU/100 g diet. All rats were fed ad libitum. There was no significant difference in the calculated protein intake between the four groups described later in this article.

Experimental protocol

Renal insufficiency was characterized by comparing results obtained in control sham-operated rats (S) and uremic rats subjected to 80% nephrectomy (Nx). Two experimental studies were performed at three and six weeks after nephrectomy. Four groups of nine rats each were studied: controls (S-3 and S-6) and nephrectomized (Nx-3 and Nx-6). For each group, 24-hour urine collections were carried out to determine creatinine, sulfate, phosphate, and calcium concentrations before sacrifice. Euthanasia was performed by intraperitoneal injection of nembutal (50 mg/kg body weight). Blood was taken from the jugular vein for the determination of biochemical parameters. The abdomen was then opened, and the whole kidney(s) was rapidly excised and cut in the sagittal plane. One half of the Nx kidney(s) or one S kidney was immediately immersed into liquid nitrogen, the other half kidney(s) for Nx or whole kidney for S rats was utilized immediately for brush-border membrane preparation. Frozen tissues were later weighed, pulverized, and freeze dried in liquid nitrogen using a Spex 6700 Freezer (Mill industries Inc., Edison NJ, USA). Renal powder was kept in liquid nitrogen, and different aliquots were used for (*1*) determination of the protein content, (*2*) RNA extraction, and (*3*) crude membrane extraction.

Techniques

Plasma and urine measurements. Plasma and/or urinary urea, creatinine, phosphate, calcium, potassium, and bicarbonate concentrations were measured by standard laboratory techniques on a Hitachi automatic analyzer (Boehringer Mannheim, Meylan, France). Inorganic plasma and urinary sulfate concentrations were determined by capillary electrophoresis as described [16] with indirect photometric detection using equipment and reagents supplied by Hewlett-Packard (Cheshire, UK). Plasma and urinary samples were analyzed twice at 1/5 and 1/10 dilutions, respectively. Sulfate concentrations were determined by peak area integration using HP^{3}DCE chemstation data-handling software. Plasma 1,25(OH)$_2$ vitamin D was determined as previously described [25]. Serum parathyroid hormone (PTH) was determined using a rat PTH immunoradiometric assay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA).

Crude renal membrane preparations

Crude renal membrane (CM) preparations were carried out by a modification of the method described by Marx, Fedak, and Aurbach [26]. One hundred to 150 mg of frozen renal powder were homogenized in 1.5 mL isotonic medium containing 0.25 mol/L sucrose, 10 mmol/L Tris, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA; pH 7.5). Homogenization was carried out using a Teflon potter ($2000 \times g$ for 1 minute). Kidney homogenates were centrifuged at $2500 \times g$ at 4°C. Centrifugation was stopped when the rotor speed reached $2500 \times g$. The supernatants were then collected and centrifuged for an additional 15-minute period at $2500 \times g$ at 4°C. The resulting supernatants were discarded, and the pellets were resuspended in 100 µL buffer solution containing 10 mmol/L Tris and 1 mmol/L EDTA (pH 7.5). Renal membranes preparations were stored at −80°C until determination of protein concentration and Western blot analysis. Proteins were measured using Pierce Coomassie protein assay reagent (Pierce Chemical Co., Rockford, IL, USA) based on the Bradford method [27].

Brush-border membrane preparations

Brush-border membrane (BBM) from kidney cortices were prepared by the Mg^{2+} precipitation method, as previously described [28]. Kidney cortex slices were homogenized in 15 mL isolation buffer (300 mmol/L mannitol, 5 mmol/L EGTA, 12 mmol/L Tris-HCl, pH 7.1), using a polytron. Twenty milliliters of H_2O (4°C) followed by $MgCl_2$ from a 1 mol/L stock solution up to a final concentration of 12.5 mmol/L were then added. The solution was kept on ice for 15 minutes and then centrifuged for 15 minutes at 4°C at $2500 \times g$. The resulting supernatant was centrifuged at $30,000 \times g$ for 30 minutes (4°C). The final pellet (BBM) was suspended in 20 mmol/L Tris-HCl, 4 mmol/L $MgCl_2$, 20 mmol/L HEPES (pH 7.5) containing 300 mmol/L mannitol. Renal BBMs were stored at −80°C until determination of protein concentration and Western blot analysis.

γ-Glutamyltransferase specific activity

The activity of γ-glutamyltransferase (γ-GT; (C2.3.2.2), an established enzyme marker of BBM, was determined as described [24] using the technique of Orlowski and Meister [29]. Specificity was assessed by measuring the activity in duplicate samples to which stop solution (acetic acid 1.5 N) was added prior the start of the enzymatic reaction.

Brush-border membrane and crude membrane proteins electrophoresis and Western blots

Experiments were performed as previously described [16]. For NaSi-1 and NaPi-2 protein determinations, BBM proteins were denatured in 95 mmol/L Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.5 mmol/L EDTA, and 100 mmol/L dithiotreitol (final concentrations). Forty micrograms of BBM proteins per lane were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel according to the method of Laemmli [30], and the samples were electrotransferred onto nitrocellulose membranes. After blocking the membrane with Tris-buffered saline (TBS; pH 7.4) containing 10% skimmed dried milk (SDM) and 1% triton X-100, membranes were incubated overnight at 4°C with a 1:5000 dilution of the rabbit polyclonal antibody specific for the rat NaSi-1 cotransporter (kindly provided by Dr. J. Biber, Zurich, Switzerland) [11, 16]. This antibody allows the detection of a specific signal for NaSi-1 cotransporter only on BBM preparations, but not on crude membranes (personal experience) [11, 16]. For NaPi-2 protein detection, membranes used for NaSi-1 protein detection were stripped and reprobed. The membranes were washed twice with phosphate-buffered saline (PBS; pH 7.5) containing 0.2% Tween, and blocked with PBS-0.2% Tween containing 10% SDM during one hour. Membranes were incubated overnight at 4°C with a 1:5000 dilution of the rabbit polyclonal antibody specific for the rat NaPi-2 cotransport (kindly provided by Drs. H. Murer and J. Biber, Zurich, Switzerland) [31] in PBS-0.2% Tween containing 5% SDM. For Sat-1 protein detection, 30 µg CM proteins were denatured in the same buffer as for BBM but without dithiotreitol, warmed at 95°C for five minutes, and electrophoresed on 10% acrylamide gels. Samples were electrotransferred onto nitrocellulose membranes, which were blocked with TBS buffer containing 1% triton X-100 and 10% SDM. Membranes were incubated overnight at 4°C with a 1:200 dilution of the mouse monoclonal antibody specific for rat Sat-1 kindly given by Dr. L.P. Karniski [12]. For β-actin protein detection, membranes initially used for either NaSi-1 or Sat-1 measurements were blocked with TBS-1% triton X-100 containing 5% SDM and incubated with a 1:10,000 dilution of monoclonal anti–β-actin antibody (Sigma, St. Louis, MO, USA). For 5′ nucleotidase protein detection, membranes initially used for NaSi-1 and Sat-1 measurements were blocked with PBS-Tween 0.05% containing 10% SDM and incubated with a 1:5000 dilution of a rabbit polyclonal anti rat 5′-nucleotidase in PBS-Tween 7.5% [32]. Antibody binding was detected by chemiluminescence using enhanced chemiluminescence (ECL) Western blotting protocol (Amersham, Life Science, Buckinghamshire, UK), as recommended by the manufacturer, using anti-rabbit or anti-mouse IgG whole antibody from donkey and sheep, as appropriate, linked to horseradish peroxidase. Stained membranes were exposed to ECL films, and quantitation of the signals was achieved by densitometric scanning using the Image Quant software package (Molecular Dynamics Inc., Sunnyvale, CA, USA)

under conditions for which linearity was observed, and signal intensity was expressed in densitometric units (DUs).

RNA extraction and ribonuclease (RNAse) protection assays (RPA)

Total RNA from 100 to 150 mg of frozen renal powder was extracted by the phenol/guanidium thiocyanate method with an "RNA plus" Kit (Quantum, Montreuil, France). RPA were performed as previously described [16, 33]. All labeled antisense probes were generated by in vitro transcription using T7 RNA polymerase (Promega France, Charbonnières, France) and $\alpha[^{32}P]$ UTP. A previously described construct containing a fragment of cDNA coding for rat NaSi-1 cloned into PCR II vector (TA cloning kit; Invitrogen, San Diego, CA, USA) was used to synthesize a NaSi-1 riboprobe of 373 bp that produced a specific protected fragment of 266 bp in the RNAse protection assay (RPA) [16]. For Sat-1, a 300 bp fragment was amplified from rat kidney cDNA using the oligonucleotides 5′-TTAAGAGCCAGGGTGATT-3′ (upper primer) and 5′-CAGCCAGCGTATGACAGG AAA-3′ (lower primer). The fragment, corresponding to nucleotides 248 to 527 in the sequence published by Bissig et al [9], was cloned into pTag vector (The Ligator; R&D Systems, Abingdon, UK). The vector was linearized with *Spe* I, and a riboprobe of 412 bp was synthesized that produce a Sat-1–specific protected fragment of 300 bp in the RPA. A previously described construct containing a fragment of cDNA coding for GAPDH cloned into Bluescript II SK+ was used to synthesize a GAPDH riboprobe of 192 bp that produced a protected fragment of 164 bp [16, 24]. A construct containing a fragment of cDNA coding for β-actin cloned in pGEM 3.2 was used to synthesize a β-actin riboprobe. The vector was linearized with *Bsu*361, and a riboprobe of 158 bp was synthesized that produced a specific protected fragment of 137 bp in the RPA.

To perform RPA, 20 μg of total RNA extracted from the rat kidneys were hybridized overnight at 50°C with 5×10^5 cpm NaSi-1 or Sat-1 and 5×10^4 cpm GAPDH and β-actin riboprobes. The radioactive mRNA hybrids were separated by electrophoresis following RNAse digestion, and the radioactivity present in the bands corresponding to the protected fragments of NaSi-1 cotransporter and Sat-1 exchanger was quantitated by electronic autoradiography (Instant Imager; Packard Instruments, Meriden, CT, USA) and expressed in radioactive counts per minute (cpm).

Expression of results

γ-Glutamyltransferase enrichment of renal BBM was calculated as the ratio of γ-GT specific activity in BBM over that in initial kidney cortex homogenates.

Transporter protein amounts were expressed in the following: (*1*) density/μg protein, defined as DUs/μg BBM (NaSi-1 or NaPi-2) or μg CM (Sat-1) proteins; (*2*) total abundance, by multiplying the density in DU/μg protein (described previously in this article) by the total amount of renal BBM or CM proteins obtained from kidneys of control rats or the remnant kidney of Nx rats; and (*3*) density/nephron, by dividing the total abundance of transporter by the number of nephrons, designated N in control rats. In Nx rats, the number of nephrons was estimated to be the fraction of residual nephrons that remained immediately after the 4/5 Nx, that is, N/5. This estimation was based on the assumption that the number of residual nephrons in Nx rats is proportional to the fraction of remnant kidney, as previously described [34]. Similarly, GFR/residual nephron in Nx rats was estimated by dividing the GFR in mL/min in Nx rats by N/5.

Similar to transporter protein amounts, NaSi-1 and Sat-1 mRNA amounts were expressed in the following: (*1*) density/μg RNA, using cpm/μg renal RNA; and (*2*) total abundance, calculated by multiplying the density obtained previously by the total amount of renal RNA obtained from kidneys of control rats or the remnant kidney of the Nx rats.

Statistical analysis

Data are presented as means ± SEM. The number of determinations (*N*) is indicated in the table and figure legends. Statistical analysis was performed using one-way analysis of variance (ANOVA), and comparisons between groups were made using Newman-Keuls test [35]. Significance was taken for *P* values < 0.05.

RESULTS
Morphometric measurements

Total body weights and weight gain per day of subtotally Nx rats were significantly lower at the time of sacrifice after the three- and six-week experimental period compared with that of the appropriate control group (Table 1). Kidney weights (in g/100 g body weight) of Nx rats were significantly higher compared with corresponding controls. Renal proteins (in mg/100 g body weight) were similar for S-3 and Nx-3 rats and were significantly higher in Nx-6 rats compared with the values in S-6 rats. Development of renal hypertrophy in Nx rats was further indicated by an increase in total renal proteins following surgery: (*1*) an approximate twofold increase in renal protein at three weeks following surgery, if one estimates that 90 mg protein were left after 80% nephrectomy (*P* < 0.001); and (*2*) a further 1.5-fold increase in renal proteins at six weeks compared with that at three weeks after surgery (*P* < 0.001).

Effect of subtotal nephrectomy on plasma biochemistry

Plasma urea and creatinine levels were significantly higher in Nx-3 rats compared with controls and were

Table 1. Morphometric measurements in control (sham-operated; S-3, S-6) and subtotally nephrectomized rats (Nx-3, Nx-6) at three and six weeks

	Experimental group			
Parameters	S-3 N = 9	Nx-3 N = 9	S-6 N = 9	Nx-6 N = 9
Body weight g	320 ± 12.5	291 ± 4.5[dg]	409 ± 15.0[fh]	355 ± 18.0[deg]
Weight gain[a] g/day	7.2 ± 0.37	6.02 ± 0.19[dg]	5.6 ± 0.23[fg]	4.4 ± 0.40[deg]
Kidney weight[b] g/100 g body weight	0.37 ± 0.01	0.48 ± 0.02[dh]	0.36 ± 0.01	0.92 ± 0.06[dh]
Renal proteins[b] mg/100 g body weight	64.5 ± 4.2	63.6 ± 3.0	68.2 ± 1.5	81.0 ± 6.2[deg]
Total renal proteins[c] mg	413 ± 27.3	185 ± 8.8[dh]	558 ± 12.5[fh]	288 ± 22.5[def]

Results are expressed as mean ± SEM.
[a] Weight gain/day was measured between the time of surgery and the time of sacrifice
[b] Results obtained for one kidney for controls and for the remnant kidney for Nx rats
[c] Results obtained for the two kidneys for controls and for the remnant kidney for Nx rats
[d] Comparisons between the Nx group (Nx-3 and Nx-6) and the corresponding control group (S-3 and S-6)
[e] Comparisons between the two Nx groups
[f] Comparisons between the two control groups
[g] $P < 0.01$
[h] $P < 0.001$

Table 2. Plasma biochemical parameters in the four groups of rats

	Experimental group			
Parameters	S-3	Nx-3	S-6	Nx-6
Urea mmol/L	5.0 ± 0.35 (3.3–6.9)	14.0 ± 1.08[a,d] (8.9–17.8)	6.0 ± 0.29 (4.2–6.9)	30.8 ± 4.63[a,b,e] (207–65.5)
Creatinine μmol/L	40.6 ± 1.22 (35–47)	65.6 ± 1.94[a,d] (54–71)	46.4 ± 1.82 (40–56)	116.4 ± 14.28[a,b,e] (86–214)
Total calcium mmol/L	2.71 ± 0.018 (2.61–2.77)	2.70 ± 0.044 (2.50–2.89)	2.57 ± 0.018 (2.47–2.65)	2.25 ± 0.118[a,b,e] (1.62–2.59)
Potassium mmol/L	3.82 ± 0.114 (3.4–4.3)	4.88 ± 0.106[a,e] (4.2–5.2)	4.23 ± 0.119 (3.7–4.6)	4.96 ± 0.260[a,d] (4.3–6.6)
Bicarbonate mmol/L	23.3 ± 0.24 (22–24)	21.1 ± 0.42[a,d] (20–24)	21.7 ± 0.18[c,d] (21–22.5)	17.33 ± 0.73[a,b,d,e] (14–21)
PTH pg/mL	135 ± 15.2 (86–190)	408 ± 73.3[a,d] (241–967)	132 ± 19.0 (69–202)	1154 ± 306.8[a,b,e] (182–3408)
Calcitriol pmol/L	409 ± 18 (323–451)	418 ± 36 (347–566)	313 ± 18 (244–373)	216 ± 52[b,d] (37–417)
Sulfate mmol/L	0.84 ± 0.038 (0.70–1.04)	1.08 ± 0.088[a,d] (0.88–1.69)	0.90 ± 0.029 (0.79–1.01)	1.42 ± 0.136[a,b,e] (1.03–2.36)
Phosphate mmol/L	2.49 ± 0.074 (2.19–2.87)	2.52 ± 0.267 (2.01–3.23)	1.92 ± 0.046[c,d] (1.76–2.17)	3.35 ± 0.360[a,b,e] (2.43–5.84)

Values are expressed as mean ± SEM, and the ranges of values are indicated in parentheses, N = 9 except for calcitriol, for which N = 6.
[a] Comparisons between CRF (Nx-3 and Nx-6) and the corresponding control group (S-3 and S-6)
[b] Comparisons between the two Nx groups
[c] Comparisons between the two control groups
[d] $P < 0.01$
[e] $P < 0.001$

further increased in the Nx-6 rats (Table 2). Total plasma calcium levels did not differ between the Nx-3 and control rats, whereas they were significantly lower in the Nx-6 rats when compared with the S-6 controls. Plasma potassium levels were slightly but significantly higher in both Nx-3 and Nx-6 rats than in control groups. Plasma bicarbonate levels were significantly lower in Nx-3 rats compared with the levels in the S-3 controls and further decreased in Nx-6 rats. Serum immunoreactive PTH levels were significantly higher in the Nx-3 rats compared with the levels in S-3 controls and were increased further in Nx-6 rats. 1,25(OH)₂D levels did not differ between Nx and control rats. However, they were significantly lower in Nx-6 rats compared with that in Nx-3 rats.

Effect of subtotal nephrectomy on sulfate and phosphate homeostasis

Plasma. Plasma sulfate levels were significantly higher in Nx-3 rats compared with S-3 controls and were increased more in Nx-6 rats (Table 2). There was no significant difference in sulfatemia between the two control groups. Plasma phosphate levels, in contrast to sulfate levels, did not differ between Nx-3 and control rats (Table 2). However, they were significantly higher in Nx-6 rats compared with that in S-6 controls (Table 2). Phosphatemia in the S-6 control group was lower than that in the S-3 control group, possibly because of physiological variation as a function of age [36].

Urine. Twenty-four–hour urine volumes were approx-

Table 3. Urine parameters in the four groups of rats

	Experimental group			
Parameters	S-3	Nx-3	S-6	Nx-6
Urine volume *mL/24 hr*	11.3 ± 1.3	$24.2 \pm 3.9^{a,c}$	9.0 ± 0.7	$30.0 \pm 1.8^{a,c}$
Creatinine clearance *mL/min/100 g body weight*	0.51 ± 0.03	$0.23 \pm 0.02^{a,c}$	0.48 ± 0.03	$0.15 \pm 0.02^{a,c}$
SO_4 excreted *μmol/24 hr/100 g body weight*	42.2 ± 3.7	$55.4 \pm 4.6^{a,b}$	40.6 ± 3.0	46.2 ± 4.5
FE_{SO_4} %	7.4 ± 0.8	$16.7 \pm 1.7^{a,c}$	6.9 ± 0.7	$17.6 \pm 1.1^{a,c}$
PO_4 excreted *mmol/24 hr/100 g body weight*	0.49 ± 0.04	0.55 ± 0.03	0.34 ± 0.03	$0.31 \pm 0.03^{a,c}$
FE_{PO_4} %	28.3 ± 2.11	$68.8 \pm 7.59^{a,c}$	27.7 ± 3.31	$49.7 \pm 2.53^{a,c}$

Values are expressed as mean $\pm$ SEM. Nine animals were studied for each group.
[a] Comparisons between CRF (Nx-3 and Nx-6) and the corresponding control group (S-3 and S-6)
[b] $P < 0.05$
[c] $P < 0.01$. Fractional excretion (FE) for SO_4 (and PO_4) was calculated following this formula:
$FE_{SO_4} = [SO_4]_{urine} \times [creatinine]_{plasma}/[SO_4]_{plasma} \times [creatinine]_{urine} \times 100$

imately twofold and threefold higher in Nx-3 and Nx-6 rats, respectively, compared with the volumes in the corresponding control groups (Table 3). As expected, creatinine clearances were lower in Nx-3 rats than those in control rats with normal renal function and even more decreased in Nx-6 rats (Table 3). There was no significant difference in creatinine clearances between the two control groups. Fractional sulfate excretion was approximately twice higher in both the Nx-3 and Nx-6 groups compared with those in the corresponding control groups ($P < 0.001$; Table 3). Sulfate excretion was significantly increased in Nx-3 compared with S-3 group but was similar in Nx-6 compared with S-6 groups (Table 3). Fractional phosphate excretion was approximately twice as high in both the Nx-3 and Nx-6 groups compared with those in the corresponding control groups ($P < 0.001$; Table 3). Phosphate excretion was similar in Nx rats compared with that in the corresponding control group.

Effect of subtotal nephrectomy on membrane fractionation

To assess whether the uremic state altered membrane fractionation, we measured the enrichment of renal BBM γ-GT specific activity in Nx and control rats (Table 4). In addition, we measured 5'-nucleotidase and β-actin protein expression in BBM and CM, since (1) 5'-nucleotidase is specifically located in BBM and (2) β-actin is a well-known ubiquitously-expressed protein. Results showed that 5'-nucleotidase expression was similar in BBM and CM prepared from kidneys from control and Nx rats at three and six weeks. β-actin protein expression was not affected by CRF in BBM preparations (Table 4). A significant decrease in β-actin expression in CM preparations was observed in Nx-3 rats compared with S-3 rats, but not in Nx-6 rats compared with S-6 rats (Table 4). Finally, the enrichment of renal BBM γ-GT–specific activity was similar in control and CRF rats three- and six-weeks post-Nx (Table 4).

Table 4. β-actin and 5'-nucleotidase expression in brush-border and crude membrane preparations, and brush-border membrane enrichment of γ-glutamyl transferase specific activity in the four groups of rats

	Experimental group			
Parameters	S-3	Nx-3	S-6	Nx-6
β-actin				
DU/μg BBM	9.2 ± 0.7 (7)	8.9 ± 0.6 (9)	8.8 ± 1.0 (7)	7.7 ± 0.5 (8)
DU/μg CM	38.1 ± 1.4 (6)	26.6 ± 3.5^a (6)	39.2 ± 1.4 (7)	34.5 ± 2.4 (8)
5' DU				
DU/μg BBM	8.7 ± 0.8 (9)	9.5 ± 0.8 (9)	11.1 ± 1.2 (9)	7.7 ± 1.2 (9)
DU/μg CM	9.6 ± 0.9 (6)	9.7 ± 0.4 (6)	9.1 ± 0.6 (7)	9.2 ± 0.5 (8)
γ-GT				
Specific activity	10.0 ± 0.3 (8)	9.3 ± 0.7 (7)	9.0 ± 0.7 (8)	10.3 ± 0.7 (8)

Abbreviations are: BBM, brush border membrane; CM, crude membrane; 5' NU, 5'-nucleotidase; DU, densitometric units; γ-GT, γ-glutamyl transferase. Specific activity is expressed in enrichment-fold. γ-GT enrichment was calculated by dividing BBM γ-GT specific activity by renal homogenate γ-GT specific activity. Western blots and γ-GT specific activity in BBM and renal homogenates measurements were performed as described in the **Methods** section. Results are the mean $\pm$ SEM. The numbers of samples are indicated by the numbers in parentheses, each from a different rat.
[a] $P < 0.01$ comparing Nx-3 and S-3

Effect of subtotal nephrectomy on renal NaSi-1 cotransporter and Sat-1 antiport protein expression

Sodium-sulfate (NaSi-1) cotransporter and Sat-1 antiport protein expression were measured by Western blotting. A significant decrease in the total renal abundance of the two transporters was observed comparing values in the Nx-3 and Nx-6 groups with that in the corresponding control groups (Fig. 1). For both transporters, the decrease was more important three weeks than six weeks after nephrectomy (Fig. 1): NaSi-1 and Sat-1 proteins both decreased by a $70 \pm 10\%$ decrease three weeks after nephrectomy ($P < 0.001$), whereas a $46 \pm 7\%$ and a $54 \pm 6\%$ decrease were observed for NaSi-1 and Sat-1 transporters, respectively, six weeks after nephrectomy ($P < 0.01$ and $P < 0.001$).
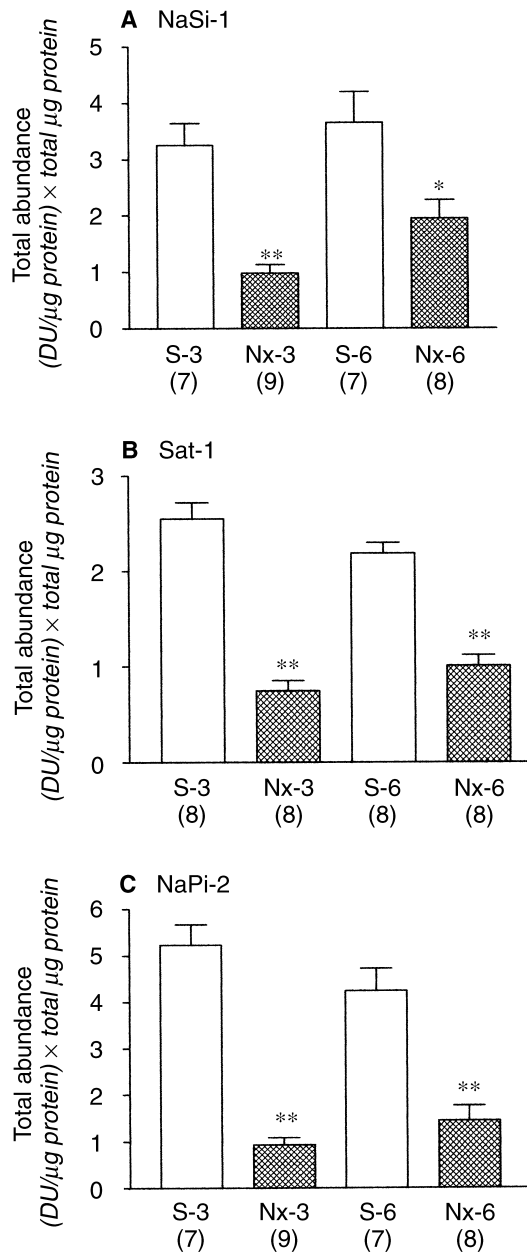
Fig. 1. Renal (*A*) sodium-sulfate cotransporter (NaSi-1), (*B*) sulfate/oxalate-bicarbonate exchanger (Sat-1), and (*C*) sodium-phosphate co-transporter (NaPi-2) total protein abundances in the four groups of rats. Protein expression was measured by immunoblotting on renal protein extracts as indicated in the **Methods** section. Forty micrograms BBM (NaSi-1 or NaPi-2) or 30 μg crude membrane (CM; Sat-1) proteins were analyzed. To quantitate transporter protein expression, the signal in the band corresponding to each transporter was quantitated by densitometry and expressed in densitometric units (DUs). Total abundance was obtained by multiplying the DU/μg BBM (NaSi-1 or NaPi-2) or μg CM (Sat-1) proteins by the total amount of renal BBM or CM proteins obtained from kidneys of control rats or the remnant kidney of the Nx rats. Results are the mean ± SEM. The numbers of samples (*N*) are indicated by the numbers in parentheses below the bars. *$P <$ 0.01 and **$P <$ 0.001 compared with the corresponding control group.

When the NaSi-1 cotransporter amount was expressed in protein density (DU/μg BBM protein), there was no significant difference between the Nx-3 and S-3 groups (Fig. 2). Moreover, NaSi-1 protein density was increased in Nx-6 rats compared with S-6 control rats (30 ± 0.6% increase, $P <$ 0.01; Fig. 2). In contrast, Sat-1 protein density was significantly decreased in both Nx-3 and Nx-6 rats compared with corresponding controls (20 ± 2% and 30 ± 3% decrease in Nx-3 and Nx-6 rats, respectively, compared with corresponding controls, $P <$ 0.01 and $P <$ 0.001; Fig. 2).

Effect of subtotal nephrectomy on BBM Na-PO₄ cotransporter protein amount

Total renal sodium-phosphate cotransporter (NaPi-2) protein abundance in BBM in Nx rats was significantly decreased compared with that in control rats (80 ± 12% and 66 ± 14% decrease in Nx-3 and Nx-6 rats, respectively, $P <$ 0.001; Fig. 1). NaPi-2 protein density (DU/μg BBM protein) was significantly decreased by approximately 40% in both Nx-3 and Nx-6 rats compared with that in corresponding control groups ($P <$ 0.001; Fig. 2).

NaSi-1 and NaPi-2 transporter protein densities and GFR in relationship to the fraction of remnant kidney

Sodium-sulfate cotransporter (NaSi-1) and NaPi-2 estimated densities per residual nephron were not significantly increased at three weeks post-Nx (Table 5). In contrast and as expected, there was a significant twofold increase in the estimated GFR/residual nephron in Nx-3 rats compared with that in controls. At six weeks, NaSi-1 protein density/residual nephron in Nx rats, but not NaPi-2 density/residual nephron was significantly increased. The estimated GFR/residual nephron in Nx-6 rats was similar to that in S-6 controls.

NaSi-1 cotransporter and Sat-1 antiport mRNA expression in kidneys from Nx and control rats was analyzed using RPA. No significant change induced by Nx was observed in total NaSi-1 mRNA abundance, although this latter tended to be higher in Nx-6 rats than in corresponding controls (Fig. 3). NaSi-1 mRNA density (cpm/μg RNA) was similar in Nx-3 and S-3 control rats, whereas the levels were significantly increased in Nx-6 rats compared with those in the controls ($P <$ 0.01; Fig. 4). In contrast to NaSi-1, total Sat-1 antiport mRNA abundance was reduced by approximately 50% in the Nx-3 and in Nx-6 rats compared with that in corresponding controls ($P <$ 0.01; Fig. 3). The Sat-1 antiport mRNA density was similar in Nx-3 and S-3 control rats (Fig. 4), while it was significantly decreased in Nx-6 rats compared with that in S-6 rats ($P <$ 0.01; Fig. 4). β-actin and GAPDH mRNA expressions were not significantly altered by CRF (Table 6).
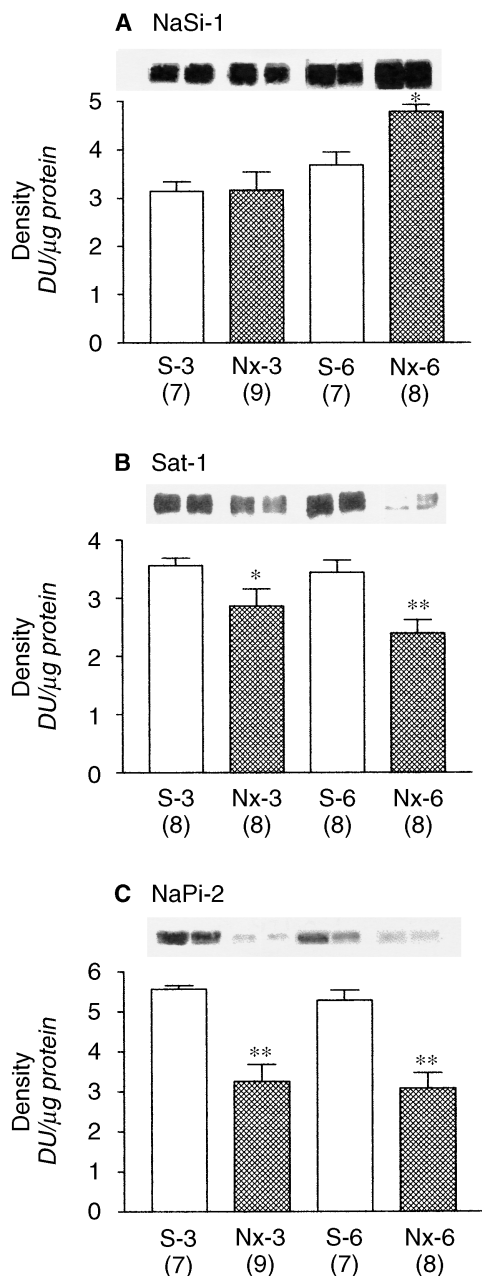
A NaSi-1

B Sat-1

C NaPi-2

Fig. 2. (A) NaSi-1, (B) Sat-1, and (C) NaPi-2 protein densities in the four groups of rats. Protein expression was measured by immunoblotting on renal protein extracts, as indicated in the **Methods** section. Forty micrograms of BBM (NaSi-1 or NaPi-2) or 30 µg CM (Sat-1) proteins were analyzed. For each group, examples of immunoblots obtained using protein extracts prepared from kidneys from two rats are shown above the bar graph. To calculate the relative density of each transporter, the signal intensity of the band corresponding to each transporter was quantitated by densitometry and expressed in densitometric units (DUs)/µg BBM (NaSi-1 or NaPi-2) or CM (Sat-1) proteins. Values are expressed as mean ± SEM. The numbers of samples (*N*) are indicated by the numbers in parentheses below the bars. *$P < 0.01$ and **$P < 0.001$ compared with the corresponding control group.

Table 5. NaSi-1 and NaPi-2 protein densities, and GFR/nephron in the four groups of rats

Group	NaSi-1	NaPi-2	GFR
S-3[a]	100 ± 13 (7)	100 ± 8 (7)	100 ± 5 (9)
Nx-3[b,c]	164 ± 21 (9)	89 ± 13 (9)	202 ± 18[d,e] (9)
S-6[a]	100 ± 15 (7)	100 ± 11 (7)	100 ± 6 (9)
Nx-6[b,c]	286 ± 39[e] (8)	169 ± 37 (8)	135 ± 28[d] (9)

[a] In control rats, protein density and GFR/nephron is the ratio of protein density or GFR (in mL/min)/total number of nephrons (*N*) in control rats. Each individual control value was divided by the mean control value to obtain 100% ± SEM.

[b] Results in Nx-rats are expressed as a percentage of mean control values obtained in control rats.

[c] To calculate the transporter densities/nephron in Nx rats, total transporter protein abundance was divided by N/5, that is, by the estimated fraction of residual nephrons that remained immediately after the 4/5 Nx (see *expression of results*, in the **Methods** section).

[d] The GFR/residual nephron in Nx rats was estimated by dividing the GFR in mL/min by N/5. The data are the mean ± SEM, *N* is indicated by the number in parentheses.

[e] $P < 0.001$ compared to controls

DISCUSSION

Although CRF has been shown to lead to marked alterations in sulfate homeostasis [1–6], the mechanisms involved at the cellular and molecular levels remained unclear. In our study, we investigated the expression of NaSi-1 and Sat-1 transporters in experimental CRF in an attempt to assess their role in the abnormalities of sulfate homeostasis observed in renal impairment. The results indicated that hypersulfatemia, in contrast to hyperphosphatemia, was observed as early as three weeks after subtotal Nx and was further increased after six weeks. This was associated with an increase in fractional sulfate excretion despite an increase in the protein density of NaSi-1 cotransporter. The likely explanation for our findings is that although NaSi-1 density is up-regulated, the total abundance of transporters, as shown in this study, is decreased as a result of the reduction in renal mass in Nx animals. The hypersulfatemia is probably caused by a failure of increased fractional sulfate excretion to compensate for filtered sulfate load. It cannot be excluded that an increase in intestinal sulfate absorption may also occur and participate in the increase of serum sulfate levels. Sat-1 antiport protein density was also decreased, suggesting a possible role for this transporter in the regulation of sulfate homeostasis.

To assess whether the changes in transporter protein expression were specific or due to a generalized reduction in kidney size, as seen in CRF, total transporter abundance and transporter protein density were compared. Interestingly, a difference was noted in the regulation of the protein density of the two sulfate transporters. NaSi-1 protein density was not diminished three weeks after Nx, and furthermore, it increased six weeks after Nx com-
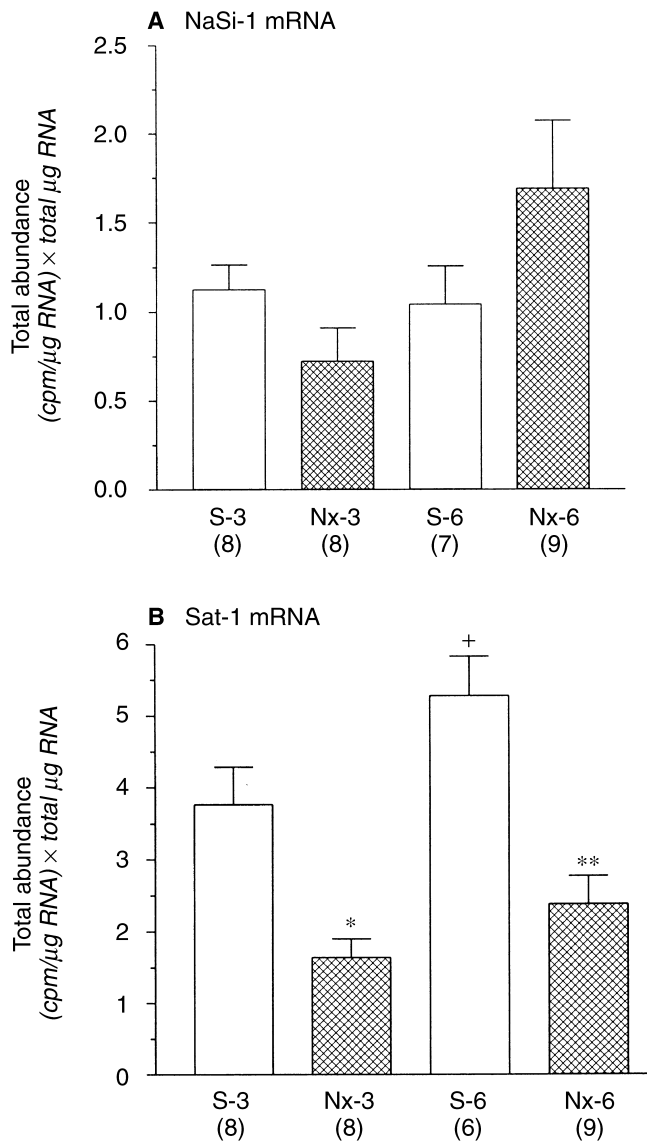
Fig. 3. Renal (*A*) NaSi-1 and (*B*) Sat-1 total mRNA abundance in the four groups of rats. The mRNA expression was measured by Rnase protection assays (RPAs) on 20 μg total renal RNA extracts, as indicated in the **Methods** section. The radioactivity in the band corresponding to each transporter was quantitated by electronic autoradiography, and total mRNA density was obtained by multiplying the radioactive counts per minute (cpm)/μg RNA by the total amount of renal RNA obtained from kidneys of control rats or the remnant kidney of the Nx rats. Results are the mean ± SEM. The numbers of samples (*N*) are indicated by the numbers in parentheses below the graphs. *P < 0.01 and **P < 0.001 compared with the corresponding control group; +P < 0.05 comparing the S-3 and S-6 control groups.

Fig. 4. Renal (*A*) NaSi-1 and (*B*) Sat-1 mRNA densities in the four groups of rats. mRNA expression was measured by RPAs on 20 μg total renal RNA extracts, as indicated in the **Methods** section. The radioactivity in the band corresponding to each transporter was quantitated by electronic autoradiography and expressed in cpm/μg RNA. Values are expressed as mean ± SEM. The numbers of sample (*N*) are indicated by the numbers in parentheses below the graphs. *P < 0.01 compared with the corresponding control group.

pared with the controls. In agreement with a preliminary report (abstract; Blancke et al, *J Am Soc Nephrol* 9:603, 1998) [24], no changes in β-actin and 5′-nucleotidase BBM expression were evidenced after both time periods, demonstrating the specificity for the increase in NaSi-1 protein density. This increase in NaSi-1 protein density could not be attributed to an alteration in membrane
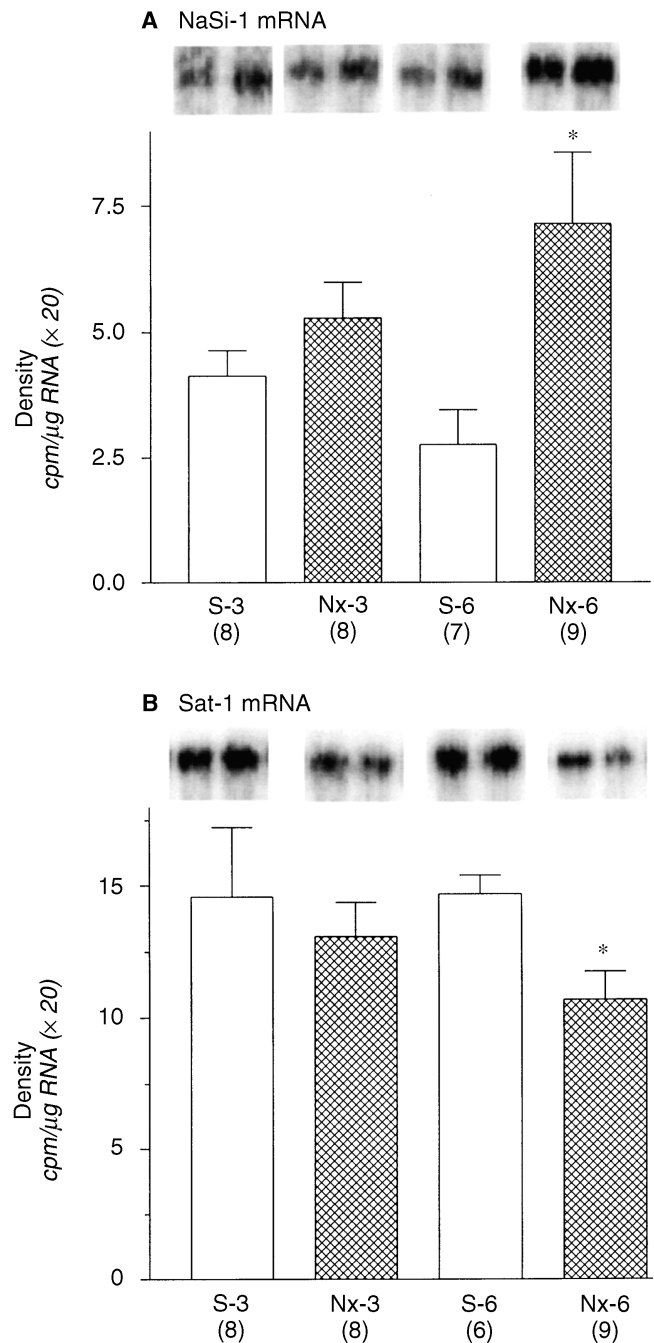
fractionation and BBM recovery resulting from the uremic state, since the enrichment of γ-GT–specific activity in BBM preparations isolated from control and Nx rats was similar in all groups. In contrast to the observed

Table 6. Expression of β-actin and GAPDH mRNA
in the four groups of rats

Parameters	Experimental group			
	S-3 (8)	Nx-3 (8)	S-6 (7)	Nx-6 (9)
β-actin	22.9 ± 1.7	25.5 ± 2.1	17.3 ± 0.8	20.9 ± 1.6
GAPDH	91.9 ± 14.0	79.2 ± 7.3	89.7 ± 9.6	76.6 ± 9.3

Values are expressed in cpm/μg total RNA, and are means ± SEM; the number of samples (*N*) is indicated by the numbers in parentheses. β-actin and GAPDH mRNA expressions were studied by RPA analysis as described in the **Methods** section. There was no significant difference for either parameter between the four experimental groups.

increase in the NaSi-1 protein density, Sat-1 density was decreased at six weeks following Nx. A small (20%) decrease in Sat-1 protein density was also observed three weeks after Nx. However, this decrease was likely not specific, as a similar decrease (30%) was also seen in CM β-actin density.

To assess the significance of the changes observed further, it was also of interest to compare sulfate metabolism and the expression of sulfate transporters versus that of phosphate homeostasis and NaPi-2 cotransporter protein. Sulfatemia was increased at a time when phosphatemia was not increased (3 weeks of renal failure). At that time, total NaPi-2 cotransporter protein abundance was also already decreased, as observed for sulfate transporters. Interestingly, however, the decrease in total NaPi-2 protein abundance was greater than that of the sulfate transporters. This finding was further documented by the observation that NaPi-2 protein density was decreased after both three and six weeks Nx, in contrast to NaSi-1 density. We had previously demonstrated a decrease in NaPi-2 protein density in a similar model of CRF [24], a finding that has been recently confirmed by Kwon et al [34]. This different regulation of the NaSi-1, Sat-1, and NaPi-2 transporters in CRF underlines the specificity of the changes demonstrated and may explain why hypersulfatemia occurs at three weeks after Nx, before an increase in serum phosphate is observed. These results are further documented when comparing the estimated densities of NaSi-1 and NaPi-2 transporters per estimated residual nephron in Nx rats. The estimated densities per residual nephron for both NaSi-1 and NaPi-2 were not significantly increased three weeks post-Nx, whereas—as expected—the estimated single-nephron GFR increased in Nx rats compared with the controls. Thus, the two transporter densities/nephron did not increase proportionally to the single nephron GFR, which likely explains the increases in FE_{SO4} and FE_{PO4} observed three weeks post-Nx. However, at this time point, the increased FE_{SO4} was probably not adequate to excrete the sulfate load (as indicated by the hypersulfatemia). Furthermore, at six weeks, NaSi-1 density/nephron increased proportionally more than single nephron GFR, a result that cannot

explain the increased FE_{SO4}, but may have also contributed to the increased sulfatemia.

The changes in NaSi-1 cotransporter mRNA were complex. NaSi-1 mRNA density was up-regulated at six weeks, consistent with the increase in the protein density at six weeks. However, no significant change in total NaSi-1 cotransporter mRNA abundance was seen, a result that confirms the possibility that the decrease in total NaSi-1 protein abundance was due to the reduction in renal protein abundance. At six weeks following nephrectomy, the decrease in Sat-1 protein amount was associated with a decrease in the Sat-1 mRNA, which was seen both for total abundance and density. No significant changes were found in β-actin and GAPDH mRNA densities in Nx animals compared with controls, in agreement with the lack of changes in GAPDH mRNA previously reported in the same model [24]. The decrease in NaPi-2 cotransporter protein in CRF has previously been shown to be accompanied by a decrease in the mRNA encoding for the protein [24].

The continual and complex interactions of electrolytes and hormonal systems that occur in CRF may be implicated in the regulation of NaSi-1 and Sat-1 expression. Down-regulation of NaSi-1 cotransporter protein and/or activity has been documented in several pathophysiological situations such as hypothyroidism [17], potassium deficiency [18], vitamin D insufficiency [16], and heavy metal intoxication [37]. Among hormonal factors involved in CRF, $1,25(OH)_2D$ deficiency and secondary hyperparathyroidism could be important players in NaSi-1 and Sat-1 regulation. We have previously shown that vitamin D status modulates renal NaSi-1 expression and sulfate homeostasis [16]. In addition, using promoter/reporter gene constructs, it was recently shown that $1,25-(OH)_2D_3$ was able to transactivate the human NaSi-1 gene (*Nas1*) promoter in opossum kidney cells coexpressing the vitamin D receptor (VDR) and human retinoid-x-receptor hRXR [38]. In the present experimental model, however, alterations in sulfatemia and sulfate transporters, in particular that of Sat-1, occurred before any changes in $1,25(OH)_2D$ serum levels. Furthermore, an increase in NaSi-1 density was observed after six weeks when $1,25(OH)_2D$ levels were low. Thus, in CRF at least, vitamin D is unlikely to be involved in the abnormalities of sulfate transport. Hyperparathyroidism is also unlikely to modulate NaSi-1 transporter expression, since results from our previous study performed in vitamin D deficient rats indicated that PTH did not regulate NaSi-1 cotransporter [16]. Moreover, recent in vivo studies have demonstrated that PTH does not modify NaSi-1 expression in renal proximal tubules [39], in agreement with earlier studies indicating that PTH did not change sulfate reabsorption by the kidney in intact and thyroparathyroidectomized rats [40]. However, we cannot exclude the possibility that hyperparathyroidism may lead to a

down-regulation of Sat-1 expression. With regard to electrolyte modulation of sulfate transport, down-regulation of proximal NaSi-1 protein and mRNA associated with an increase in fractional sulfate excretion has been described in chronic potassium depletion [18]. Whether hyperkalemia leads to an increase in NaSi-1 cotransporter protein remains to be established. In our model, however, hyperkalemia occurs at three weeks, while no change was observed in NaSi-1 density. As discussed for hyperparathyroidism, on the other hand, hyperkalemia may be implicated in Sat-1 regulation. Another factor in the regulation of sulfate transporters may be the acidosis observed in CRF. It has been reported that physiological changes of blood pH influence the renal handling of sulfate [41, 42]. An increased concentration of proton in plasma has been shown to stimulate the reabsorption of sulfate. It was postulated that this effect could also be mediated by hypobicarbonatemia [42]. In our study, the up-regulation of NaSi-1 observed at six weeks could be due to the acidosis associated with CRF, since at this time the animals had significantly lower serum bicarbonate concentrations. The hypobicarbonatemia also could alter Sat-1 expression since Sat-1 transports bicarbonate. In addition, down-regulation of Sat-1 protein may be a response to the hypersulfatemia. It may contribute to the abnormalities in oxalate metabolism observed in CRF, as Sat-1 provides a pathway for oxalate transport [43, 44].

Very little is known about the clinical significance and potential harmful effects of chronic hypersulfatemia. Dietary protein restriction is clinically recommended in CRF and has been shown to reduce symptoms [45–48]. It is interesting to note that a reduction in protein intake also leads to a reduction in circulating sulfate levels [14, 15, 49]. Hypersulfatemia, as seen in CRF, could therefore be considered as a "uremic toxin." Increased serum sulfate has been shown to alter the sulfation of many endogenous substances and hormones, which might affect their metabolic kinetics [50–52]. Finally, increased sulfate concentrations may lead to a reduction in ionized calcium concentration. Some studies suggest that calcium–sulfate interaction may be as important as the calcium–phosphate interaction and may influence calcium-regulating hormones such as PTH, thus contributing to the pathogenesis of renal osteodystrophy [20, 21].

In conclusion, these results demonstrate that both NaSi-1 and Sat-1 total protein expressions are decreased in CRF, which may contribute to the increased fractional sulfate excretion. However, this increase in fractional sulfate excretion is not sufficient to maintain sulfate homeostasis. Strikingly, in contrast to the reduction in activity or expression usually described for enzymes, transporters, and channels expressed in proximal tubular cells [24, 34, 53–55], and demonstrated in the present study for Sat-1 expression, NaSi-1 cotransporter density was increased in CRF. The consequences of the two opposite

changes in sulfate transporters on intracellular sulfate metabolism are yet unknown. Similarly, the significance of the difference in NaSi-1 and NaPi-2 densities remains to be determined, but may explain why hypersulfatemia occurs earlier than hyperphosphatemia in CRF.

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