JCI The Journal of Clinical Investigation

Isolation from human calcium oxalate renal stones of nephrocalcin, a glycoprotein inhibitor of calcium oxalate crystal growth. Evidence that nephrocalcin from patients with calcium oxalate nephrolithiasis is deficient in gamma-carboxyglutamic acid.

Y Nakagawa, … , S Deganello, F L Coe

J Clin Invest. 1987;79(6):1782-1787. https://doi.org/10.1172/JCI113019.

Research Article

We have determined that the organic matrix of calcium oxalate kidney stones contains a glycoprotein inhibitor of calcium oxalate crystal growth (nephrocalcin) that resembles nephrocalcin present in the urine of patients with calcium oxalate stones and differs from nephrocalcin from the urine of normal people. Pulverized calcium oxalate renal stones were extracted with 0.05 M EDTA, pH 8.0; nephrocalcin eluted in five peaks using DEAE-cellulose column chromatography, and each peak was further resolved by Sephacryl S-200 column chromatography. Four of the five DEAE peaks corresponded to those usually found in nephrocalcin from urine; the fifth eluted at a lower ionic strength than any found in urine. Amino acid compositions and surface properties of nephrocalcins isolated from kidney stones closely resembled those of nephrocalcins isolated from urine of stone-forming patients: they differed from normal in lacking gamma-carboxyglutamic acid residues, and in forming air-water interfacial films that were less stable than those formed by nephrocalcin from normal urine.

Isolation from Human Calcium Oxalate Renal Stones of Nephrocalcin, a Glycoprotein Inhibitor of Calcium Oxalate Crystal Growth

Evidence That Nephrocalcin from Patients with Calcium Oxalate Nephrolithiasis Is Deficient in γ-Carboxyglutamic Acid

Yasushi Nakagawa,*‡ MaryAnn Ahmed,‡ Susan L. Hall,‡ Sergio Deganello,*§ and Fredric L. Coe*‖

**Nephrology Program, Department of Medicine, ‡Department of Biochemistry and Molecular Biology, ‖Department of Pharmacology and Physiology, University of Chicago, Chicago, Illinois 60637; and §Institute of Mineralogy, University of Palermo, Italy*

Abstract

We have determined that the organic matrix of calcium oxalate kidney stones contains a glycoprotein inhibitor of calcium oxalate crystal growth (nephrocalcin) that resembles nephrocalcin present in the urine of patients with calcium oxalate stones and differs from nephrocalcin from the urine of normal people. Pulverized calcium oxalate renal stones were extracted with 0.05 M EDTA, pH 8.0; nephrocalcin eluted in five peaks using DEAE-cellulose column chromatography, and each peak was further resolved by Sephacryl S-200 column chromatography. Four of the five DEAE peaks corresponded to those usually found in nephrocalcin from urine; the fifth eluted at a lower ionic strength than any found in urine. Amino acid compositions and surface properties of nephrocalcins isolated from kidney stones closely resembled those of nephrocalcins isolated from urine of stone-forming patients: they differed from normal in lacking γ-carboxyglutamic acid residues, and in forming air–water interfacial films that were less stable than those formed by nephrocalcin from normal urine.

Introduction

Humans (1) and other mammals (2) excrete in their urine an acidic glycoprotein that strongly inhibits the growth of calcium oxalate crystals, contains γ-carboxyglutamic acid (GLA),[1] and may account for at least some of the well-known ability of urine to prevent calcium oxalate crystal growth (3). We have purified and described this inhibitor (1–3), which we have called nephrocalcin (NC) by analogy with osteocalcin, a bone protein that also contains GLA. NC exhibits microheterogeneity in eluting from DEAE-cellulose at four different ionic strength regions that we have called A–D; the NC each of the four fractions has a molecular weight of 14,000 after dissociation into its monomeric form by incubation with 0.05 M EDTA at pH 7.9, but is aggregated in polymeric forms in urine (3).

Patients with calcium oxalate renal stones produce NC that differs from that produced by normal individuals (4). All four fractions, A–D, lack GLA, normally present in amounts of two to three residues per molecule of NC in the A–C fractions (1, 5). C and D fractions, which occupied 50% of the total, have a lower affinity for the calcium oxalate crystal than normal C and D fractions. And, whereas normal NC from the A–C fractions forms stable monolayers at the air–water interface, with collapse pressures > 40 dyn/cm, all four fractions from patients form unstable monolayers with collapse pressures of 25–30 dyn/cm. These abnormalities distinguish the NC from patients from normal NC and may contribute to renal stone formation.

In this study, we have sought to confirm our findings in patients by determining whether NC can be extracted from calcium oxalate renal stones and, if so, whether its abnormalities match those of NC we have isolated from the urine of patients. Specifically, our study tests three predictions about NC in calcium oxalate kidney stones: (*a*) Although NC from all four fractions from the urine of stone-forming patients adsorbs to calcium oxalate crystals with dissociation constants in the range of 0.1–6×10^{-6} M (1, 4, 5), and therefore could become incorporated into growing stones, the NC with highest affinity should bind preferentially to the stones; (*b*) NC from stones should be deficient in GLA; and (*c*) NC from stones should form unstable monolayers at the air–water interface regardless of its affinity, inasmuch as both low- and high-affinity NC from patients has this abnormality. To test whether NC exists in stones, and, if so, to characterize its properties, we have extracted the organic matter out of kidney stone matrix, and have characterized it. We were successful in isolating NC, and find that it lacks GLA, forms extremely unstable monolayers, as does NC purified from urine of patients with stones, and has a normal affinity for the calcium oxalate crystal surface.

Methods

Materials. Calcium oxalate kidney stones either were a gift from Dr. L. H. Smith, Mayo Clinic, Rochester, MN, or were collected at the Kidney Stone Center of the University of Chicago, Chicago, IL. All stones contained > 80% calcium oxalate monohydrate, as determined by x-ray crystallography.

EDTA (disodium salt, J. T. Baker Chemical Co., Phillipsburg, NJ), Tris (Fisher Scientific Co., Pittsburgh, PA), and other chemicals were analytical grade and used without further purification. Tris-HCl buffer solution for surface activity measurement was prepared using Tris (ultrapure grade, Aldrich Chemical Co., Milwaukee, WI) and sodium chloride (Gold label, Aldrich Chemical Co.). GLA monoammonium salt was obtained from Calbiochem-Behring Corp. (San Diego, CA). DEAE-cellulose (DE-52, Whatman Inc., Clifton, NJ) was recycled with 0.5 N HCl and 0.5 N NaOH before equilibrating in a buffer solution. Calcium oxalate monohydrate crystals were prepared in our laboratory (5). Water used in the experiments was deionized by a mixed ion-exchange bed and distilled using glass distillation equipment. Dialysis tubing (Spectro/Por I, mol wt cutoff 8,000; Spectrum Medical Industries, Los Angeles, CA) was prepared by boiling in 2% $NaHCO_3$ and 10 mM EDTA for 20 min,

Address reprint requests to Dr. Coe, Renal Section, Box 28, University of Chicago, 5841 S. Maryland Avenue, Chicago, IL 60637.

Received for publication 8 December 1986.

1. *Abbreviations used in this paper:* GLA, γ-carboxyglutamic acid; NC, nephrocalcin.

J. Clin. Invest.

0021-9738/87/06/1782/06 $1.00
Volume 79, June 1987, 1782–1787

then boiling in deionized distilled water, and storing in 50% ethanol at 4°C.

Purification. 3 g of pulverized kidney stone were suspended in 50 ml of 0.05 M EDTA, pH 8.0, and magnetically stirred for 4 d at 4°C. The suspension was centrifuged (SS-34 rotor, DuPont-Sorvall, Newtown, CT) at 10,000 rpm for 20 min, and the supernatant was dialyzed against 6 liters of water for 24 h with three changes. The precipitate was resuspended in 50 ml of 0.05 M EDTA and stirred for 4 d at 4°C. This extraction process was repeated eight times until no more NC was detected. The dialyzed extract was adjusted to 0.05 M NaCl, pH 7.3, then subjected to DEAE-cellulose column (2 × 15 cm) chromatography using a linear NaCl gradient from 0.1 to 0.4 M in 0.05 M Tris-HCl, pH 7.3 (400 ml each). Subsequent Sephacryl S-200 column chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) was used for purification (1).

DEAE-cellulose column chromatography and Sephacryl S-200 column chromatography used for the purification of urine NC were carried out in the same manner as reported previously (4, 5). Elution of protein from a column was monitored by measuring absorbance at 230 nm, and protein concentration was determined by the alkaline hydrolysis method (1, 2) using bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) as a standard. Ionic strengths of NaCl elution gradients were monitored by a conductivity meter (CDM 2e, Radiometer, Copenhagen, Denmark) calibrated with 0.1 M KCl aqueous solution.

Inhibitory activity. Calcium oxalate crystal growth inhibitory activity was measured by using the [^{14}C]oxalate method (1–5). Briefly, calcium oxalate seed crystals were incubated in a metastable supersaturated calcium oxalate solution at 37°C with or without NC while stirring; samples were removed at intervals and filtered, and ^{14}C activity in the filtrate was determined by liquid scintillation counting (LS-230, Beckman Instruments, Inc., Palo Alto, CA). For monitoring inhibition during separation processes, the ^{14}C level after 40 min of incubation was taken as an index of inhibition; higher ratioactivity levels indicated a greater inhibition of calcium oxalate crystal growth (1–5).

Dissociation constants. The dissociation constants of the NCs were determined by use of a spectrophotometric method (6). An aqueous solution of 10 mM Tris-HCl containing 90 mM NaCl was adjusted to pH 7.2 with 4 N HCl. Both 1.0 mM sodium oxalate and 1.0 mM $CaCl_2 \cdot 2H_2O$ were prepared in this buffer. Purified monomeric NC (mol wt 14 × 10^3) was dissolved in water to a concentration between 10^{-6} and 10^{-8} M. Aliquots of 1–100 μl of the test solution were added to the assay solution. The calcium oxalate chemical concentration product, 10^{-6} M^2, is 25.0-fold above the equilibrium concentration product of a solution allowed to equilibrate for 48 h with pure calcium oxalate monohydrate crystals (7).

Calcium oxalate monohydrate crystals were prepared as previously described (5), and the product was verified using infrared spectrophotometry (FT-IR, Nicolet Instrument Corp., Madison, WI), by finding characteristic strong absorptions at 660 and 590 cm^{-1}. The seed crystal slurry was made by suspending 8.0 mg of the solid calcium oxalate monohydrate crystals in 10 ml of deionized distilled water, allowing the mixture to equilibrate overnight at room temperature with stirring, then bringing it to 37°C, and equilibrating for 1 h.

Time-course measurements of absorbance of the assay solution at 214 nm were made using a Lambda 5 or 7 spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) interfaced to an Apple IIe computer by an Adalab data acquisition system (Interactive Microware Inc., State College, PA). In this solution, the absorbance of oxalate greatly exceeds that of calcium chloride, so changes of absorbance reflect loss of oxalate due to calcium oxalate crystal growth. The sodium oxalate and $CaCl_2$ solutions were equilibrated at 37°C; a 1-ml aliquot of each was transferred to a cuvette in a cell holder thermostated at 37°C by a Haake constant-temperature, circulating bath (Buchler Instruments Inc., Saddle Brook, NJ), and magnetically stirred. When the baseline had stabilized, an aliquot of inhibitor solution (1–100 μl) was added to the assay solution. The stability of the baseline was again examined, then crystal growth was initiated by the addition of 30-μl seed crystal slurry, and the absorbance was monitored for 400 s at 214 nm.

The rate of crystallization is proportional to the concentration of binding sites on the crystal surface (s) and the concentration of both the calcium ion (Ca^{2+}) and oxalate ion (Ox^{2-}) above the saturation limit of aqueous calcium oxalate. Let $C = [Ca^{2+}] = [Ox^{2-}]$; if ($s$) remains constant over the course of the reaction, the rate law is:

$$v = -dC/dt = ks(C - C_\infty)^2, \quad (1)$$

where k = the rate constant, C_∞ is the saturation concentration, and v is the reaction velocity. By integrating, and substituting absorbance (A) for oxalate concentration using Beer's law, we obtain:

$$1/(A_\infty - A) = [t(ks/e)] + [1/(A_\infty - A_o)], \quad (2)$$

where A_∞ is the absorbance at the point of saturation, A_o is initial absorbance, t is time, and e is the molar extinction coefficient of oxalate. Eq. 1 can be rewritten in terms of optical absorbance:

$$(A_\infty - A) = e \times (v/ks)^{0.5}. \quad (3)$$

Substituting Eq. 3 into Eq. 2, we obtain a form of the integrated rate law with velocity as the dependent variable:

$$1/(v)^{0.5} = [(ks)^{0.5} \times t] + [e/((ks)^{0.5} \times (A_\infty - A_o))]. \quad (4)$$

By least square fit of $1/(v)^{0.5}$ vs. t, we obtain as the intercept the value of $1/(v)^{0.5}$ at $t = 0$, from which v_i is calculated by inversion and squaring.

The dissociation constant is calculated using the scheme:

$$(s) + (I) \rightleftharpoons (x), \quad (5)$$

where (s) is the concentration of binding sites, (I) is the inhibitor concentration, and (x) is the concentration of the inhibitor–crystal complex. From this scheme we define the dissociation constant:

$$K = (s)(I)/(x) \quad (6)$$

and define

$$s_o = s + x, \quad (7)$$

where s_o is the total concentration of sites in both bound and unbound forms, and $I \cong I_o$, the concentration of inhibitor in the solution. Using Eq. 1 to relate s and v, we derive the equation:

$$v = v_o/(1 + I/K), \quad (8)$$

rearranged to the Langmuir isotherm-type form:

$$v/(v_o - v) = K/I, \quad (9)$$

where v and v_o are initial velocity with and without inhibitor present, respectively, and the dissociation constant, K, is determined using a least squares fit of $1/I$ vs. $v/(v_o - v)$.

Properties of NC at the air–water interface. Amphiphilic properties of purified NC were measured using a Lauda film balance (Brinkmann Instruments Co., Westbury, NY) (5). Proteins were layered on 0.01 M Tris-HCl containing 0.1 M NaCl, pH 7.2, after contaminating surfactants in the solution were removed by passing air through the buffer solutions (4). Protein solutions were prepared in the same buffer and dialyzed against 1 liter of the same buffer overnight before measurement.

Other measurements. Molecular weights were estimated by elution profiles from a Sephacryl S-200 column calibrated using BSA, ovalbumin, soybean trypsin inhibitor, and ribonuclease as standards. A calibration standard of molecular weight was prepared from K_{av} vs. elution volume (8).

Amino acid compositions were determined using a Beckman Instruments amino acid analyzer, 118CL, with W-3P resin and lithium citrate buffers. Proteins were hydrolyzed for 24 h at 110°C in an evacuated sealed tube containing 6 N HCl. GLA content was determined by alkaline hydrolysis (9). Carbohydrate compositions were analyzed by gas chromatography (5).

Samples of the stones were pulverized and analyzed by powder diffraction using a Debye camera (Philips, Eindhoven, Netherlands) with a nominal diameter of 114.6 mm (10). Typical exposure time was 8 h at 35 kV and 15 mA, using Mn-filtered FeK radiation. To enhance our

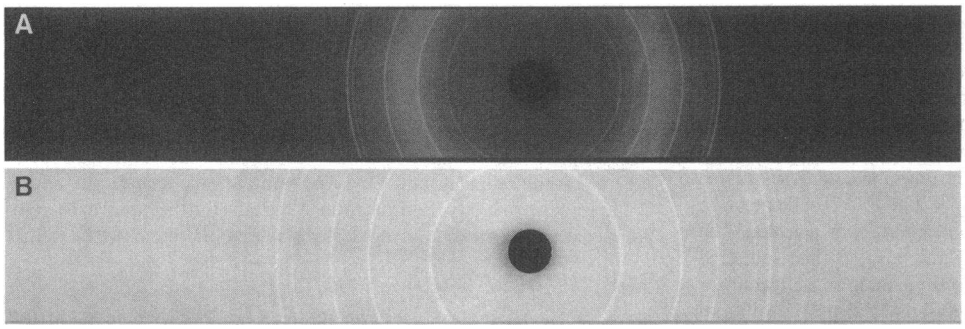

Figure 1. Powder x-ray diffraction pattern. (*A*) Calcium oxalate renal stone before EDTA extraction; (*B*) the same stone after the eighth extraction. Experimental details are described in Methods.

ability to detect phases present in only minor amounts, an individual small crystalline aggregate, measuring < 40 μm in cross section, was separated from a stone before EDTA treatment. That aggregate appeared by plane and polarized optical microscopy to consist of the phases that had not shown themselves satisfactorily in the x-ray films taken of the whole stone powder. The material was mounted on a Gandolfi camera (Officina Elettrotecnica di Tenno, Tenno, Italy) with a nominal diameter of 114.6 mm, and evacuated at ~ 0.1 torr for 2 h before exposure to x-rays. Powder diffraction patterns were generated using the Gandolfi mode (7 h at 35 kV and 16 mA, using Mn-Filtered FeK radiation).

Powder x-ray diffraction patterns (Fig. 1) taken of stones before extraction showed two diffraction lines, at 6.56 and 4.03 Å, in addition to those expected from calcium oxalate monohydrate itself (11). The film taken with the Gandolfi camera (Fig. 2) identified the 6.56-Å diffraction as belonging to anhydrous uric acid (12), in that the entire pattern of this crystal was visible. It also reveals traces of what we suspect was allopurinol or an allopurinol derivative (diffractions = 7.4, 6.2, 4.29, 3.45 Å). The 4.03-Å diffraction line was not explored using the Gandolfi camera, but probably is associated with organic proteinaceous material, inasmuch as this is the usual case in biological specimens. After the eighth extraction (Fig. 1 *B*) only the diffraction lines of calcium oxalate monohydrate were left, accompanied by an extremely faint trace of the line (diffraction = 6.56 Å), from anhydrous uric acid.

Results

Like NCs isolated from kidney tissue (2), urine (4), and cell culture medium (5), stone NC exhibited microheterogeneity in eluting from DEAE-cellulose in multiple peaks (Table I). The first urinary NC peak, which we have termed the A peak (4), elutes at 12–22 mS; however, the first kidney stone inhibitor fraction has a peak that elutes at ionic strength 5–11 mS, which we designate as pre-A. Inhibitors in regions A (12–22 mS), B (23–29 mS), C (30–39 mS), and D (40–45 mS), the locations of urinary inhibitor peaks (4), were also eluted from the DEAE-cellulose column. Successive stone extractions yielded NC in mainly the pre-A, B, and C peaks (Table I); and after the eighth extraction, no inhibitory activity was found. The protein from all eight extractions was pooled for each of the five fractions (Table I) to provide material for further study; from the protein concentrations in each pooled fraction (Table I), determined by alkaline hydrolysis, the total NC protein recovered was 2.66 mg, and amounts in each fraction ranged from 312 to 860 μg.

Each pooled NC peak was further purified by a Sephacryl S-200 column (2 × 110 cm) with 0.05 M Tris-HCl containing 0.2 M NaCl and 0.02% NaN_3, (pH 7.3). As we have found with urine NC, inhibitor molecules could be detected in up to four peaks (1, 2, 4, 5) corresponding to molecular weights of 68,000, 48,000, 23,000, and 14,000, respectively. The distribution ratios of these molecular weight fractions varied from preparation to preparation. Urine NC also shows these four peaks (2, 4, 5), and when peaks are incubated with EDTA, all dissociate to 14,000 mol wt. We did not attempt such dissociation here.

Amino acid compositions of each fraction were analyzed by acid hydrolysis and alkaline hydrolysis. All amino acid residues were calculated based on a mol wt of 14,000. As we observed in urinary NC, all fractions had a high content of acidic amino acids, and low aromatic and basic amino acid content (Table II). GLA contents of pre-A, A, and B were 0.1, 0.2, and 0.1 residues per molecule, respectively, and none in the C and D fractions. The carbohydrate content of fraction B (Table III) was similar to that of urine NC (4).

Surface activities of each of the five kidney stone NC peaks were measured by a Lauda film balance using 135 μg of each protein, the same amount of protein that we used to study urinary NC (4). None of the five fractions (Fig. 3) showed a pattern of film compression and collapse like that of normal urine NC; the force rose only when the surface was compressed far below 250 cm^2, whereas urine NC produced increasing force when the surface was compressed to this value. This difference is illustrated in the figures, which compare stone NC with urine NC results, redrawn for reference to our previously published measurements (4). There was no clear collapse point, indicating that a stable monolayer was not present (13). Given the amount of protein we used, the absence of a collapse pressure and the late and small rise in force with compression are most compatible with the idea that the protein dissolved; the amount of protein was so large that, if it had formed a stable monolayer, the force should have risen and collapse would have been observed.

The dissociation constants of the pooled NC peaks isolated from kidney stones were in the same range as urine NC (Fig. 4) from normal subjects, $1–5 \times 10^{-7}$ M. The fact that the experimental data fit closely to the Langmuir-type isotherm equation for each of the five fractions (Fig. 4, *A–E*) indicates that the assumptions of the equations were not being violated by the protein crystal interactions we observed; however, the *y* intercept is greater than 0 (Fig. 4), clearly indicating that at very high

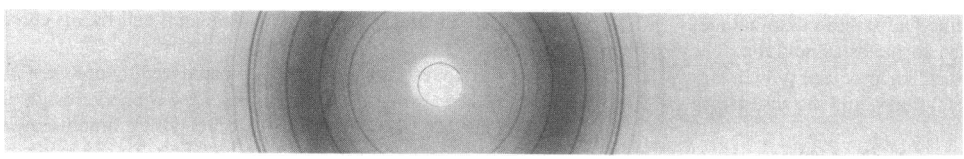

Figure 2. Gandolfi camera pattern. Powder x-ray diffraction pattern generated from a small crystalline aggregate (~ 40 μm) of the "extra" phase presented in Fig. 1 *A*.

Table I. Distribution of Glycoproteins in Kidney Stone Extracts

Extraction	Fraction (*mS*) Pre-A (5–11)	A (12–22)	B (23–29)	C (30–39)	D (40–45)
	%	%	%	%	%
1	—	10.7	24.6	41.9	23.3
2	69.4	30.6	—	—	—
3	—	2.9	36.5	60.6	—
4	1.0	—	43.4	26.1	29.7
5	12.7	—	55.8	31.4	—
6	14.5	—	42.0	43.6	—
7	56.0	—	22.7	21.3	—
8	—	31.1	53.3	15.5	—
Total protein (μg)	400	420	672	860	312
Percent of total	15.8	15.8	25.2	32.3	11.7

Values are percent of activity in each fraction.

concentrations of NC, when $1/I$ approaches 0, $[v/(v_o - v)] \neq 0$, in violation of the predictions of the Langmuir assumptions (Eq. 9).

Discussion

Our main results strongly confirm our previous ones (4), in that they are what we predict from the properties of NC in urine of stone formers. NC from kidney stones, like NC from the urine of patients who form kidney stones, lacks GLA and forms monolayers at the air–water interface which are much less stable than those formed by NC from urine of normal humans or from medium from a cultured renal cell line (5). The lack of GLA and the inability to form stable monolayers may be linked, because normal NC from peak D lacks GLA and forms a less stable monolayer than normal NC from the A–C regions of DEAE elution (2, 4, 5). The normal dissociation constants of kidney stone NC despite the absence of GLA are also consistent with our past results in urine NC, inasmuch as normal D proteins (5) and patient urine A and B NC (4) have dissociation constants of $\sim 1\text{–}3 \times 10^{-7}$ M despite a lack of GLA. Overall, NC of stone formers, from urine and kidney stones seems to be deficient in GLA, and has weak amphiphilic properties, but may have normal affinity to the calcium oxalate monohydrate crystal.

The fact that kidney stone NC has normal dissociation constants for its C and D fractions, even though the C and D fractions of NC from the urine of stone formers have dissociations of 6.5 and 1.5×10^{-6} M (4), is consistent with the idea that patients make some high-affinity C and D NC that selectively adsorbs to stone crystals and was recovered in our experiment. Possibly, dissociation constants are not as critical a determinant of how protective a NC is against stone formation as are the presence of GLA and an ability to form stable monolayers, a conjecture that requires further study.

We have used Langmuir-type equations to calculate dissociation constants, and within the range of concentrations of NC

Table II. Amino Acid Composition of NC Isolated from Kidney Stone

Fraction	Pre-A	A	B	C	D
Lys	8.1	5.6	4.3	1.3	6.8
His	4.4	2.2	1.7	2.8	3.4
Arg	4.2	6.4	4.8	4.3	2.0
Asp	12.0	14.0	10.5	13.8	10.6
Thr	8.4	9.8	7.4	4.3	7.4
Ser	10.2	8.2	6.2	7.9	9.8
Glu	9.1	13.4	10.1	5.4	12.8
Pro	8.1	5.8	4.4	6.3	6.4
Gly	9.1	10.8	8.1	15.8	10.0
Ala	12.5	9.2	6.9	9.4	11.2
Val	8.9	9.4	7.1	7.4	6.4
Ile	4.2	5.4	4.1	3.4	4.2
Leu	8.1	9.2	7.1	7.1	9.0
Tyr	0.5	1.4	1.1	0.5	1.2
Phe	2.9	4.2	3.2	2.8	3.0
Gla	0.1	0.2	0.1	0	0

Values are number of residues in NC molecules with a molecular weight of 14,000.

Table III. Carbohydrate Composition of Fraction B

	wt%
Mannose	1.6
Galactose	1.4
Glucose	3.9
Galactosamine	1.2
Glucosamine	1.0
Fucose	+
N-Acetylneuraminic acid	0.7

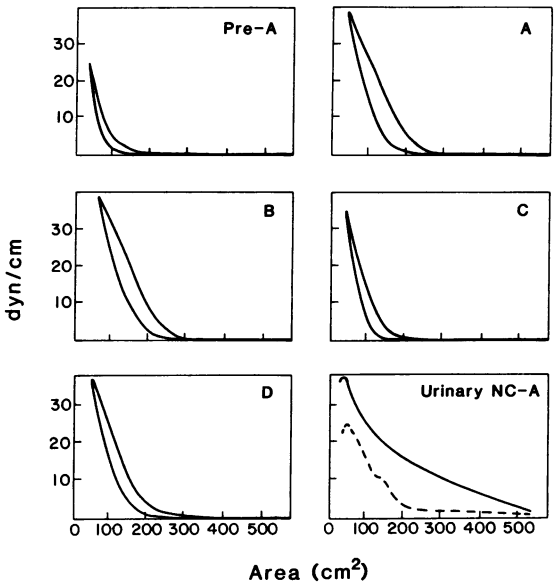

Figure 3. Force–area curves of NC isolated from calcium oxalate renal stones. Protein, 135 μg, was spread over Tris-HCl buffer (pH 7.3), and compressed and expanded between 600 and 20 cm^2 at 2.2 cm^2/s. Results from normal urine NC-A (*solid line*) and NC-A from urine of patients with calcium oxalate kidney stones (*dashed line*) (4) are reproduced in the lower right hand panel for comparison.

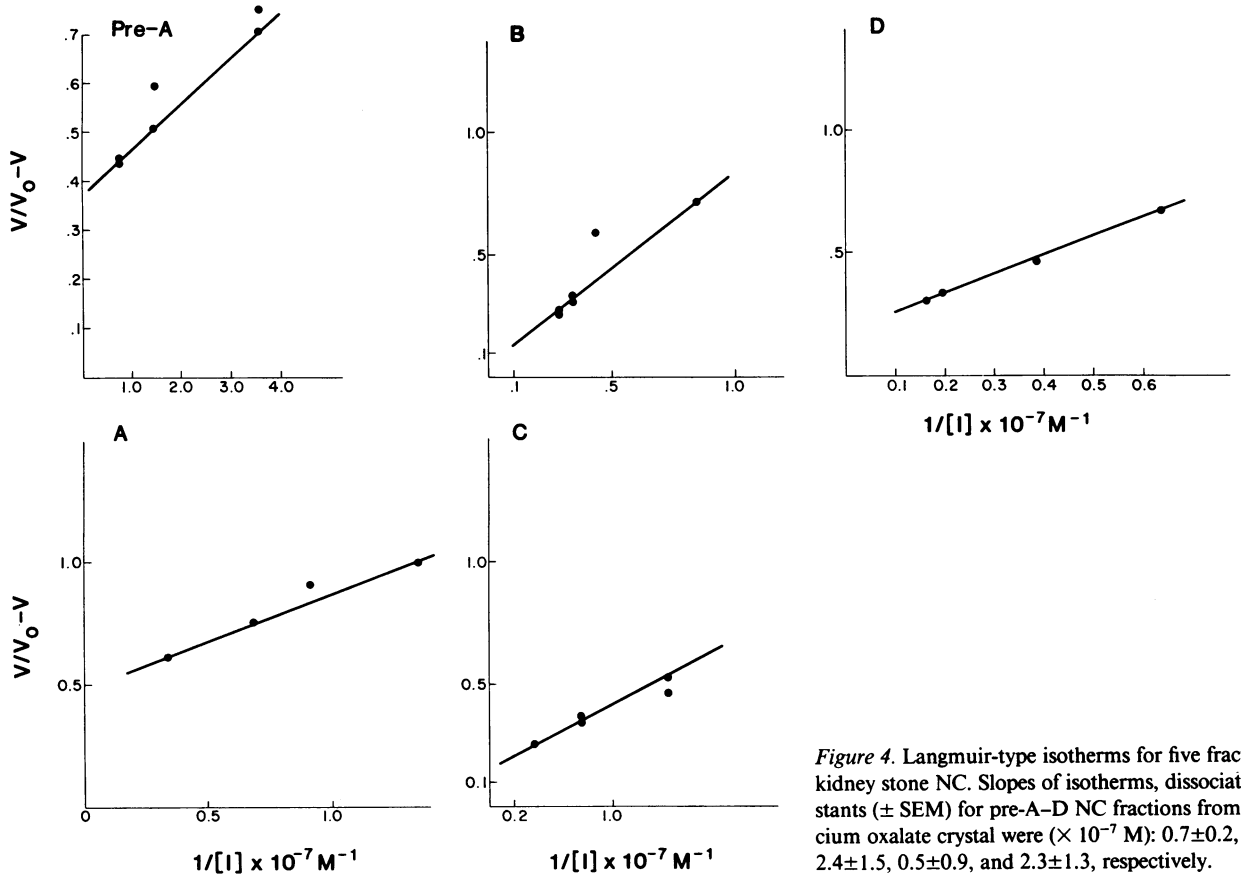

Figure 4. Langmuir-type isotherms for five fractions of kidney stone NC. Slopes of isotherms, dissociation constants (± SEM) for pre-A–D NC fractions from the calcium oxalate crystal were ($\times 10^{-7}$ M): 0.7±0.2, 3.6±1.3, 2.4±1.5, 0.5±0.9, and 2.3±1.3, respectively.

that we used here, the assumptions from which the equations derive are not clearly being violated because there is a good linear fit of $v/(v_o - v)$ on $1/I$. However, this equation clearly cannot be used at concentrations of NC high enough to force 1/I to 0. The y intercepts of Fig. 4 are > 0, whereas Eq. 9 predicts an intercept of 0. Our comparisons are limited to the range over which we have actually documented linearity.

The fractional distribution of NC in stones seems consistent with other estimates of proteins in stones. We have isolated a total of 2.7 mg of NC from 3 g of calcium oxalate renal stone, 0.1% by weight. Warpehoski et al. (14) found ~ 2–3% of kidney stone was organic material, and 1.2% protein might be present. Lian et al. (15) extracted 2.6–5.3% of nondialyzable protein from calcium oxalate renal stones. Because both reports (14, 15) describe protein extracted from kidney stone without further purification, it is difficult to compare their results to ours. However, if we assume that 3% of organic materials are extractable (14), 90 mg of organic materials could be extracted from 3 g of kidney stone, and based on this number at least 3% of the organic matrix ought to be NC.

The biochemical characteristics of stone and urine NC are similar, but not identical. Kidney stone NC can be eluted in five peaks from a DEAE-cellulose column using a linear NaCl gradient, whereas urine NC elutes in four peaks (4, 5); but the four peaks that do correspond, A through D, appear in approximately equal percentages in NC from the two sources. Molecular weights of stone NC, 63,000, 48,000, 23,000, and 14,000, are as we observed in urinary NC (4, 5). Amino acid compositions of all NC peaks were high in aspartic acid and glutamic acid, and low in aromatic amino acid and basic amino acid residues. The carbohydrate compositions of the B fractions were similar and in agreement with those of urinary inhibitors reported before (1, 4, 5).

Lian et al. (15) have reported that the kidney stone matrix contains a high percentage of aspartic acid and glutamic acid and that GLA and *O*-phosphoserine are present in calcium oxalate and hydroxyapatite renal stones. We have not quantitatively analyzed phosphoserine because of its instability (1), but we observed the presence of *O*-phosphoserine during the course of analysis. Lian et al. (15) have partially purified an EDTA extract of calcium oxalate stones by gel filtration and have found elution peaks of optical density at 280 nm corresponding to molecular weights of 49,000–17,000 containing variable amounts of GLA residues. These peaks could have contained aggregated NC. Warpehoski et al. (14) also reported a high aspartic acid and glutamic acid content, and the presence of GLA in the organic matrix of calcium oxalate renal stones. They eliminated the possibility of the Tamm–Horsfall glycoprotein being in the organic matrix. We calculated the number of GLA residues based on a molecular weight of 14,000, or about 100 amino acid residues in the protein moiety; however, other groups expressed one to three GLA residues per 1,000 amino acid residues. Thus our determination of GLA residues agrees with others.

Overall, the present data support our previous observations. Like urine NC from stone formers, stone NC proteins lack GLA, and form weak interfacial monolayers. The weak monolayer formation and loss of GLA could be linked, if the GLA were necessary to stabilize the structure of the molecule so that hydrophobic and hydrophilic regions separate spacially and make the molecule amphiphilic. How the amphiphilic nature of nor-

mal NC is related to its effects on crystals, and what it may contribute to natural defense against stones is completely unknown at this time. The present results contrast with our urine results, in that C and D NC from stones have normal affinity for the crystal surface as determined by the inhibition isotherm plots. This probably reflects selective adsorption of high-affinity NC onto stones from urine in which low-affinity C and D NC may predominate, but the matter requires new experiments for resolution. Because the results using stones collected from many unselected patients are consistent with those using urine from different patients, it seems probable that GLA deficiency and a weakening of amphiphilicity are real and characteristic features of NC produced by patients with calcium oxalate stones, and that those abnormal molecular features must somehow be at least part of the reason for their stone disease.

Acknowledgments

We wish to thank Professor F. J. Kézdy for his valuable suggestions and discussions. We thank also Mrs. A. Abram for her assistance at the initial stage of this work.

This research was supported by U. S. Public Health Service grants IP50 AM 20585 and AM 33949.

References

1. Nakagawa, Y., V. Abram, F. J. Kézdy, E. T. Kaiser, and F. L. Coe. 1983. Purification and characterization of the principal inhibitor of calcium monohydrate crystal growth in human urines. *J. Biol. Chem.* 258:12594–12600.

2. Nakagawa, Y., V. Abram, and F. L. Coe. 1984. Isolation of calcium oxalate crystal growth inhibitor from rat kidney and urine. *Am. J. Physiol.* 247:F765–F772.

3. Coe, F. L., H. C. Margolis, L. H. Deutsch, and A. L. Strauss. 1980. Urinary macromolecular crystal growth inhibitors in calcium nephrolithiasis. *Miner. Electrolyte Metab.* 3:268–275.

4. Nakagawa, Y., V. Abram, J. H. Parks, H. S.-H. Lau, J. H. Kawooya, and F. L. Coe. 1985. Urine glycoprotein crystal growth inhibitors: evidence for a molecular abnormality in calcium oxalate nephrolithiasis. *J. Clin. Invest.* 76:1455–1462.

5. Nakagawa, Y., H. C. Margolis, S. Yokoyama, F. J. Kézdy, E. T. Kaiser, and F. L. Coe. 1981. Purification and characterization of a calcium oxalate monohydrate crystal growth inhibitor from human kidney tissue culture medium. *J. Biol. Chem.* 256:3936–3944.

6. Nakagawa, Y., S. L. Hall, S. Park, F. J. Kézdy, and F. L. Coe. 1986. A spectrophotometric assay for calcium oxalate crystal growth inhibition. *Fed. Proc.* 45:1726.

7. Ito, H., and F. L. Coe. 1977. Acidic peptide and polyribonucleotide crystal growth inhibitors in human urine. *Am. J. Physiol.* 233:F455–F463.

8. Determan, H. 1968. Molecular weight determination. *In* Gel Chromatography. Springer-Verlag, New York. 105–129.

9. Hauschka, P. V. 1977. Quantitative determination of γ-carboxyglutamic acid in proteins. *Anal. Biochem.* 80:212–223.

10. Deganello, S., and C. Chou. 1984. The uric acid-whewellite association in human kidney stones. *Scanning Electron Microsc.* 11:927–933.

11. A. S. T. M. Powder Diffraction File, Card Number 20-231. American Society for Testing Materials, Philadelphia.

12. A. S. T. M. Powder Diffraction File, Card Number 21-1959. American Society for Testing Materials, Philadelphia.

13. Gaines, G. L., Jr. 1966. Properties of monolayers. *In* Insoluble Monolayers at Liquid–Gas Interfaces. John Wiley & Sons, New York. 136–207.

14. Warpehoski, M. A., P. J. Buscemi, D. C. Osborn, B. Finlayson, and E. P. Goldberg. 1981. Distribution of organic matrix in calcium oxalate renal calculi. *Calcif. Tissue Int.* 33:211–222.

15. Lian, J. B., E. L. Prien, Jr., M. J. Glimcher, and P. M. Gallop. 1977. The presence of protein-bound γ-carboxyglutamic acid in calcium-containing renal calculi. *J. Clin. Invest.* 59:1151–2257.