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Urinary matrix calculi consisting of microfibrillar protein in patients on maintenance hemodialysis

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Urinary matrix calculi consisting of microfibrillar protein in patients on maintenance hemodialysis. In seven patients on maintenance hemodialysis, *de novo* recurrent renal stone formation was observed. In all patients, the underlying disease was glomerulonephritis, with or without the nephrotic syndrome. All patients had considerable persistent proteinuria. The stones consisted predominantly of protein, as revealed by amino acid analysis, and had a negligible carbohydrate and lipid content. Only in some specimens, X-ray diffraction and scanning electron microscopy revealed the presence of small amounts of whewellit (calcium oxalate monohydrate) and/or uric acid. In semithin sections, the stones had a laminated texture and exhibited structural anisotropy under polarized light. With transmission electron microscopy, they were found to consist of peculiar microfibrils. The proteinaceous material differed from fibrin or Tamm-Horsfall-protein, as indicated by ultrastructure, carbohydrate analysis, and amino acid analysis. Symptomatic *de novo* matrix stone formation constitutes another complication of dialyzed patients which has not been reported so far.

Calculs urinaires constitués de protéines microfibrillaires chez des malades en hémodialyse chronique. Chez sept malades en hémodialyse chronique, la formation récidivante de calculs urinaires a été observée. Chez tous les malades, la maladie initiale était une glomérulonéphrite avec ou sans syndrome néphrotique. Tous les malades avaient une protéinurie importante. Les calculs étaient essentiellement constitués de protéine, comme l'indiquait l'analyse des acides aminés, et avaient un contenu négligeable en hydrates de carbone et lipides. Dans quelques échantillons seulement la diffraction X et la microscopie électronique à balayage ont révélé la présence de faibles quantités d'oxalate de calcium monohydrate et/ou d'acide urique. Sur les coupes semifines les calculs avaient un aspect laminé et étaient anisotropiés en lumière polarisée. En microscopie électronique à transmission il a été observé des microfibrilles particulières. La substance protéique diffère de la fibrine ou de la protéine de Tamm-Horsfall d'après l'étude ultrastructurale, l'analyse des hydrates de carbone, et l'analyse des acides aminés. La formation de novo de calculs, accompagnée de syndromes de lithiase, constitue une complication non encore rapportée à ce jour au cours de la dialyse chronique.

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Urinary matrix calculi are an unusual clinical problem [1–6]. Stones consisting of an organic matrix, devoid of a mineral phase, have been found in patients with proteus infection [7]. Furthermore, in some patients without urinary tract infection, matrix stones were observed which consisted of fibrin-like proteinaceous material [1–2] or mucoprotein [3–4].

The following report describes the occurrence of matrix stones in proteinuric patients with glomerulonephritis who were in terminal renal failure and were treated with hemodialysis. Such stones exhibited distinctive laminated structure and consisted of a peculiar microfibrillar proteinaceous material.

Methods

Serum concentrations of calcium and inorganic phosphate were measured with a Technicon autoanalyzer. Vitamin 25(OH)-D was measured by the method of Belsey [8] and immunoreactive PTH (iPTH) was measured by a radioimmunoassay with a carboxyterminal antibody (AVI/2) [9]. Urinary concentrations of calcium and magnesium were measured by atomic absorption spectophotometry. Urinary inorganic phosphate was measured by the Fiske-Subarow method; and urinary protein, by the Biuret method. Urinary oxalate was measured by isotachophoresis before and after treatment of urine with oxalate decarboxylase (E.C. 4.1.1.2.). The injection volume was generally 2 to 4 μl. The leading electrolyte solution was 7.5 mmoles of hydrochloric acid, 1 mmole of sodium chloride, 0.25% hydroxypropylmethylcellulose (HPMC) (pH, 2.3); the terminating electrolyte solution was 10 mmoles of formic acid. Samples were run at 350 μA and detected at 150 μAmp. Recovery was 98 ± 6% up to a concentration range of 0.5 mM. The measurement

was carried out using the LKB Tachophor (LKB Co., Bromma, Sweden).

Stones were retrieved by having the patients urinate through gauze; stones were preserved in the patients' own urine at 4° C until they were analyzed. For X-ray diffraction analysis, a small aliquot of a stone sample was attached to a capillary glass rod and was analyzed according to the Debye-Scherrer procedure as described by Krischner [10]. For scanning electron microscopy, 1-μl samples were attached to a holder, subjected to the freeze-drying method (−158° C, 10^{-7} Torr), coated with gold and analyzed with a Jeol JS 1 apparatus, as previously described [11]. For transmission electron microscopy examination, the stones were fixed in phosphate-buffered 3% glutardialdehyde solution (pH, 7.4) for 2 hr, postfixed in 1% osmiumtetroxyde for 1 hr, and embedded in araldite. Semithin sections were stained with toluidine blue and paraphenylendiamine, crystal violet, and congo red; ultrathin sections were stained with lead citrate and uranyl acetate. Immunofluorescent studies of cryostat-cut sections were carried out with goat antihuman immunoglobulin and monospecific rabbit antihuman IgA, IgG, IgM and with rabbit anti-fibrinogen (Behringwerke Co., Marburg). For chemical analysis of amino acids, the samples were washed in saline (4 × 3 ml), dried (40° C) for 36 hr, hydrolyzed with 6 N hydrochloric acid at 110° C for 18 hr, evaporated at low pressure in a rotation evaporator at 40° C, taken up in 4 ml of sodium citrate buffer (pH, 2.2), and analyzed on the amino acid analyzer (Biotronic Co., München). For detection of gammacarboxyglutamic acid [12], material was extracted (10 mM EDTA; pH, 8.0), dialyzed, and submitted to partial acid hydrolysis (6 M hydrochloric acid; 45° C) for 24 hr, respectively (total of 96 hr). The hydrolysates were then analyzed by high-voltage paper electrophoresis [13].

To exclude the presence of major amounts of soluble serum proteins contaminating the matrix material that was subjected to the above analysis, we washed homogenized matrix extensively, treated it with sodium dodecylsulfate, and subjected it to disc electrophoresis [14]. No protein bands with an electrophoretic mobility greater or smaller than that of serum albumin could be demonstrated with this procedure. The detection limit of this method is 10 μg protein/sample. For determination of lipids, samples were extracted with chloroform and methanol (2:1) for 1 hr, cholesterol was determined with the Liebermann-Burchard method [15], and phospholipids were determined after Zilversmit [16] with the test kit of Boehringer Mannheim Co. (Mannheim). For determination of carbohydrates, samples were incubated in acetoanhydride for 6 hr, and the supernatant remaining after centrifugation was evaporated, washed three times in absolute methanol, desiccated, washed again four times with distilled water, and dried for 3 days in a vacuum. The residue was hydrolyzed in 1.5 ml ethanol/hydrochloric acid for 24 hr at 90° C neutralized with silver carbonate, reacted with silylizing reagent, and separated by gas chromatrography [17].

Clinical observations. In the dialysis center of the Department of Internal Medicine, Heidelberg, renal stone formation was observed in seven hemodialyzed patients, or approximately 5% of the total population of dialysis patients treated during the last 2 years. The pertinent clinical data is summarized in Table 1. All patients were male. In all patients, the underlying renal disease was glomerulonephritis with or without the nephrotic syndrome. All patients had considerable proteinuria of the nonselective glomerular type, as revealed by disc electrophoresis of the urine [15]. None of the patients had a history of nephrolithiasis prior to hemodialysis or a family history of nephrolithiasis. The patients did not take any medication other than aluminum oxide by mouth and polyvitamin capsules, particulary no drugs that are excreted in the urine. None of the patients had vitamin D therapy at the time of renal colic; three of the patients (patients 3, 4, and 6) had taken vitamin D prior to the stone episode. Repeated tests for bacteriuria were consistently negative, and urinary tuberculosis was excluded by acid-fast stain and culture. Computer tomography of the kidneys was performed in all patients, but nephrocalcinosis could not be demonstrated by this procedure. Small cysts could be shown, however, in two of the patients (2 and 4) with sonography, and in five of the patients with computer tomography. Retrograde urologic examination was carried out in all but two patients; no urologic abnormalities, particularly no vesicoureteral reflux or calyceal deformities, could be demonstrated. None of the patients had a history of analgesic abuse. All patients presented with colicky pain with ($N = 4$) or without ($N = 3$) macrohematuria. Three patients presented with vomiting, and six patients with nausea. The colicky episodes lasted from 10 hr to 8 days with a median of 2 days. All stones were passed spontaneously and surgery was never required. With the exception of one patient (patient 6), stones could be recovered in the urine for further analysis.

Results

Stone analysis. The size of the stones ranged from 0.5 to 500 mg (wet weight). In the majority of stones, X-ray diffraction analysis showed an amorphous phase, which comprised more than 80 to 95% of the material; in one patient (patient 3), a small interference could be identified as whewellit (calcium oxalate monohydrate), and in two patients (2 and 5), uric acid and some ammonium hydrogen urate was found; no inorganic material was identified in the stones of the other patients.

The carbohydrate, lipid, and amino acid composition is given in Table 2. Carbohydrate content was consistently ≤ 1% in all stones studied, and cholesterol and phospholipid content was 5 to 10%. The major component was protein. Amino acid analysis revealed notable concentrations of dicarboxylic acids (aspartic acid and glutamic acid) and diamino acids (lysine and arginine) and complete absence of hydroxyproline. The presence of gammacarboxyglutamic acid could be established qualitatively, but its amount was not quantified.

Immunofluorescent studies of washed specimens were negative for immunoglobulins and fibrinogen with the exception of one patient who showed faint staining for fibrinogen.

Scanning electron microscopy of the untreated stone surfaces and the fractured stone surfaces showed that the major constituent of the stones was organic material which exhibited a fibrillar texture (Fig. 1).

In semi-thin sections, the stones were seen to consist of concentric lamellae with alternating density (Fig. 2). The material exhibited pronounced affinity for congo red and was anisotropic under polarized light (green birefringence). No metachromasia was seen with crystal violet.

Transmission electron microscopy (Fig. 3) re-

Table 1. Clinical data[a]

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Controls[b]
Age, *years*	21	37	38	31	54	49	24	24 to 59
Renal disease	GN	GN	GN + NS	GN	GN + NS	GN + NS	GN	—
No. of renal stones	4	7	51	28	3	2	8	—
Side of colic	Rt	Rt	Rt + Lt	Lt	Rt + Lt	Rt	Rt + Lt	—
Serum chemistry:								
Calcium,[c] *mEq/liter*	4.73 ±0.12	4.93 ±0.15	4.63 ±0.16	4.48 ±0.31	4.48 ±0.13	4.65 ±0.18	4.43 ±0.20	4.61 ±0.60
Inorganic phosphate,[e] *mg/dl*	4.75 ±0.36	5.77 ±0.68	4.75 ±0.46	5.17 ±0.87	4.33 ±0.38	5.83 ±0.34	5.28 ±1.14	6.10 ±1.70
25 (OH)-D,[d] *nM/liter*	134	105	350	310	—	230	72	183 ± 41
Immunoreactive PTH,[e] *pmoles/liters*	153	120	150	60	—	282	290	160 ± 54
Urinary findings:								
Volume, *ml/24 hr*	480	431	510	320	138	710	640	460 ± 103
Osmolality, *mOsm/kg*	240	295	256	300	324	326	360	309 ± 28
pH	5.0	5.0	6.0	5.0	5.0	5.0	5.0	
Calcium, *mmoles/liter*	0.76	0.69	0.48	0.91	0.58	0.7	0.8	0.9 ± 0.21
Magnesium, *mmoles/liter*	2.83	2.94	3.58	4.47	2.83	1.64	6.2	2.16 ± 0.45
Inorganic phosphate, *mmoles/liter*	4.1	8.9	12.6	4.3	11.5	11.6	8.6	6.0 ± 3.3
Uric acid, *mmoles/liter*	1.0	1.47	1.35	1.47	2.29	1.32	2.3	1.3 ± 0.5
Oxalate, *mmoles/liter*	0.24	0.35	0.67	0.23	1.79	0.26		0.34 ± 0.14
Protein, *mg/dl*	212	155	190	115	67	186	283	83 ± 37

[a] Abbreviations are: GN, glomerulonephritis; NS, nephrotic syndrome; Rt, right; Lt, left
[b] Controls were patients on maintenance hemodialysis without nephrolithiasis (N = 10).
[c] Mean ± SD of five determinations.
[d] Normal range for 25(OH)-D is 50 to 300 nmoles/liter.
[e] Normal range for iPTH is 2 to 30 pmoles/liter.

vealed more or less tightly packed interwoven nonbranching microfibrils. The width of the individual fibrils varied between 100 and 160 Å. If the fibrils were sectioned in an appropriate plane, a corkscrew-like twisted appearance was noted. Only occasionally, amorphous, nonelectron-dense substances were observed between such fibrillar structures.

Discussion

The above clinical observations demonstrate that recurrent renal matrix stone formation constitutes a complication of maintenance hemodialysis that has not been recognized before. Such stone formation occurred only in patients with a history of glomerulonephritis and persistent marked proteinuria of the nonselective glomerular type. Chemical analysis, X-ray diffraction analysis, and scanning electron microscopy demonstrated that mineral material, that is, calcium oxalatemonohydrate (whewellit), and uric acid, was present only in some patients and constituted only a minor fraction of the stone material. Because stones were not examined immediately after their passage, it is possible that such mineral material as was detected was not deposited in vivo. In view of the low urinary excretion rates of calcium oxalate, it is interesting, however, that calcium oxalate can precipitate at all in the urine of patients with end-stage renal failure. Urinary oxalate concentration was found to be remarkably high in some patients, so that the calcium oxalate formation product was apparently transgressed. [18].

Chemical analysis, semithin section microscopy, transmission electron microscopy, and scanning electron microscopy clearly demonstrate that the major constituent of stones is amorphous organic matrix, which consists predominantly of protein. It is unlikely that contaminating amino acids, polypeptides, or soluble serum proteins interfered to a major extent with the chemical analysis of the matrix material, because the specimens were washed exhaustively and because no protein band comigrating with high- or low-molecular-weight serum proteins could be detected with SDS polyacrylamidgel electrophoresis [14]. Immunofluorescence was usually negative for immunoglobulins and for fibrinogen. Lipids, particularly cholesterol, were present only as a minor contaminant and were presumably due to inclusion of some cellular detritus.

The very low carbohydrate content is remarkable because this finding presumably excludes a major contribution of Tamm-Horsfall protein, which has a carbohydrate content over 10% [19] or a contribution of other mucoproteins. The stones did not consist of necrotic renal tissue, as documented by light and electron microscopy. Coacervation of organic material secondary to urinary tract infection, particularly proteus infection [7], can be excluded by the consistent absence of bacteriuria both prior to and during the stones episodes and by the failure to demonstrate bacteria in the stones. Furthermore,

Table 2. Analysis of stones

	Stone 1	Stone 2	Stone 3	Stone 4	Stone 5
Dry weight, *mg*	1.98	1.73	0.67	3.91	0.43
Protein, *mg*	0.78	1.38	0.48	2.45	0.32
Carbohydrate, *mg*	0.01	0.10	0.10	0.02	0.10
Cholesterol, *mg*	0.146	0.023	0.051	0.120	0.020
Phospholipids, *mg*	0.076	0.017	0.021	0.043	0.010
Amino acid content, %					
Aspartic acid	12.0	13.5	12.6	12.0	12.0
Threonine	5.7	4.8	5.7	4.7	5.1
Serine	7.7	9.4	8.6	6.8	6.1
Glutamic acid	12.6	11.9	12.0	13.1	14.0
Proline	4.3	4.8	4.4	4.0	3.7
Glycine	6.1	5.6	5.9	4.6	3.5
Alanine	5.5	4.0	5.1	3.5	3.7
Cystein/cystine SO_3H	0.3	2.0	0.5	3.2	3.1
Valine	6.6	7.0	6.9	6.0	5.8
Methionine	0.5	0.2	0.2	0.1	0.5
Isoleucine	3.2	3.9	3.0	3.7	3.2
Leucine	8.2	7.6	7.9	7.8	8.1
Tyrosine	3.8	4.0	3.9	5.0	5.2
Phenylalanine	4.9	5.5	4.8	6.3	6.1
Lysine	7.0	6.7	7.0	8.1	7.8
Histidine	3.3	3.5	3.5	4.4	3.8
Arginine	5.3	5.0	5.3	6.9	7.0
Unidentified	1.1	—	0.7	—	1.2

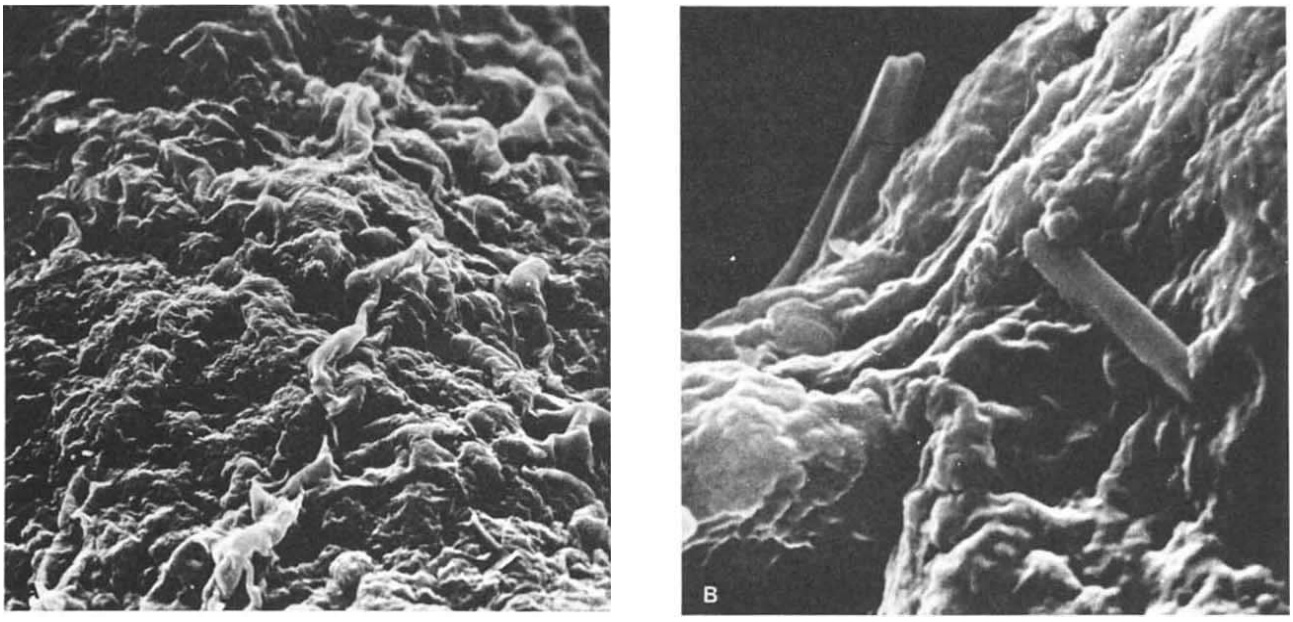

Fig. 1. *Surface scanning electron microscopy of urinary matrix calculi of patient 3: Fractured stone surface.* **A** Note exclusive presence of organic matrix, which presents a corrugated surface; the material appears to consist of twisted strings. **B** Two mineral crystals exhibit the peculiar appearance of whewellit protruding over the matrix surface. (Magnification, ×1000)

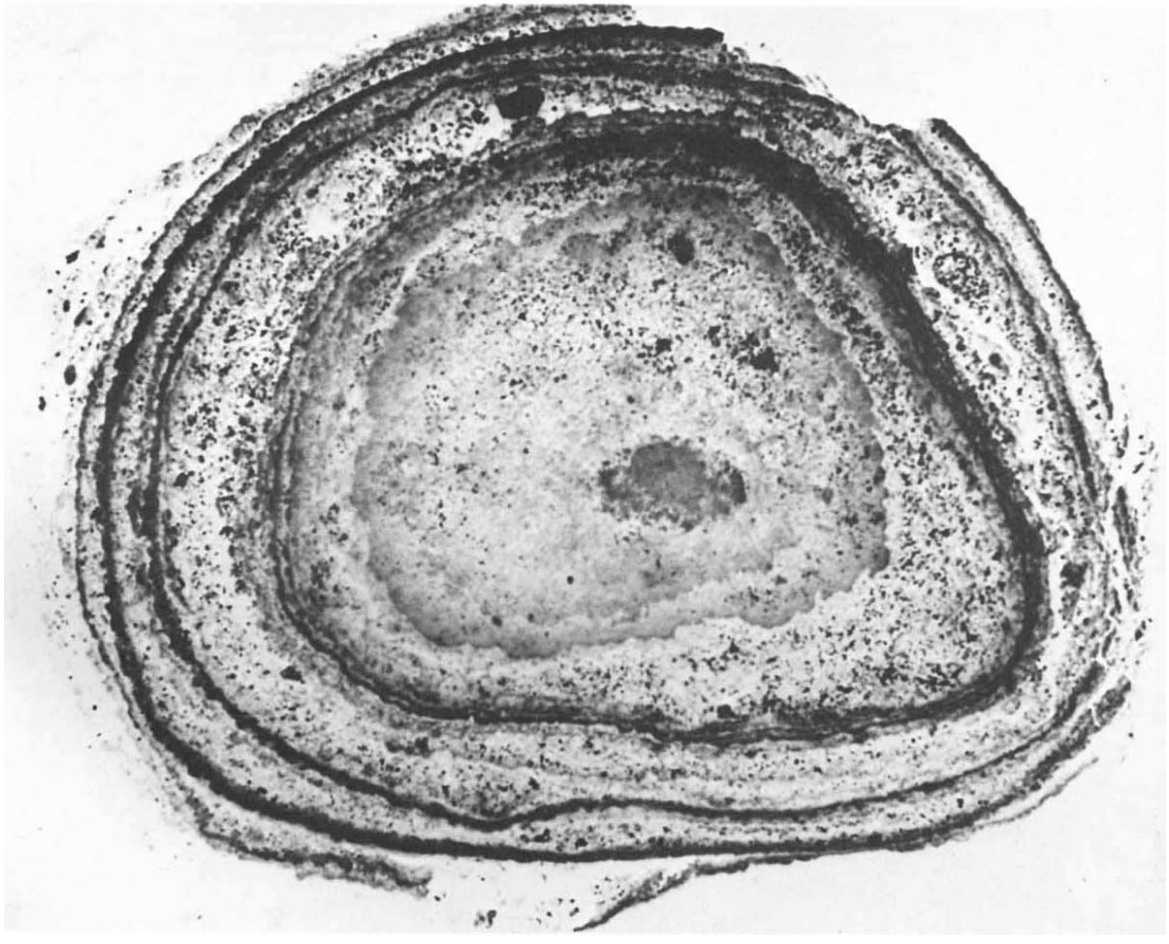

Fig. 2. *Semithin section (800 nm) of urinary matrix calculus of patient 4.* Note lamellar composition of stone. (Magnification, ×250; toluidine blue and paraphenylendiamine stain).

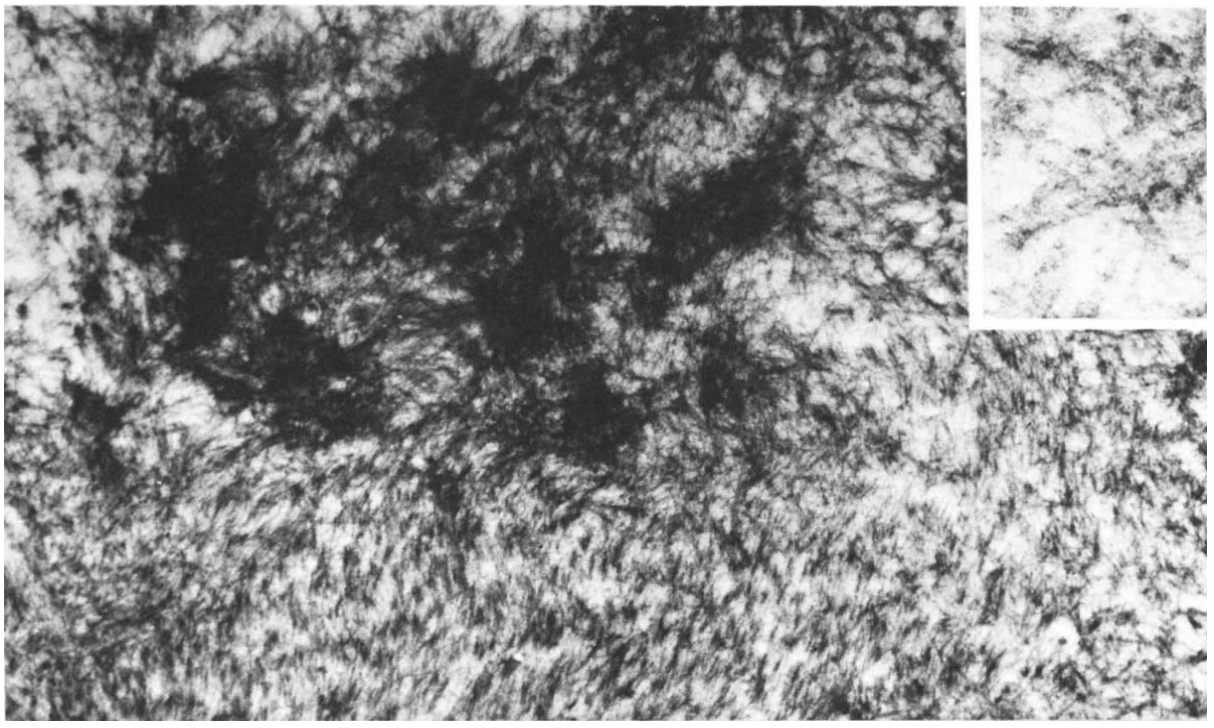

Fig. 3. *Transmission electron microscopy of urinary matrix calculus of patient 4.* Note tightly packed interwoven nonbranching microfibrils (diameter, 100 to 160 Å). (Magnification, ×4300; insert, ×100,000).

the low carbohydrate content of the stone specimens would also argue against an infectious origin because such stones have notoriously high carboxyhydrate contents. In addition, the ultrastructural appearance of the microfibrils and the absence of hydroxyproline on amino acid analysis makes it unlikely that the material is derived from the basal membrane-like material that is excreted in normal urine [20].

The presence of a major proportion of mature or immature fibrin in the stones appears unlikely in view of the almost consistently negative staining for fibrin with immunofluorescence, in view of the lack of the typical striation of fibrin and in view of the observed ultrastructural appearance. The diameter and density of packing of the microfibrils did not correspond to the characteristics of fibrin. Furthermore, the amino acid analysis of the material did not agree with the reported amino acid composition of fibrinogen [21].

The nature of the protein material remains unknown. There was some similarity with known characteristics of amyloid, because the specimens exhibited structural anisotropy (revealed after congo red staining under polarized light) and microfibrillar structure (demonstrated with transmission electron microscopy). The specimens, however, failed to consistently show metachromasia with crystal violet, and beta-pleated structure could not be demonstrated with X-ray diffraction analysis of demineralized matrix (data not given).

The highly ordered structure with concentric lamellae suggests that the material arose by intermittent slow growth from a supersaturated solution. It is of note that in 1894 similarly lamellated protein stones were described by Peipers [1], who found such material in microcysts which were present in the "contracted kidneys" of a patient dying from uremia. Recently, such cysts have also been described in dialyzed patients [22], and computer tomography in our seven patients with stones revealed the presence of such microcysts in five patients. It is tempting to speculate that prolonged presence of urine with high protein content in stagnant fluid of cysts, perhaps in the presence of continued reabsorption of sodium and water, may provide a nidus for stone formation. The fibrillar texture of the protein material is puzzling, but it has recently been reported that in urinary casts anisotropic fibrillar protein material can frequently be demonstrated [23] (Anders G, personal communication). Various proteins have been shown to assume microfibrillar structure upon exposure to partial proteolysis [24], and such proteolysis might con-

ceivably occur in tubular urine with known protease activity [25]. The relevance of these considerations, if any, for matrix stone formation remains to be established.

The true incidence of matrix stone formation in patients on maintenance hemodialysis is unknown. Although in the present study we observed one stone episode per 160 patient-months, no such stone episodes were observed in the same center for over 5 years prior to the present study. The remarkably high incidence may be due to chance clustering or else may reflect greater awareness of the problem. The latter argument is particularly relevant, because in some patients stones could only be demonstrated after the patients were asked to void their urine through gauze. Otherwise, stones may easily escape the notice of patient and physician. In addition, stones ought to be preserved in saline or the patients own urine, because they may rapidly crumble and dissolve when exposed to air.

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