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Research Article

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The Presence of Protein-Bound γ -Carboxyglutamic Acid in Calcium-Containing Renal Calculi

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ABSTRACT The amino acid γ -carboxyglutamic acid (Gla) is found in four blood-clotting proteins, in a bone protein, in kidney protein, and in the protein present in various ectopic calcifications. This paper reports the presence of Gla in the EDTA-soluble, nondialyzable proteins of calcium-containing renal calculi including calcium oxalate, hydroxyapatite, and mixed stores of apatite and struvite (MgNH_4PO_4). Calculi composed of pure struvite and those composed of only uric acid or cystine do not contain Gla. From calcium oxalate and hydroxyapatite stones, a protein of about 17,000 daltons was obtained which contained about 40 residues of Gla per 1,000 amino acids. The amino acid composition of this protein had no apparent relationship to the Gla-containing bone protein or to the similarly-sized F_1 fragment of prothrombin which contains about 64 residues of Gla per 1,000 amino acid residues. The Gla-rich protein in calcium-containing renal stones thus may be a different Gla-containing protein. These data as well as other studies demonstrating the presence of Gla in pathologically calcified tissues not normally containing Gla suggest that the Gla-containing proteins may be of considerable pathophysiological significance.

INTRODUCTION

γ -Carboxyglutamic acid (Gla)¹ has recently been found in the four vitamin K-dependent blood-clotting proteins (1-3), in proteins from bone (4, 5), and in another plasma protein, protein C, the function of

which is presently unknown (6). In proteins, this amino acid is produced by a posttranslational carboxylation of specific glutamic acid residues in the precursor protein by a vitamin K-dependent and bicarbonate ion-requiring reaction which has been studied in liver (7) and kidney (8) microsomes. The formation of Gla in the chicken bone protein is also vitamin K-dependent (9) and bicarbonate ion-requiring (10). In prothrombin, these Gla residues act as calcium-binding sites essential for the vital calcium- and phospholipid-dependent conversion of prothrombin to thrombin. If this carboxylation reaction is inhibited in vivo by coumarin-type anticoagulants or by vitamin K deficiency, the prothrombin protein, which is deficient in γ -carboxyglutamic acid, exhibits only weak, relatively nonspecific interaction with calcium ions, and the conversion to thrombin in the normal clotting cascade sequence is greatly retarded (11).

Since the Gla residues in the blood-clotting proteins appear to have calcium-binding properties essential for their normal physiological function, it appears possible that other proteins which are involved in other functions involving calcium ions may also contain this specific amino acid. Using a simple and reliable method developed in our laboratory for the quantitative measurement of Gla in proteins (4), we report the presence of Gla both in proteins of the organic matrix of only those renal calculi containing calcium and in certain urinary proteins.

METHODS

Renal calculi. Five types of urinary calculi, of which the major crystalline constituent (greater than 95%) was characterized by polarization microscopy and X-ray diffraction as calcium oxalate, hydroxyapatite, magnesium ammonium phosphate (struvite), mixtures of hydroxyapatite and struvite, uric acid, and cystine, were utilized in these studies. Except for the struvite stones which were obtained

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¹Abbreviations used in this paper: Gla, γ -carboxyglutamic acid; SDS, sodium dodecyl sulfate.

TABLE I
Amino Acid Composition* of the EDTA-Soluble,
Nondialyzable Proteins of Renal Calculi

Amino acids	Calcium oxalate†	Hydroxyapatite	Struvite	Apatite struvite‡	Uric acid‡	Cystine‡
<i>residues/1,000 amino acids</i>						
Hyp	—	—	—	—	—	—
Asp	307	212	237	171	165	103
Thr§	50	50	55	61	54	50
Ser§	78	76	63	109	78	61
Glu	202	179	172	152	150	110
Pro	39	26	45	57	32	53
Gly	60	82	55	111	107	217
Ala	53	66	56	89	71	105
Half Cys	4	9	15	3	11	14
Val	26	40	49	45	42	40
Met	7	7	2	1	7	—
Ile	19	19	28	21	29	24
Leu	53	56	71	49	69	56
Tyr	20	27	20	16	33	35
Phe	13	33	33	28	27	25
Lys	20	41	50	61	47	47
His	22	34	26	9	31	25
Arg	25	37	31	16	33	31
Gla¶	25	23	25	0.9	14	0.7

* Amino acid content determined by 6 N HCl hydrolysis except for Gla.

† Protein extracted from pooled stones.

§ Includes O-phosphoserine and O-phosphothreonine.

^{||} Glu content corrected by subtraction of Gla.

¶ Determined by alkaline hydrolysis.

from dogs,² the rest of the material was obtained from adult human patients. In typical preparations of stone matrix protein from calcium oxalate, mixed stones of apatite-struvite, and uric acid, 25 g of stones was obtained by pooling stones from 10 to 15 patients. The hydroxyapatite-containing calculi which were obtained from a single patient amounted to 37 g distributed among seven stones. An unusually large cystine stone weighing 160 g and a pool of small cystine stones from two patients were examined independently.

Preparation of the matrix proteins. The stones were ground to a powder using mortar and pestle and extracted with 0.5 M EDTA, pH 8.0 at 5°C for 48 h, with the latter procedure repeated twice. The centrifuged supernates were dialyzed exhaustively in Spectrophor I (Bausch and Lomb, Scientific Optical Products Div., Rochester, N. Y.) against water and then freeze dried.

Hydrolysis conditions. (a) Gla content: to assay for the presence of Gla, 5 mg of protein was hydrolyzed in 0.5 ml of 2 M KOH *in vacuo* for 24 h in alkaline-resistant tubes (model 7280, Corning Glass Works, Corning, N. Y.). The hydrolysate was neutralized to pH 7 with 70% perchloric acid at 0°C and centrifuged to remove KClO₄. (b) total amino acid content: 1 mg of protein was hydrolyzed in 1 ml of

constantly-boiling 6 N HCl *in vacuo* for 24 h at 105°C. (c) Phosphoserine content: for the routine detection of O-phosphoserine, 3 mg of protein was hydrolyzed in 4 N HCl for 6 h *in vacuo* at 105°C (12). A timed series of hydrolyses (from 1 to 24 h) was also carried out to determine the destruction of O-phosphoserine under the conditions used. When these data were extrapolated to zero time, it was found that, in 4 N HCl at 105°C for 6 h, approximately 70% of the O-phosphoserine was recovered. Similar studies of O-phosphothreonine revealed that only 30% of the O-phosphothreonine content was recovered after similarly timed hydrolysis.

Amino acid analysis. A Beckman/Spinco model 121 amino acid analyzer (Beckman Instruments, Inc. Spinco Div., Palo Alto, Calif.) equipped with a single column (0.28 × 33.0 cm) of Beckman AA-20 resin and an integrator system was used. (a) Gla program: for the complete separation of Gla from taurine and cysteic acid, a short program was run employing a stepped series 0.2 M citrate buffer from pH 2.70 to 3.44. The position of Gla was determined with an authentic sample obtained from Dr. Richard Hiskey of the University of North Carolina at Chapel Hill. The amount of Gla was quantitated using the color factor for Gla obtained with the authentic compound. The content of glutamic acid was obtained by subtracting the number of Gla residues from the total number of glutamic acid residues found after hydrolysis in 6 N HCl for 24 h at 108°C. (b) Total amino acid analysis was performed using the standard Beckman program. (c) Phosphoserine program: O-phosphoserine and O-phosphothreonine were separated from cysteic acid, and taurine employing 0.2 M sodium citrate buffer, pH 1.6.

Isolation of Gla and decarboxylation to glutamic acid. Alkaline hydrolysates of 25 mg of the EDTA-soluble proteins from calcium oxalate stones with putative Gla were applied to a preparative amino acid column (90 × 6 cm) containing Technicon Chromobeads type A resin (Technicon Instruments Corp., Tarrytown, N. Y.) and equipped with a split stream collecting 97% in 2.5-ml fractions and monitoring 3% by ninhydrin. The Gla was eluted with pH 2.5, 0.2 M citrate buffer and found to be in one tube. Part of the fraction was adjusted to pH 1.0 with 1 N HCl on ice and divided into equal aliquots which were placed in sealed ampoules and heated in a boiling water bath for 5, 10, 15, 20, and 40 min. The decarboxylation reaction was stopped at the specified times by placing the sealed ampoule on ice. Each aliquot and a portion of the original fraction were analyzed for its Gla and glutamic acid contents.

Fractionation of the EDTA extract of calcium oxalate and calcium hydroxyapatite stones. 150 mg of EDTA-extracted material from either calcium oxalate or hydroxyapatite stones were dissolved in 5 ml of 0.05 M NH₄HCO₃ buffer and applied to a Sephadex G-100 column (Pharmacia Fine Chemicals, Inc., Div. of Pharmacia, Inc., Piscataway, N. J.) 2.5 × 90 cm equilibrated in 0.05 M NH₄HCO₃ at room temperature. The sample was eluted with the same buffer at 15 ml/h in 3-ml fractions. Each fraction was read in a Zeiss spectrophotometer (Carl Zeiss, Inc., New York) at 230 nm. Sodium dodecyl sulfate (SDS) gel electrophoresis was performed by the method of Shapiro et al. (13). Molecular weights were determined in 10 and 12% acrylamide gels which were stained in Coomassie Blue.

Analysis of urine samples. 24-h urines were collected from three adults with no family history of renal calculi and from three patients who are recurrent stone formers. Urines were dialyzed exhaustively against water in Spectrophor I and lyophilized. The material was analyzed for Gla by 2 N KOH hydrolysis for protein content by standard amino acid analysis.

² Pure MgNH₄PO₄ do not exist in humans, probably because of the higher levels of calcium in human urine than in dog urine.

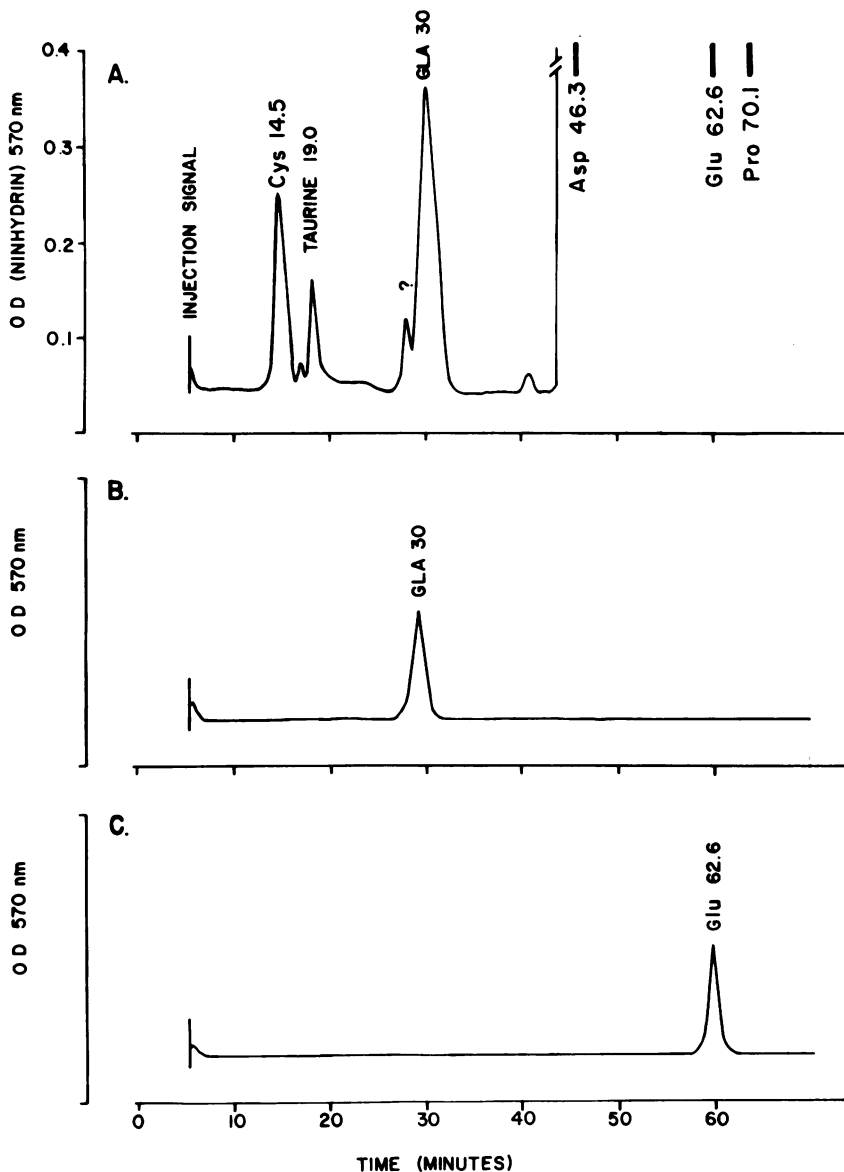


FIGURE 1 Beckman 121-M amino acid elution profiles. The initial elution buffer of 0.2 M sodium citrate, pH 2.7 resulted in separation of Gla eluting at 30 min from cysteic acid eluting at 14.5 min. (A) 2 N KOH hydrolysis for 22 h at 106°C of the EDTA-soluble, nondialyzable protein from calcium oxalate stones. (B) An aliquot of the putative Gla isolated on a preparative amino acid column after alkaline hydrolysis of the EDTA extract. (C) Conversion of Gla to glutamic acid is indicated. An aliquot of the putative Gla was heated in 0.5 N HCl for 1 h at 100°C before amino acid analysis.

RESULTS

The yield of nondialyzable extractable protein after three 48-h EDTA extractions ranged from 2.6 to 5.3% of the total dry weight of the original stone in the case of the calcium oxalate stones, 7.8% for hydroxyapatite stones, and 0.4–0.9% for pure magnesium ammonium phosphate, uric acid, cystine, and apatite-struvite

stones. The amino acid composition of the EDTA-soluble, nondialyzable extracts obtained from the various calculi are shown in Table I. The nondialyzable extracts obtained from different pools of calcium-oxalate stones showed varying aspartic acid contents ranging from 212 to 307 residues/1,000 total amino acid residues. The Gla content of these extracts from calcium oxalate stones ranged from 19.5 to 25 resi-

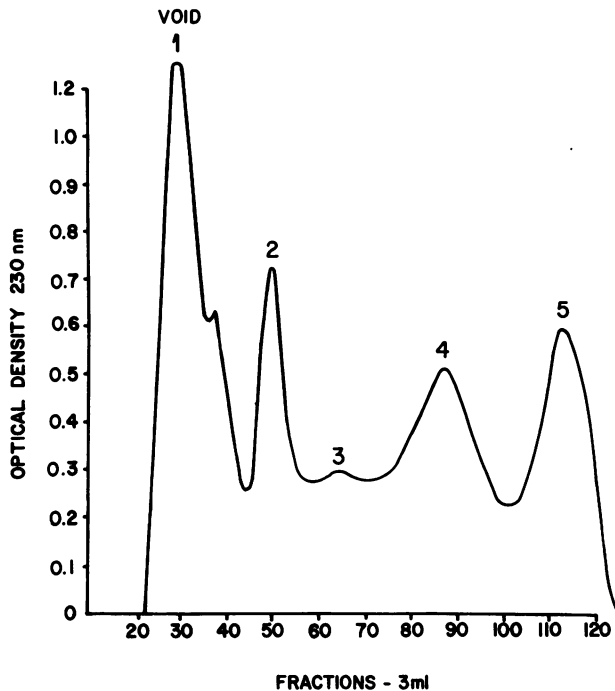


FIGURE 2 Gel filtration chromatography. 150 mg of the EDTA extract from calcium oxalate stones was separated into five fractions on Sephadex G-100 in 0.1 M NH_4HCO_3 eluting with the same buffer at a flow rate of 12 ml/h.

dues/1,000 total amino acid residues. The extracts obtained from apatite-struvite stones contained from 12 to 22 residues of Gla/1,000 total amino acid residues, whereas the EDTA extract of the hydroxyapatite stones contained 25 residues of Gla/1,000 amino acid residues. The uric acid stones and the cystine stone pools contained either no Gla or only trace amounts of Gla. Pure magnesium ammonium phosphate stones of canine origin were also found to contain only trace amounts of Gla (<1 residue/1,000 amino acids).

Fig. 1A demonstrates the ninhydrin-positive peak at the position of Gla obtained from the alkaline hydrolysate of the EDTA-soluble, nondialyzable proteins from the calcium oxalate stones. The putative Gla peak disappears if the alkaline hydrolysate is boiled at $\text{pH} < 1$ for 1 h before amino acid analysis. The identity of this peak as Gla was confirmed by isolation of a sufficient quantity of the amino acid which converted to glutamic acid when subjected to heat- and acid-catalyzed decarboxylation. The putative Gla fraction, isolated on the preparative column from a 25-mg hydrolysate, is observed to be free from the other acidic components when chromatographed in a Beckman 121-M analyzer (Fig. 1B). When this fraction was heated to 100°C at $\text{pH} 1.0$, it was converted only to glutamic acid (Fig. 1C).

The half-time conversion of Gla to glutamic acid under these conditions was the same (9 min) as found with authentic Gla.

Gel filtration of the EDTA extract from calcium oxalate stones on Sephadex G-100 yielded five fractions (Fig. 2). Different pools had similar but not identical profiles with differences observed in the relative yields of peaks 2 and 5. The Gla-rich fraction, peak 4, usually accounted for 25% of the protein content in the EDTA extract.

The percent protein in each fraction, its Gla, phosphoserine, and partial amino acid composition are summarized in Table II. A significant amount of O-phosphoserine and O-phosphothreonine were found in the lower molecular weight or retarded G-100 fractions. The marked destruction of O-phosphothreonine combined with the relatively small amount present, precluded an accurate calculation of its content. Qualitatively, it appeared to be present at a concentration less than 10% of the O-phosphoserine. The apparent molecular weight of the major component in each fraction is also given in Table II, and the SDS dics gels of each fraction are shown in Fig. 3. Peak 5 traveled with the buffer front and stained very poorly. The Gla-rich, peak 4 fraction (with 44 residues/1,000) has an apparent molecular weight of 17,000 daltons.

The soluble, nondialyzable proteins in the EDTA extract from hydroxyapatite stones was similarly fractionated by gel filtration on a Sephadex G-100 column. A fraction eluting in the same area as peak 4 from calcium oxalate was again found to be the Gla-rich component. Disc gel electrophoresis showed the fraction to be a protein of approximately 15,000 daltons. The protein has a slightly lower Gla content than the

TABLE II
Distribution, Molecular Weight, and Partial Amino Acid Content of the G-100 Fractions of Calcium Oxalate EDTA-Soluble Proteins

	Peak				
	1 (Void)	2	3	4	5
Distribution by protein content, %	38	20	16	23	3
Mol wt of major component	ND*	49,000	23,000	17,000	ND*
Amino acids, residues of amino acid/ 1,000 total amino acid residues					
Asp	109	144	180	290	260
Glu	123	153	187	188	190
Gla	0	5	12	39	11
O-Phosphoserine	1.5	4	10	13	9

* Not determined.

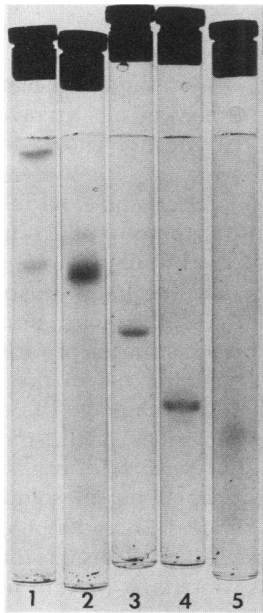


FIGURE 3 Dodecyl sulfate electrophoresis. 10% acrylamide-dodecyl sulfate gels of the Sephadex G-100 peaks 1-5 from the fractionation of the EDTA extract from calcium oxalate stones.

calcium oxalate protein (Table III) but a strikingly similar amino acid composition. Table III compares the amino acid composition of the calcium oxalate and hydroxyapatite stone "matrix" protein with two known Gla-containing proteins of similar Gla contents: the F₁ fragment of prothrombin (residues 1-156) and the EDTA-soluble, nondialyzable Gla-containing protein obtained from chicken bone (osteocalcin).

After the identification of Gla in calcium-containing renal calculi, the nondialyzable urinary proteins from three stone patients with a history of recurrent stone disease and three normals were analyzed for Gla content. Gla was found to be present ranging from 4.3 to 6.4 residues/1,000 amino acids in the nondialyzable protein of both normal and stone-forming patients. A dog urine was similarly analyzed and contained 7.2 Gla residues/1,000 amino acids in the nondialyzable urinary proteins. Isolation of the Gla-containing urinary proteins is in progress.

DISCUSSION

In general, the overall amino acid composition of the EDTA-soluble, nondialyzable proteins associated with varying types of renal calculi is quite similar, confirming an earlier report by Spector et al. (14). These investigators also noted that there were significant differences in the amino acid composition of certain of the partially purified proteins derived from

the calcium oxalate, apatite struvite, and uric acid stones, perhaps related to the differences in the structure of the crystalline component of the stones.

In the present study, another very distinct difference between the proteins derived from the various renal calculi was detected, namely, that only the proteins associated with calcium-containing calculi contained Gla. The protein in the calcium oxalate and the hydroxyapatite stones had a consistently high level of Gla, whereas the proteins associated with uric acid, cystine, and struvite did not contain Gla. The calculi composed of both struvite and hydroxyapatite contained a variable amount of Gla roughly in proportion to the percentage of hydroxyapatite present. Thus it appears that the Gla content correlates with the calcium content of the stones and not with the total

TABLE III
Amino Acid Composition of Several GLA-Containing Proteins

Amino acids	Protein from			Human prothrombin F ₁ fragment§
	Hydroxy-apatite stone*	Calcium oxalate stone*	Chicken bone†	
	<i>residues of amino acid/1,000 total amino acid residues</i>			
Asp	309	290	108	90←
Thr	41	44	8	64←
Ser	55	65	45	71
Glu	190	188	134	84←
Pro	25	38	97	64
Gly	34	41	98	71
Ala	41	46	120	64
Cys	3	7	3	64←
Val	37	29	56	57
Met	15	12	2	6
Ile	25	20	22	25
Leu	41	52	66	64
Tyr	37	27	51	26
Phe	28	18	37	26
His	18	24	19	13
Lys	20	22	8	32
Arg	19	24	61	96←
Try ^{**}	11	5	<1	19
Gla ^{**}	39	44	60	64
O-Phosphoserine	9	13	8	0

Arrows indicate significant differences.

* Separated from the nondialyzable proteins in the EDTA extract from hydroxyapatite and calcium renal stones by gel filtration.

† Separated by gel filtration on Sephadex G-100.

§ Magnusson et al. (3).

^{||} Includes O-phosphothreonine.

[¶] Corrected by subtraction.

** Determined by alkaline hydrolysis.

content of matrix protein or with the presence of Gla in the urine. The analysis of the pure struvite (MgNH_4PO_4) stones, such as are found in dogs (and not in humans), shows an absence of Gla which is consistent with the above speculation. Although this is a cross-species comparison, we have shown that dog urine contains Gla (*vide infra*).

The presence of Gla in calcium-containing stones is consistent with the known calcium-binding properties of protein-bound Gla (1, 2). The additional presence of O-phosphoserine in the proteins of these renal stones may not only be important in the binding of the calcium ions but also in fixing their stereochemical configuration, especially if the carboxyl groups are in close juxtaposition to the O-phosphoserine residues as they are in the enamel proteins (15).

To date, Gla has been identified as a component of the vitamin K-dependent blood-clotting factors (3), in a plasma protein, protein C (6), and in the EDTA-soluble, nondialyzable proteins of chicken and bovine bone (4, 5). The major protein components of the calcium-containing stones is relatively rich in this unique calcium-binding amino acid, to the same degree as the F_1 fragment from prothrombin (64 residues/1,000) (3) and the protein from bone (60 residues/1,000). A comparison of the amino acid composition of the purified Gla-containing protein of renal calculi with the other known Gla-containing proteins, however, shows marked differences in the amino acid composition, particularly in the aspartic acid and arginine content. This makes it highly unlikely that the Gla-containing protein(s) of renal calculi represent any of the known Gla-containing proteins such as prothrombin or the bone protein. Thus it seems most likely that the Gla-containing proteins extracted from renal calculi represent still another distinct class of proteins containing Gla.

Although the preliminary results have demonstrated the presence of Gla-containing protein(s) in the urine of normal and stone-forming patients, the relationships among the proteins excreted in the urine, the known serum and bone proteins, and the proteins obtained from renal calculi are unclear. In this regard, the recent finding that kidney microsomes synthesize a protein containing Gla is of great significance (8).

It is not possible at this time to ascribe a function to the Gla-containing proteins of renal calculi. Gla-containing proteins have been detected in the ectopic calcifications of soft tissues which do not normally contain Gla (16). In both this instance and in the case of the Gla-containing proteins associated with the bone matrix, it is not clear whether the protein is associated with the initiation of calcification, whether it represents an inhibitor of mineralization, or whether it arises by virtue of the fact that the Gla-containing proteins are adsorbed to the calcium-containing

calculi much as they may be fractionated on columns of hydroxyapatite due to the binding of the Gla residues to calcium (4). On the other hand, although the exact biochemical mechanisms involved in the formation of renal calculi are not known either in general or for any of the specific types of renal stone, it is clear from both theoretical and experimental considerations that a solution to solid-phase transformation is unlikely to occur only by simple homogeneous nucleation (17). It has not yet been established whether the first fragments of the solid mineral phase are initially formed on the surfaces of various microscopic or ultrastructural components in the kidney, as suggested by Caulfield and Schrag (18), or are nucleated in solution by interactions of the renal calculi components (Ca^{++} , $\text{PO}_4^{=}$, uric acid, oxalate) with specific macromolecular components in solution (19), but in any event, the process is undoubtedly initiated by heterogeneous nucleation. Thus it is tempting to speculate that the Gla-containing protein may serve as the nucleation substrate, although an equally plausible argument can be advanced that they function in just the opposite way. Similar considerations (16) have evolved concerning the possible role of the phosphoproteins in the initiation of calcification in enamel (15), bone (20), and dentin (21).

Further investigations on the nature of the Gla-containing protein of renal calculi, their site of synthesis, their Ca^{++} -interaction properties, and the number, configuration, and interaction properties of the bound calcium ions (17), as well as their relationship to the proteins in the urine containing Gla, may shed light on their physiological role.

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