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Research Article

A state of supersaturation of urine with respect to brushite is considered to be important in the formation of renal stones composed of calcium phosphate. 56 supersaturated urine specimens and 44 undersaturated specimens were incubated with collagen (Sigma collagen). Most of the supersaturated specimens calcified the collagen, whereas none of the undersaturated ones did so. Among samples which calcified the collagen, whereas none of the undersaturated ones did so. Among samples which calcified the collagen, the activity product of Ca^{++} and $HPO_4^{=}$ after incubation with collagen was essentially the same as that after incubation of the same specimen with brushite; it usually differed from that obtained after incubation with octacalcium phosphate or hydroxyapatite. The molar calcium-to-phosphorus ratio of the solid phase in collagen was approximately 1. These results suggested that the solid phase formed in collagen is brushite. This conclusion was confirmed by the direct identification of brushite in collagen by X-ray diffraction.

Calcification of Collagen by Urine In Vitro: Dependence on the Degree of Saturation of Urine with Respect to Brushite

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ABSTRACT A state of supersaturation of urine with respect to brushite is considered to be important in the formation of renal stones composed of calcium phosphate. 56 supersaturated urine specimens and 44 undersaturated specimens were incubated with collagen (Sigma collagen). Most of the supersaturated specimens calcified the collagen, whereas none of the undersaturated ones did so. Among samples which calcified the collagen, the activity product of Ca^{++} and $HPO_4^=$ after incubation with collagen was essentially the same as that after incubation of the same specimen with brushite; it usually differed from that obtained after incubation with octacalcium phosphate or hydroxyapatite. The molar calcium-to-phosphorus ratio of the solid phase in collagen was approximately 1. These results suggested that the solid phase formed in collagen is brushite. This conclusion was confirmed by the direct identification of brushite in collagen by X-ray diffraction.

INTRODUCTION

A state of supersaturation of urine with respect to the constituents of the stone is essential for the development of a renal calculus, no matter what theory is invoked for the pathogenesis of that form of stone (1–6). In previous communications (5, 6), urine specimens from patients with idiopathic hypercalciuria and recurrent renal calculi were shown to be usually supersaturated with respect to brushite ($CaHPO_4 \cdot 2H_2O$). The urine specimens from normocalciuric subjects were usually undersaturated except at a high urinary pH. These observations led us to consider brushite as the solid phase which governs the formation of renal stones composed of calcium phosphate, and may lead to the development of other calcium-containing renal stones (6).

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In this schema for the pathogenesis of renal calculi, brushite is considered to constitute the nidus and to regulate the subsequent growth of the nidus into a stone, whether that nidus is formed by spontaneous precipitation (3) or under the influence of an organic matrix (1, 2). In this communication we shall provide experimental evidence that brushite is the solid phase which is formed from urine in an organic matrix.

METHODS

Estimation of the degree of saturation of urine with respect to brushite. The method for the calculation of activity product of Ca^{++} and $HPO_4^=$ (K_{sp}), presented in detail previously (5, 6), was based on that of Levinskas (7). This consisted of first estimating the ionic strength (μ) of urine from its cation composition (6). The activity coefficients (γ) of Ca^{++} and $HPO_4^=$ at the specified μ was then obtained by graphic analysis. The concentration of $HPO_4^=$ was also determined by graphic analysis from urinary pH, total phosphate concentration, and μ. K_{sp} was then calculated from the equation: $K_{sp} = \alpha_{Ca^{++}} \cdot \alpha_{HPO_4^=} = [Ca^{++}] \cdot \gamma_{Ca^{++}} \cdot [HPO_4^=] \cdot \gamma_{HPO_4^=}$ where α is the activity and the bracket [ ] indicates molar concentrations.

The degree of saturation of urine with respect to brushite was calculated as follows (6). K_{sp} of urine was calculated before and after incubation with brushite (~~$CaHPO_4 \cdot 2H_2O$~~, Mallinkrodt). The ratio of the activity product of urine before incubation ($K_{sp,i}$) with the activity product of urine supernatant after incubation ($K_{sp,f}$) represented the degree of saturation of urine. A value of $K_{sp,i}/K_{sp,f}$ of 1 indicated saturation, greater than 1 supersaturation, and less than 1 undersaturation.

Calcification of collagen. The preparation of collagen employed (Sigma collagen, Sigma Chemical Co., Lot No. 58B-0770) was the same as that used by Wadkins (8). It was prepared according to the method of Einbinder and Schubert (9). 75 mg of collagen was incubated in 15 ml of urine at 37°C for 48 hr under constant stirring (with a magnetic stirrer). After 0.5 hr, 6 hr, and after 24 hr of incubation, the pH of urine was adjusted to the original pH with 0.1 N HCl or 0.1 N NaOH. The final pH after 48 hr of incubation did not differ from the original pH by more than 0.1 U. Visible precipitate was not observed during the incubation.

Approximately 10 ml of urine without collagen was decanted from each sample. Occasionally, small collagen fiber particles were also decanted; they were removed by filtration through Whatman No. 1 paper. The calcification of collagen was determined from the change in the concentration of calcium in the medium after incubation with collagen. A decrease in calcium concentration represented the uptake of calcium by collagen (calcification), whereas an increase in calcium concentration represented the release of calcium from collagen. Since the error in the chemical determination of calcium is approximately 2.5%, the uptake of calcium of less than 5% was considered to be not significant. Sigma collagen contained 0.03 mg of Ca per 75 mg of dry collagen; in the standard incubation studies (75 mg of collagen in 15 ml of urine), the maximum release of calcium from collagen was thus 0.03 mg/15 ml of urine or 2 mg/liter of urine. The quantity of calcium released usually represented less than 5% of the total calcium concentration of the original urine, and therefore did not significantly alter the results of calcium uptake by collagen. In selected experiments, the uptake of calcium was determined directly; collagen was ashed and analyzed for calcium. There was no significant difference in the calcium uptake determined by the two methods.

When there was an uptake of calcium by collagen, there was an uptake of phosphate as well. However, the percentage change in the concentration of phosphate after incubation with collagen was much less than that of Ca, because of higher initial urinary concentrations of phosphate. Further, Sigma collagen contains a significant quantity of phosphate (0.46 mg of phosphorus per 75 mg of collagen) some of which probably diffused into the medium during incubation. For these reasons, the calcium uptake rather than phosphate uptake was employed to study the calcification process.

Qualitatively similar results were obtained when urine specimens were incubated with collagen prepared from Achilles tendon by the method of Thomas and Tomita (10). Unlike Sigma collagen, this preparation has been shown to give a positive response to the silver nitrate staining technique of von Kossa when it undergoes calcification at pH 7.4 (8, 10). The uptake of calcium from an artificial medium at pH 7.4 by the two preparations of collagen was also found to be qualitatively similar (8).

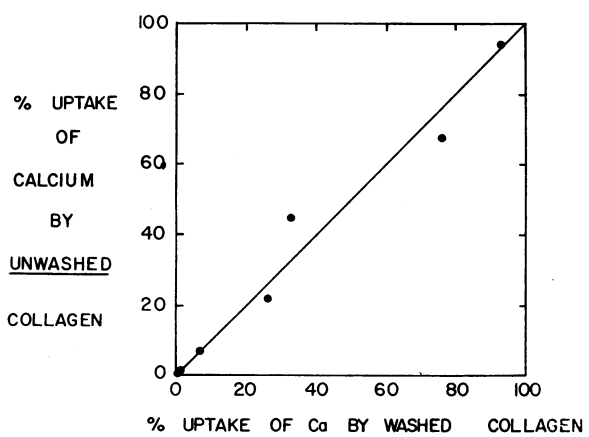

FIGURE 1 Comparison of calcium uptake by the unwashed Sigma collagen and by the washed collagen. The diagonal line represents the equal uptake of collagen by the two collagen preparations. Most of the experimental points were near this line of equality. Collagen concentration was 75 mg of dried collagen per 15 ml of urine.

TABLE I
Patient Data

Patient	Age	Sex	Diagnosis
	yr		
Group I			
1. A. D.	22	F	Primary hyperparathyroidism
2. E. B.	47	F	Primary hyperparathyroidism
3. S. D.	51	F	Idiopathic hypercalciuria
4. E. S.	30	F	Idiopathic hypercalciuria
5. C. H.	23	F	Idiopathic hypercalciuria
6. B. P.	12	F	Idiopathic hypercalciuria
7. G. P.	44	M	Idiopathic hypercalciuria
8. P. N.	47	M	Idiopathic hypercalciuria
9. R. B.	46	M	Idiopathic hypercalciuria
Group II			
1. J. G.	39	M	Recurrent nephrolithiasis
2. L. E.	41	M	Recurrent nephrolithiasis
3. E. G.	34	M	Recurrent nephrolithiasis
4. H. B.	41	M	Recurrent nephrolithiasis
5. N. M.	42	M	Recurrent nephrolithiasis
6. E. R.	57	F	Recurrent nephrolithiasis
7. L. P.	20	F	Recurrent nephrolithiasis
8. J. D.	69	M	Recurrent nephrolithiasis
9. A. Z.	40	M	Recurrent nephrolithiasis
Group III			
1. V. G.	22	F	Normal volunteer
2. V. S.	24	F	Normal volunteer
3. A. E.	20	F	Normal volunteer
4. L. B.	40	M	Normal volunteer
5. R. B.	49	F	Essential hypertension
6. W. G.	8	M	Adrenogential syndrome
7. H. T.	45	F	Idiopathic osteoporosis
8. J. R.	31	M	Idiopathic osteoporosis
9. N. S.	26	F	Amenorrhea
10. E. M.	37	F	Adrenal insufficiency (treated)
11. T. P.	56	M	Fibrogenesis imperfecta ossium
12. E. B.	47	F	Status postparathyroidectomy

Studies were also performed with washed Sigma collagen. Sigma collagen was dialyzed with distilled water at 10°C for 1 hr. The washed preparation contained 0.03 mg of P and 0.02 mg of Ca per 75 mg of collagen instead of 0.46 mg of P and 0.03 mg of Ca per 75 mg of the unwashed preparation. The uptake of calcium from urine specimens by the two preparations of collagen was quantitatively similar (Fig. 1). Studies to be reported hereon were performed entirely with unwashed Sigma collagen.

Identification of the solid phase in collagen. Six supersaturated urine specimens (with respect to brushite) were each incubated separately with collagen, brushite, octacalcium phosphate (OCP),[1] and hydroxyapatite (HA) (Gee and Dietz) (11) at 37°C for 48 hr. After 0.5 hr, 6 hr, and

[1] Kindly provided by Dr. Walter Brown of the American Dental Association.

after 24 hr of incubation, the pH of urine was adjusted to the original pH with 0.1 N HCl or 0.1 N NaOH. The activity product of Ca^{++} and $HPO_4^{=}$ in the urine supernatant was calculated at the final pH of urine.

15 specimens of calcified collagen (from 15 separate urine specimens) were ashed, redissolved in 1.2 N HCl, and analyzed for calcium and phosphorus. The molar ratio of calcium and phosphorus (Ca/P) was determined for each specimen. Five specimens of calcified collagen were washed in distilled water, freeze-dried, and analyzed by X-ray diffraction.[2]

Chemical determinations. The techniques for the measurements of magnesium, phosphorus (P),[3] soduim, potassium, ammonium, and pH were previously described (6). Calcium was precipitated as the oxalate, redissolved, and determined by titration with ethylenediaminetetraacetate. Recovery of calcium from urine was greater than 99%.

Clinical data. Nine patients with hypercalciuria[4] and a recurrent history of passing calcium-containing stones (group I), nine patients with normal urinary calcium and recurrent nephrolithiasis (group II), and twelve subjects with normal urinary calcium, without nephrolithiasis (group III) were evaluated (Table I). Group I consisted of two patients with primary hyperparathyroidism (parathyroid hyperplasia) and seven patients with idiopathic hypercalciuria (12). Each of the patients in group II gave a history of urinary tract infection during the course of his disease. Group III consisted of normal volunteers and patients with essential hypertension, the adrenogenital syndrome, idiopathic osteoporosis, amenorrhea, adrenal insufficiency (treated), fibrogenesis imperfecta ossium (13), and status postparathyroidectomy for parathyroid hyperplasia. The patients in groups I and II had passed multiple renal calculi consisting of pure calcium phosphate or of mixed calcium phosphate and oxalate, as shown by X-ray diffraction. The mean age of patients in group I was 36 yr, in group II, 43 yr, and in group III, 34 yr. These patients had received a diet each day containing 400–600 mg of calcium for 5 or more days before and throughout the period of urine collections. 1–17 24-hr urine specimens were obtained from each patient. The specimens were collected in plastic containers at 10°C without acid or preservative; those with pH greater than 6.7 were preserved under oil. None of the specimens studied showed visible precipitation. 35 specimens from patients in

[2] Kindly performed by Dr. David Eanes of the National Institute of Dental Research.

[3] Urinary orthophosphate will be expressed in units of elemental phosphorus.

[4] Hypercalciuria is here defined as urinary calcium excretion exceeding 200 mg/day on a diet containing approximately 400 mg/day.

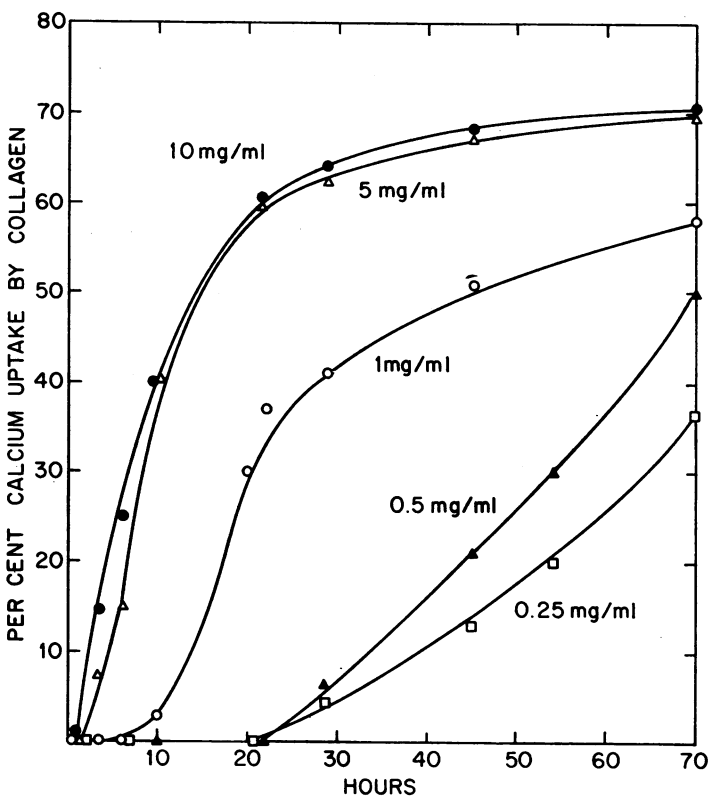

FIGURE 2 Effect of collagen concentration on the uptake of calcium by collagen. Different amounts of collagen were incubated in 200 ml of urine which was supersaturated with respect to brushite (activity product ratio of 2.58 and pH 6.84). At various times during incubation, 1 ml of urine was removed for measurement of calcium. The uptake of calcium by collagen is shown. (The urine specimen was from patient E. B. in group I.)

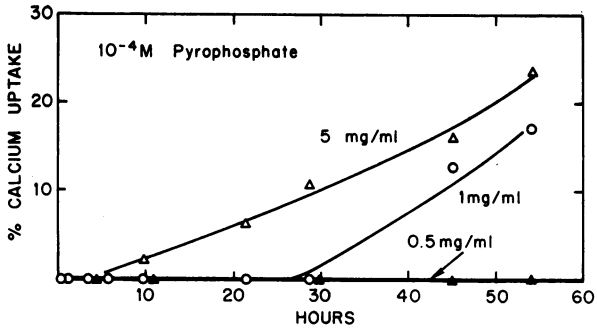

FIGURE 3 Effect of pyrophosphate on the uptake of calcium by collagen. To the same urine specimen (as in Fig. 2), pyrophosphate was added (20 μmoles/200 ml of urine). At all concentrations of collagen, pyrophosphate inhibited the uptake of calcium.

group I, 51 specimens from patients in group II, and 14 specimens from subjects in group III were evaluated for the determination of the degree of supersaturation with respect to brushite, and for calcification of collagen.

RESULTS

Effect of changing concentration of collagen on the uptake of calcium. Uptake of calcium from a supersaturated urine (with an activity product ratio of 2.58 and pH of 6.84) was determined at different concentrations of collagen, ranging from 0.25 mg to 10 mg of collagen per ml of urine (Fig. 2). Increasing the amounts of collagen decreased the time required to reach the limiting velocity. At collagen concentration of 5 mg/ml, there was a rapid uptake of calcium during the first 24 hr, followed by a slow uptake during the subsequent 46 hr. The uptake of calcium was almost complete by 48 hr. For this reason, urine specimens were evaluated for collagen calcification at the collagen concentration of 5 mg/ml (or 75 mg/15 ml) and 2 days of incubation.

Inhibition of calcification by pyrophosphate. At lower concentrations of collagen, there was a latent period of many hours before calcification took place (Fig. 2). This may have resulted from the presence in urine of inhibitors of calcification such as pyrophosphate (14). To evaluate this problem further, pyrophosphate was added to urine specimens (0.1 μmole/ml of urine). After the pH had been adjusted to the original pH, the urine specimen was incubated with different amounts of collagen as before. The uptake of calcium was markedly inhibited at all concentrations of collagen (Fig. 3). The latent period before calcification was much more marked in the presence of pyrophosphate. This period may represent the time required for the "inactivation" of the inhibitors of calcification.

Dependence of collagen calcification on the degree of saturation of urine with respect to brushite. Each urine specimen was incubated with both collagen (5 mg/ml) and brushite (2 mg/ml). Calcium uptake by collagen and the degree of saturation of urine with respect to brushite ($K_{sp,i}/K_{sp,r}$) at the final pH of urine (after incubation

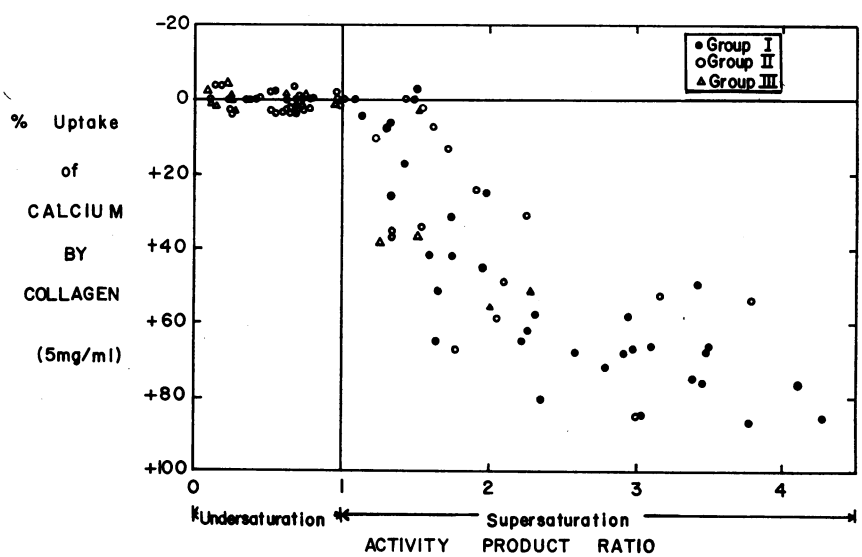

FIGURE 4 Dependence of calcium uptake by collagen on the degree of saturation of urine with respect to brushite. The uptake of calcium by collagen is presented as the decrease in the concentration of calcium in urine after incubation with collagen. None of the undersaturated urine specimens (activity product ratio of less than 1) calcified the collagen, whereas most of supersaturated urine specimens did so. Collagen concentration was 5 mg/ml.

with collagen) were determined.[5] Fig. 4 shows the relationship between calcium uptake by collagen and the activity product ratio. There was no significant uptake of calcium from urine specimens which were undersaturated with respect to brushite (activity product ratio of less than 1). However, a significant uptake of calcium occurred from most of urine specimens which were supersaturated (activity product ratio of greater than 1). The uptake of calcium by collagen was greater from the more supersaturated urine specimens.

8 of 56 supersaturated urine specimens did not calcify collagen, probably because of inhibition of nucleation by the inhibitors of calcification (8, 14). These specimens were only slightly supersaturated with respect to brushite (activity product ratio of less than 1.7). The more supersaturated urine specimens invariably calcified the collagen. Selected urine specimens were incubated with a lower concentration of collagen (0.5 mg/ml) for a shorter period (24 hr instead of 48 hr). The uptake of calcium from the same urine specimens was considerably less at the lower concentration of collagen (Fig. 5). However, most of urine specimens which calcified the collagen at the higher concentration of collagen also calcified it at the lower concentration. Thus, the pattern of calcium uptake at the lower collagen concentration was qualitatively similar to that occurring at the higher collagen concentration. Since collagen calcification is completely inhibited by 10^{-4}M pyrophosphate (at 0.5 mg/ml, Fig. 3), the "inhibitor activity" of most urine specimens examined is probably less than that of this concentration of pyrophosphate.

[5] Since there was only a small change in pH with collagen incubation, there was only a small or insignificant change in the activity product ratio when the final pH rather than the initial pH was employed.

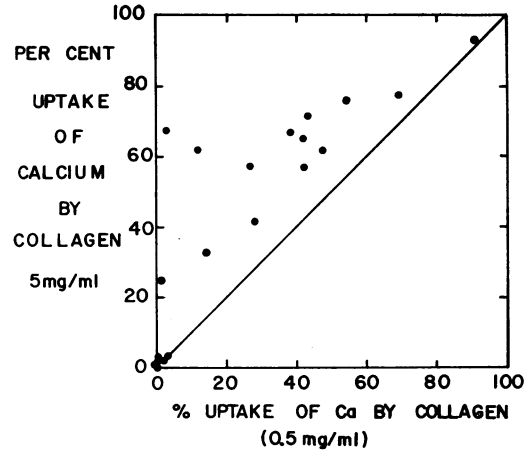

FIGURE 5 Comparison of calcium uptake by two concentrations of collagen. Uptake of calcium by collagen at two different concentrations (5 mg/ml and 0.5 mg/ml) was determined. The diagonal line represents the equal uptake of calcium at the two collagen concentrations. All the experimental points were above this line, indicating that calcium uptake at the higher collagen concentration was greater than that at the lower collagen concentration. Two specimens calcified the collagen only at the higher collagen concentration.

Identification of the solid phase in collagen. From 48 urine specimens which calcified the collagen, the activity product of Ca^{++} and $HPO_4^{=}$ (K_{sp}) was calculated after incubation with brushite (2 mg/ml) and after incubation with collagen (5 mg/ml). K_{sp} was plotted against the final pH of urine (at the completion of incubation) (Fig. 6). The values of K_{sp} after incubation with collagen were within the range of those after incubation with brushite. The solubility product of urine specimens after incubation with collagen was essentially the same as

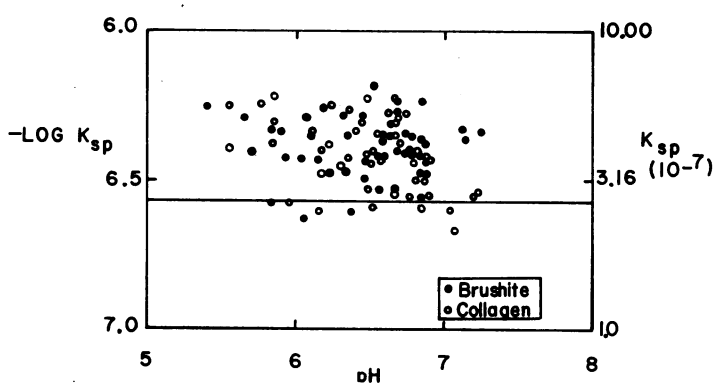

FIGURE 6 Activity product of Ca^{++} and $HPO_4^{=}$ of urine after incubation with collagen or with brushite. The activity product of urine after incubation with brushite (closed circles) was generally greater than the theoretical activity product of brushite (represented by horizontal line). The activity product of urine after incubation with collagen was not significantly different from that after incubation with brushite.

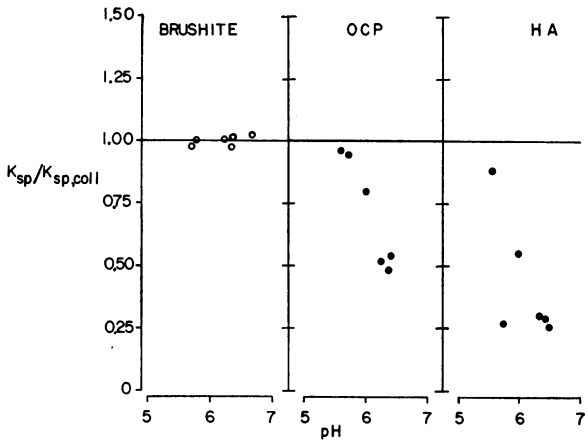

FIGURE 7 Comparison of the activity product of Ca^{++} and $HPO_4^{=}$ after incubation of urine with collagen, brushite, octacalcium phosphate, and hydroxyapatite. The activity product after incubation with collagen ($K_{sp,coll}$) was equal to that obtained after incubation with brushite. However, at pH greater than 5.7, $K_{sp,coll}$ was considerably higher than the activity product obtained after incubation with octacalcium phosphate or hydroxyapatite. In this study, 75 mg of collagen, and 30 mg each of brushite, octacalcium phosphate, and hydroxyapatite were incubated with 15 ml of urine.

that after incubation with brushite. This is more clearly demonstrated in Fig. 7. Six urine specimens were each incubated separately with collagen, brushite, octacalcium phosphate, and hydroxyapatite. The activity product of Ca^{++} and $HPO_4^{=}$ of samples after incubation with collagen ($K_{sp,coll}$) was equal to that obtained after incubation with brushite; the ratio of activity products was approximately 1. However, at pH greater than 5.7 $K_{sp,coll}$ was considerably higher than the activity product obtained after incubation with octacalcium phosphate or hydroxyapatite. These results indicate that the solid phase formed in collagen is brushite, and not OCP or HA.

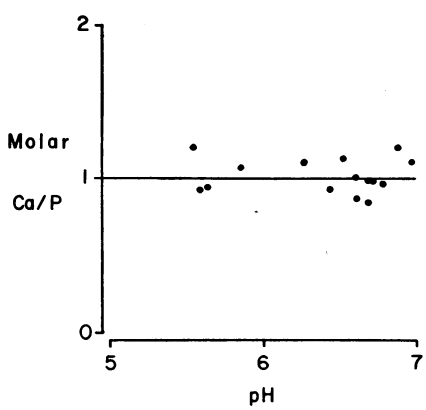

FIGURE 8 Chemical composition of the solid phase in collagen. 15 specimens of calcified collagen were analyzed for Ca and P content. The Ca/P approximated the theoretical value of 1 for brushite. 75 mg of collagen was incubated for 2 days in 15 ml of urine specimens which were supersaturated with respect to brushite. Each preparation of collagen showed an uptake of calcium from urine of more than 5%. The pH represents the final urinary pH.

Below pH 5.7, K_{sp} of OCP and HA approached that of brushite (Fig. 7). This suggests that the stable phase of calcium phosphate at this range of pH is brushite. Similar dependence on pH of crystal solubility has been shown for hydroxyapatite by other workers (15).

Additional evidence for the presence of brushite in collagen was obtained by direct chemical analysis and by X-ray diffraction. 15 specimens of calcified collagen were analyzed for Ca and P content. The Ca/P varied from 0.84 to 1.20 (Fig. 8). The mean Ca/P was 1.015 ±0.025 (SEM), which approximated the theoretical value of 1 for brushite. Five specimens of collagen, obtained from urine with the final pH of 5.5 to 6.6, were analyzed by X-ray diffraction. The predominant phase was brushite; octacalcium phosphate, hydroxyapatite, or calcium oxalate was not detected.

Comparison of studies in the three groups. Eight of nine patients with hypercalciuria (group I) usually excreted urine which was supersaturated with respect to brushite and which calcified the collagen (Table II). In the remaining patient, the mean activity product ratio was 1.02; none of the specimens from this patient calcified the collagen. This patient had the lowest value of urinary calcium and pH. Of the 35 urine specimens examined from this group, 32 were supersaturated and 30 calcified the collagen.

In six of nine patients with normal urinary calcium and stones (group II), the mean activity product ratio was slightly greater than 1 or not significantly different from 1. The remaining three patients excreted undersaturated urines. In this group, 19 out of 51 specimens were supersaturated, and 14 calcified the collagen.

In subjects with normocalciuria and without stones, four specimens were purposely allowed to become supersaturated by the addition of $CaCl_2$ or by raising the pH (group III-B). Three of these specimen calcified the collagen. In the remaining 10 specimens (unaltered) from nine subjects (group III), only one specimen was supersaturated. The urinary calcium content in this specimen was the highest in this group.

Similar dependence of collagen calcification on the activity product ratio was demonstrated in the three groups (Fig. 5). In each group, none of the undersaturated urine specimen calcified the collagen, whereas most of the supersaturated specimens did so.

DISCUSSION

The existence of urinary peptides possessing inhibitory powers against biological calcification is now recognized, largely through the work of Howard, Thomas,

TABLE II

Summary of Studies in the Three Groups

Patient	Urinary Ca	Urinary P	pH	Activity product ratio	Ca uptake by collagen	Total No. samples	Number super-saturated	Number with collagen calcification
	mg/day	*mg/day*			%			
Group I								
1. A. D.	279 ±38	669 ±33	6.49 ±0.20	2.06 ±0.48	+50.3	3	3	3
2. E. B.	224 ±12	507 ±11	6.67 ±0.05	2.82 ±0.31	+60.7	5	5	5
3. S. D.	247 ±48	671 ±65	6.59 ±0.12	2.35 ±0.44	+51.8	7	6	5
4. E. S.	235 ±16	830 ±95	6.50 ±0.03	2.64 ±0.26	+64.2	8	8	8
5. C. H.	214 ±17	1046 ±142	6.23 ±0.16	2.36 ±0.52	+41.2	7	6	6
6. B. P.	208 ±10	669 ±137	5.46 ±0.17	1.02 ±0.47	−1.3	2	1	0
7. G. P.	352	1026	5.88	1.96	+45.2	1	1	1
8. P. N.	340	822	6.03	1.33	+26.0	1	1	1
9. R. B.	221	735	6.17	1.31	+7.2	1	1	1
						Total = 35	32	30
Group II								
1. J. G.	109 ±15	950 ±105	6.52 ±0.15	1.27 ±0.25	+19.6	17	7	7
2. L. E.	91 ±9	1109 ±174	6.51 ±0.19	0.90 ±0.16	+2.5	7	3	1
3. E. G.	191 ±30	872 ±88	5.86 ±0.20	1.14 ±0.17	+4.7	5	3	2
4. H. B.	150 ±13	1034 ±129	6.28 ±0.10	1.04 ±0.26	+14.4	12	4	3
5. N. M.	167 ±33	745 ±69	5.56 ±0.25	0.61 ±0.45	−1.5	3	1	0
6. E. R.	199	250	6.67	1.34	+36.5	1	1	1
7. L. P.	181 ±24	512 ±16	5.31 ±0.06	0.73 ±0.05	−0.5	2	0	0
8. J. D.	49 ±12	601 ±84	5.81 ±0.19	0.08 ±0.01	0	3	0	0
9. A. Z.	154	924	6.30	0.71	0	1	0	0
						Total = 51	19	14
Group III								
3. A. E.	135	929	6.42	0.99	+1.0	1	0	0
4. L. B.	91	420	5.50	0.12	+1.0	1	0	0
5. R. B.	94	566	5.32	0.10	−2.0	1	0	0
6. W. G.	11	592	6.08	0.25	0	1	0	0
7. H. T.	194	891	6.42	1.52	+36.9	1	1	1
9. N. S.	168	887	6.13	0.74	0	1	0	0
10. E. M.	132	1091	5.93	0.27	+4.0	1	0	0
11. T. P.	105	317	5.70	0.21	−4.5	1	0	0
12. E. B.	124 ±48	245 ±35	5.90 ±0.37	0.43	−0.5	2	0	0
						Total = 10	1	1
Group III-B.								
1. V. G.	154	586	6.64*	1.25	+38.5	1	1	1
2. V. S.	262*	737	6.35	2.28	+51.6	1	1	1
8. J. R.	1070*	884	5.93	2.00	+56.0	1	1	1
9. N. S.	168	887	6.65*	1.53	+3.0	1	1	0
						Total = 4	4	3

Urinary calcium, P, pH, and activity product ratio are presented as mean ±SEM. Calcium uptake is 100 [1 − (calcium concentration in urine after collagen incubation/calcium concentration in urine before incubation)]. Positive value indicates uptake and negative value release of calcium by collagen. Ca uptake by collagen is presented as the mean of total number of urine specimens examined in each patient. The numbers and initials of patients in each group correspond to those in Table I. The final pH after incubation of urine with collagen is presented. Activity product ratio was calculated at the final pH. Group III-B comprised four specimens from subjects with normocalciuria and without stones, which were modified by increasing the urinary pH or the calcium concentration (shown by asterisks).

and associates (1, 16–18). These workers demonstrated that urine from patients with recurrent renal calculi (calcium containing) usually calcifies rachitic cartilage, whereas that from normal subjects does not. This was later attributed to the presence in the normal urine of peptides which markedly inhibit calcification. These urinary peptides have been shown to be equally effective in preventing calcification of modified collagen (8). The collagen therefore represents a simpler and more readily available preparation for testing the propensity of urine for calcification than the rachitic cartilage.

However, the exact role of the urinary peptides in the pathogenesis of renal stones is not clear. Unfortunately, the studies of inhibition are of limited biological significance, since they were performed with altered urine specimens (adjusted to pH 7.4, and diluted to a specific gravity of 1.01) (16). Further, the precise determination of the degree of saturation of urine with respect to brushite or other solid phase was not performed (1, 16–18). Thus these studies do not clearly exclude the possibility that the calcification of rachitic cartilage by "stone-forming urine" results from the state of supersaturation of urine rather than from a "deficiency" of the inhibitors of nucleation.

We have attempted to resolve the above difficulties in our study. The propensity of unaltered urine to calcify collagen and the degree of saturation of that urine with respect to brushite were measured (6). Our results emphasize the importance of the state of supersaturation of urine to initiate the calcification process. None of the undersaturated urine specimens calcified the collagen, whereas most of the supersaturated ones did so, regardless of the collagen concentration. Inhibitors of calcification probably do not play a significant role in *this* calcification system, since only a few supersaturated urine specimens failed to calcify the collagen. The pattern of collagen calcification by urine specimens of patients with stones was similar to that by specimens from subjects without stones. This observation is consistent with the recent results of Robertson, Hambleton, and Hodgkinson (19), who questioned the absence or reduction of urinary peptides inhibitors as etiologically important in the formation of renal stones.

This study provides further experimental evidence for our hypothesis of renal stone formation, wherein brushite plays a regulatory role (5, 6). Our results suggest that the state of saturation of urine with respect to brushite determines whether calcification of the organic matrix (here assumed to be represented by collagen) will occur. When calcification takes place, the results suggests that the solid phase formed in the matrix is brushite. Among supersaturated samples, the activity product of Ca^{++} and $HPO_4^{=}$ after collagen incubation was equivalent to that of brushite, and not that of octacalcium phosphate and hydroxyapatite. The Ca/P of the solid phase in collagen approximated that for brushite of 1. Finally, the predominant phase in collagen was identified as brushite by X-ray diffraction. Thus brushite may play an important regulatory role in the *matrix theory* of stone formation.

In this schema for the pathogenesis of renal stones consisting of calcium phosphate, passage of urine supersaturated with respect to brushite is critical in the formation of stone. A state of supersaturation may allow the formation of the brushite nidus. If supersaturation persists the nidus may become stabilized and eventually grow into a stone. Brushite may eventually be transformed into other calcium phosphates, particularly when it is exposed to an alkaline medium. The brushite nidus may also serve as a nucleus for other calcium-containing renal stones (20, 6). This hypothesis is supported by our observation that none of the undersaturated urine specimens (with respect to brushite) calcified the cartilage, even though some of them were supersaturated with respect to calcium oxalate, octacalcium phosphate, or hydroxyapatite (4) (Fig. 7). Thus, the formation of a brushite nidus may be required before the development of other calcium-containing stones. The formation of renal stones of calcium phosphate and calcium oxalate among patients with hypercalciuria, many of whom excrete persistently supersaturated urine with respect to brushite, is therefore not surprising.

In studies in progress, brushite will be shown to play an equally important role in the precipitation theory of stone formation.

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