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Tamm-Horsfall Mucoprotein Promotes Calcium Phosphate Crystal Formation in Whole Urine: Quantitative Studies

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Summary. Fresh samples of human urine adjusted to pH 6.8 were rapidly evaporated at 37 °C to 1,250 mosmol/kg. The calcium phosphate precipitated was washed and the calcium measured by flame atomic absorption spectrophotometer. This procedure was found to be reasonably reproducible. When macromolecules were first removed by ultrafiltration the precipitated calcium was reduced by 76.9%. Addition of human Tamm-Horsfall mucoprotein (T-H) to the urine ultrafiltrates prior to evaporation largely but not completely restored the precipitated calcium. Clumping of crystals was studied quantitatively by passage through a nylon mesh. Mean retention on the mesh was strikingly reduced after ultrafiltration and increased by addition of T-H to the ultrafiltrates. These findings support the view that T-H triggers calcium phosphate crystal formation and clumping in whole urine.

Key words: Tamm-Horsfall mucoprotein, Calcium phosphate, Crystalluria.

Introduction

Stone matrix may be important in the pathogenesis of stone formation. Pursuing this idea, Hallson and Rose [1] by using a rapid urinary evaporation technique [2] showed that calcium phosphate crystal formation in whole urine was promoted by the presence of Tamm-Horsfall mucoprotein (Uromucoid). They also noted that the presence of Tamm-Horsfall protein caused clumping of the precipitate of calcium phosphate. However, the methods used by these authors were only qualitative and moreover, the rapid evaporation technique had not then been fully assessed. Therefore, it was decided firstly to develop a quantitative technique for measuring calcium phosphate crystal formation in urine and secondly to study thereby the effect of uromucoid on calcium phosphate crystal formation in whole urine.

Materials and Methods

All urine studies were performed at 37 °C. Fresh urine samples from normal subjects shown to be crystal free were adjusted to pH 6.8, the osmolalities measured, and the samples then evaporated to 1,250 mosmol/kg and incubated for 1 h as previously described for studies on calcium oxalate precipitation [3]. After incubation the samples were centrifuged for 10 min at 3,000 rpm, the supernatant discarded and the precipitate washed to remove urine (hence dissolved calcium salt) adhering to the calcium phosphate precipitate. Three wash liquids were tested namely, deionized water, phosphate buffer pH 8 and phosphate buffer pH 7. The precipitate was washed twice with 0.5 ml of the above fluids. Phosphate buffer pH 7 was eventually selected for all subsequent work. The washed precipitate was dissolved in 1.5 ml of 1 MHCl and the calcium in the dissolved precipitate measured by atomic absorption spectrophotometry [4].

To test for particle size of the calcium phosphate precipitate a sieving procedure was used with a nylon sieve of pore size 10 μm. A comparison was made of retention on the sieve of the crystals formed after evaporation of whole urine or the ultrafiltrate of urine with and without added uromucoid (35 mg/l) prepared as previously described [3]. The scheme of investigation of urine at pH 6.8 is illustrated in Table 1.

Results

1. Variation in Urinary pH. Twenty urine samples were adjusted to pH 6.80 and then each pH was measured after incubation for 1 h. The range of rise was from 0–0.4 pH units with a mean of 0.12 (6.92 S.D. ± 0.18).

2. Final Osmolarities. The osmolarity achieved was compared with that intended (1,250 mosmol/kg) in 20 samples of urine subjected to the evaporation procedure. The mean achieved value was 5.6 mosmol/kg higher than intended with S.D. of 14.72.

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Table 1. Scheme for quantitative studies of crystal clumping

	50 ml urine	50 ml U/F[a]	50 ml U/F & Uromucoid
Evaporate to 1,250 mosmol/kg	YES	YES	YES
Centrifuge	YES / NO	YES / NO	YES / NO
Sieve	YES	YES	YES
Measure calcium on sieve	YES	YES	YES
Wash precipitate	YES	YES	YES
Dissolve precipitate	YES	YES	YES
Measure calcium in dissolved precipitate	YES	YES	YES

[a] Ultrafiltrates of urine

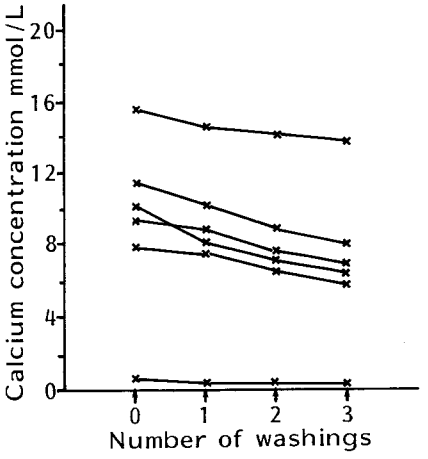

Fig. 1. The effect of washing and re-washing of precipitates of calcium phosphate with deionised water upon measured calcium contents after dissolving in acid

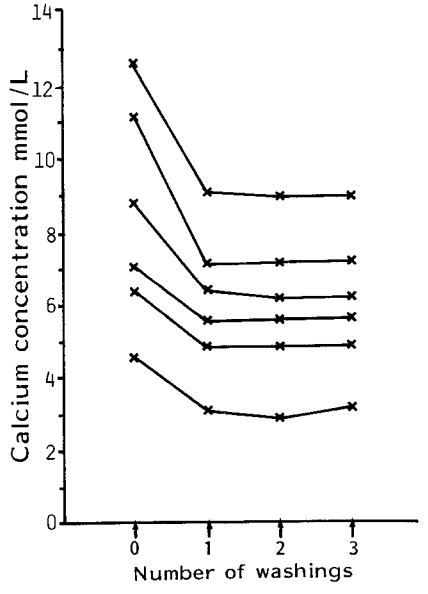

Fig. 2. As for Fig. 1 but washing with phosphate buffer at pH 7

3. Washing the Precipitate. Six samples were studied washing each precipitate three times using 0.5 ml of deionized water. As shown in Fig. 1 there was progressive dissolution of calcium from the precipitate on each washing which thus rendered deionized water unsuitable. Phosphate buffer pH 7 was tested similarly and the result (Fig. 2) showed that there was no loss of calcium from the precipitate in the second and third washes. Phosphate buffer pH 7 was considered most suitable and used for washing in subsequent experiments.

4. Reproducibility of the Method. Twenty fresh urine samples from 13 male hospital staff members were studied. Each sample was divided into two portions and each evaporated separately and the precipitate collected and analysed for calcium as described above. The percentage mean difference in calcium concentration between the pairs of results was 5.68 S.D. ± 4.37. No sample was rejected.

5. Effect of Incubation Time on the Precipitate. Ten samples of whole urine and the ultrafiltrate of urine from 7 healthy individuals were used. After evaporation to 1,250 mosmol/kg each sample was divided into 6 equal portions. The first portion was not incubated the second incubated for $^1/_2$ h at 37 °C, the third for 1 h, the fourth for 2 h, the fifth for 3 h and the sixth for 4 h. The results in Fig. 3 show that an increasing incubation time leads to a progressive rise in urinary calcium phosphate precipitation until 3 h when precipitate bulk becomes fairly constant. In the further studies 1 h was chosen as a convenient period of incubation for experimental purposes.

6. Effect of Ultrafiltration on Total Precipitation. Twenty urine samples were subjected to ultrafiltration as described above and as shown in Table 2, the mean reduction of precipitated calcium was 76.9%. Although the distribution of the values are non-Gaussian it can be seen that there was a clear fall in every case and the statistical significance of

Table 2. Calcium concentration mmol/l in the case of whole urine and ultrafiltrate of urine with and without uromucoid from total precipitate and precipitate on sieve

Initials		(A) Total precipitate			(B) Precipitate on sieve		
		Whole Urine	U/F Urine	U/F + Uromucoid added	Whole Urine	U/F Urine	U/F + Uromucoid added
1	GPK	35.4	10.1	30.3	8.8	0.75	8.1
2	JK	37.4	7.1	30.3	4.4	0.42	3.5
3	CW	46.5	1.8	21.0	21.0	0.2	17.4
4	DS	32.3	1.5	18.8	25.3	2.2	19.2
5	JK	11.1	6.3	10.6	7.4	1.3	6.4
6	GPK	10.1	2.5	7.6	10.6	1.5	7.9
7	GM	33.3	15.2	26.3	25.3	1.9	21.5
8	SS	2.8	0.89	1.8	0.58	0.11	0.42
9	SS	18.7	2.5	9.1	3.5	0.38	3.5
10	ROM	39.4	1.0	10.1	4.3	0.50	4.0
11	RM	11.5	1.0	10.6	1.7	0.76	2.8
12	PH	9.9	6.5	7.9	0.52	0.10	0.42
13	RM	23.7	1.1	12.6	10.6	0.21	8.4
14	SS	1.1	0.63	1.5	0.15	0.05	0.31
15	GG	29.3	12.9	21.5	20.2	1.4	15.4
16	RS	1.4	0.78	1.2	0.21	0.05	0.16
17	CW	25.3	8.8	25.3	25.3	0.89	16.2
18	DS	24.5	9.1	18.9	8.6	1.1	5.2
19	CS	18.2	6.6	16.7	1.7	0.12	1.3
20	SS	6.1	0.25	5.8	5.1	1.0	4.5
	MEAN	20.89	4.82	14.83	9.24	0.75	7.32

Table 3. Derived data from Table 2. Percentage of precipitate retained on Sieve

	Whole Urine	U/F Urine	U/F + Uromucoid added
1	25.0	7.4	26.7
2	11.8	5.9	11.4
3	45.1	17.8	83.1
4	78.1	142	102
5	66.5	20.1	60.1
6	105	60.1	104
7	78.8	12.6	81.7
8	20.6	12.4	23.5
9	18.9	15.1	38.4
10	10.9	49.5	39.8
11	14.4	74.5	26.5
12	5.2	1.5	5.3
13	44.6	18.4	66.6
14	13.2	7.9	20.3
15	69.0	10.9	71.8
16	15.0	6.4	13.1
17	100	10.1	64.0
18	35.3	12.5	27.6
19	9.1	1.8	7.6
20	83.3	40.4	78.3

the fall was verified by using Friedmann's non-parametric ranking test ($p < 0.01$).

7. Effect of Adding Uromucoid on Total Precipitate. Out of 20 samples used above, the mean gain of precipitated calcium on re-adding uromucoid to ultrafiltrate of urine was 59.5% relative to the whole urine precipitate. Using Friedmann's non-parametric ranking test the difference between ultrafiltrate of urines and ultrafiltrate plus uromucoid was found to be significant ($p < 0.01$)

8. Effect of Ultrafiltration on Crystal Clumping. The mean retention of calcium phosphate precipitated on the sieves is shown in Tables 2–3. Out of 20 samples studied there was a striking fall in percentage retention in 16. There were increases in 4 but in 3 of these (numbers 10, 11, and 20) the absolute values shown in Table 2 were so low that they were probably rather inaccurate and can be ignored. The fourth [4] is not explained. Using all the data the mean retention was 44.2% in the case of whole urine and 15.6% in the case of ultrafiltrates of urine and this effect was significant ($p < 0.01$) using Friedmann's non-parametric ranking test.

9. Effect of Adding Uromucoids on Crystal Clumping. There was a striking increase in retention of calcium phosphate on adding uromucoid to the ultrafiltrates of the urine. Three of the four exceptions are explained as above. The mean calcium phosphate crystal retention was 50.9% for ultra-

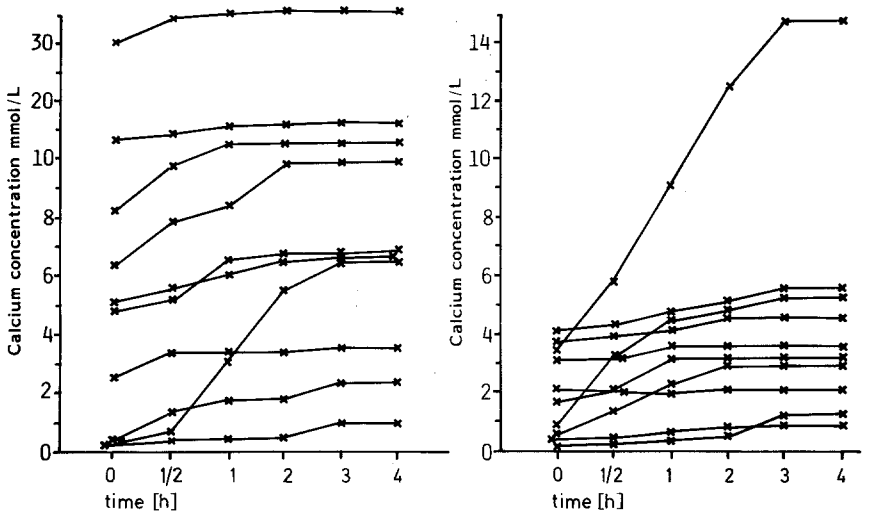

Fig. 3. The effect of duration of incubation after evaporation of urine and ultrafiltrates upon calcium concentration in dissolved precipitates. *Left*, whole urine; *right*, ultrafiltrates of urine

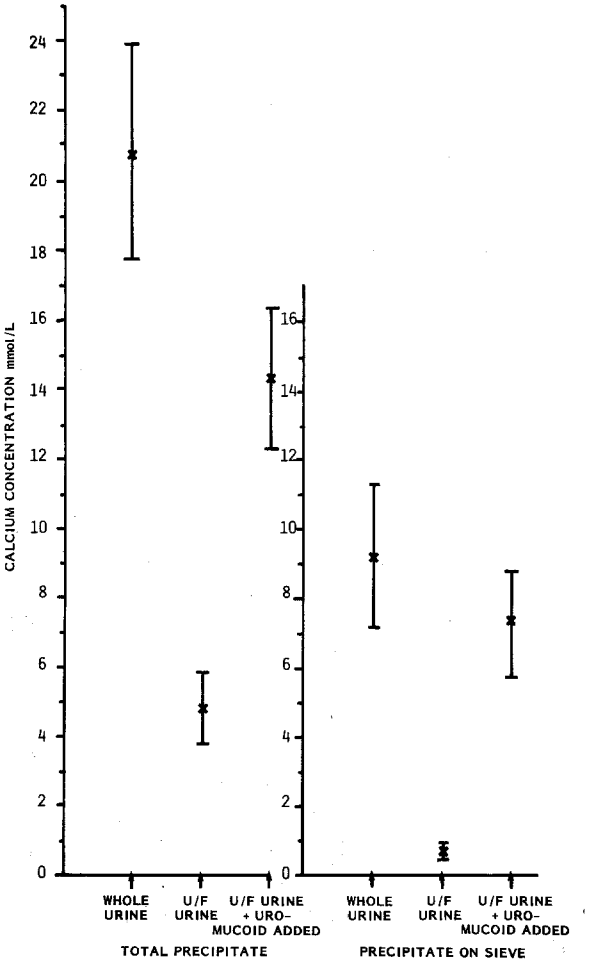

Fig. 4. Calcium concentrations (means and SEM) in dissolved whole precipitates and precipitates retained on sieves after evaporation of whole urine and urine ultrafiltrates with and without addition of uromucoid

filtrates of urine with uromucoid added compared with 15.6% in case of ultrafiltrate of urine alone. This effect again was significant ($p < 0.01$) using Friedmann's non-parametric ranking test. All the results are shown in Table 2 and Fig. 4.

Discussion

As found by earlier work [5, 6] pH 6.8 proved convenient for producing calcium phosphate precipitates, which were thought to be free from calcium oxalate for the following reasons. First, the precipitates from 6 samples were analysed for oxalate [7] and none could be detected. Second, on microscopic examination of the precipitate no calcium oxalate crystals could be seen at pH 6.8. Third, the ratio of phosphate: oxalate in urine is about 100:1 and the ratio of weights of precipitates is of the same order so that oxalate ought to be negligible. Sörenson phosphate buffer (pH 7) was found to be satisfactory for washing the precipitate and using a minimal volume it proved capable of removing excess urine calcium whilst preventing dissolution of the precipitate.

Measuring calcium from the dissolved precipitate gave a measure of calcium phosphate crystals in the precipitate. The technique was quantitative, reproducible, simple and rapid, the whole process could be finished within two hours so that no preservative was required.

Two main points were found. Firstly, when uromucoid was removed from urine by ultrafiltration the calcium phosphate precipitate fell to 76% of that in whole urine. On addition of purified uromucoid to the ultrafiltrates of urine this fall was reversed. Secondly, as previously described [2] it was clearly observable not only by light microscopy but even to the naked eye that the calcium phosphate from whole urine was clumped whereas that from ultrafiltrate of urine was not. The clumping phenomenon was quantitated by the nylon sieve studies which showed that the proportion of bound or clumped calcium phosphate of size more than 10 μ was greater in whole urine than in ultrafiltrate of urine by a factor of 3. On addition of uromucoid to ultrafiltrate of urine so as to give a normal urinary concentration, the total precipitate was found to be virtually restored to that found in the whole urine, while on the sieve retention was increased by readdition of uromucoid to ultrafiltrate of urine.

This finding is also in agreement with Boyce [8] who demonstrated that fraction I mucoprotein, recovered from calculous disease urine, binds calcium avidly forming an insoluble complex, and on addition of phosphate ions, progressive formation of calcium phosphate crystals follows the appearance of calcium mucoprotein precipitate. The explanation of the effect of uromucoid on clumping calcium phosphate crystals could be that by concentrating the urine to high osmolarity uromucoid molecules become aggregated into an insoluble polymeric form upon which calcium phosphate crystals may grow and become entrapped. A similar process could operate in vivo since within such a uromucoid mesh a mixture of uromucoid and calcium phosphate crystals could adhere high in the renal tubules and so lead to stone formation.

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