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Pyrophosphate Does not Influence Calcium Oxalate or Calcium Phosphate Crystal Formation in Concentrated Whole Human Urine

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Summary. 1) Low pyrophosphate urine was generated by passage through a nylon coil bearing immobilised inorganic pyrophosphatase. High pyrophosphate urine was made by addition of inorganic pyrophosphate. 2) Urine samples of low, normal, and high pyrophosphate content were rapidly evaporated at 37 °C to 1,050 or 1,250 mosmol/L and the crystals formed studied by microscope, isotope and chemical methods. 3) Urinary pyrophosphate levels had no significant effect upon calcium oxalate crystals formed in whole urine at pH 5.3 or 6.1, or calcium phosphate crystals formed at pH 6.8.

Key words: Crystalluria, Pyrophosphate, Whole urine.

Introduction

Pyrophosphate was thought by Fleisch and Bisaz to be an inhibitor of calcium oxalate [3] and calcium phosphate [2] crystal formation. It has been confirmed that pyrophosphate inhibits both growth and aggregation of calcium oxalate crystals in simple aqueous solutions [4, 14] and that pyrophosphate inhibits calcium phosphate formation. There is considerable doubt, however, about whether or not pyrophosphate plays a significant part in preventing crystal formation in urine. Some authors [1, 22] found that calcium oxalate crystal growth was retarded in human urine by pyrophosphate while others [13] found that it played only a small part. Part of the difficulty may be that methods have differed very greatly, some using simple aqueous solutions and some very dilute urine. Some have measured total growth of crystals and some crystal aggregation. Furthermore, they are all open to the criticism that they were not performed under the conditions that relate to urine under in vivo conditions. The systems in simple aqueous solutions take no account of the physiological effect of Tamm-Horsfall mucoprotein in triggering calcium salt precipitation as occurs when water is removed from dilute urine to yield concentrated urine [5, 17]. The systems using whole urine to which are added calcium and oxalate ions were found to yield abnormal crystals which did not resemble the envelope calcium oxalate dihydrate crystals occurring naturally in the urine of stone formers [7]. With the introduction of the technique of rapid evaporation of whole urine (7) it became possible to assess the role of potential inhibitors and promotors of calcium oxalate and calcium phosphate crystal formation in whole urine in a way that overcomes the above objections. In this test system water is rapidly abstracted from dilute urine and this would seem to be analogous to the action of collecting tubules of the kidney in removing water from dilute urine. Perhaps as a result of this similarity the envelope calcium oxalate crystals formed look just like those found in urine of some normal subjects and of stone formers [7].

Two methods of assessing crystal formation have been used, as previously described [8]. The method with ^{14}C-oxalate is fully quantitative and measures total crystal growth but gives no indication of crystal size, shape or aggregation. These features, however, can be observed with the microscope method, although only semi-quantitatively. The two methods are therefore complementary. Using these methods it has been possible to demonstrate in whole urine the roles of magnesium [8] and of citrate [11] in inhibiting calcium oxalate crystal formation and the lack of effect of urate [10].

This paper describes similar studies with pyrophosphate, using immobilised enzyme phosphatase to make low pyrophosphate urine while avoiding contamination by foreign substances.

Methods

To make low-pyrophosphate urine, 35 ml samples were cycled for 120 minutes (pH 6.5, 37 °C) through nylon coils bearing immobilised pyrophosphatase (EC 3.6.1.1,). Pyrophosphate in the urine was

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Table 1. Urine pyrophosphate levels after evaporation (μmol/L)

	pH 5.3 (isotope)			pH 5.3 (microscope)			pH 6.1 (isotope)			pH 6.1 (microscope)			pH 6.8 phosphate tests		
	Low	Normal	High	Low	Normal	High	Low	Normal	High	Low	Normal	High	Low	Normal	High
Mean	14.2	69.7	189.6	12.3	53.3	173.6	15.4	54.2	174.1	13.2	49.5	170.2	7.9	42.6	163.1
S.D.	7.31	42.8	41.4	6.4	23.8	24.3	6.9	19.3	19.4	8.2	18.6	18.6	4.4	14.8	14.5
S.E.	2.1	12.4	12.0	1.9	6.9	7.0	2.0	5.8	5.8	2.4	5.4	5.4	1.3	4.3	4.2
N.	12	12	12	12	12	12	12	11	11	12	12	12	12	12	12

measured enzymatically (20) before and after this treatment. A further control sample (pH 6.5, 60 ml) was passed for the same time through a nylon coil free from immobilised pyrophosphatase (dummy coil). The pH of the samples was then adjusted to 5.3 or 6.1 to study calcium oxalate crystals or, to 6.8 for calcium phosphate. Sufficient sodium pyrophosphate was added to a 25 ml aliquot of urine from the dummy coil to raise the concentration after evaporation by 120 μmol/L. Second and third 25 ml aliquots consisted of dummy coil and low-pyrophosphate urine respectively. The three aliquots of urine with high, natural and low contents of pyrophosphate were evaporated to the same osmotic concentration (1,050 mosmols/kg was used for calcium oxalate crystals and 1,250 mosmols/kg for calcium phosphate crystals). Following evaporation each sample was incubated for one hour at 37 °C and centrifuged at 37 °C. Calcium oxalate crystals were determined by the semi-quantitative microscope method or the fully quantitative isotope method previoulsy described [17]. In the calcium oxalate tests sufficient sodium oxalate was added just before evaporation to raise the final concentration after evaporation by 0.11 mmol/L. The microscope estimation also gave the opportunity to look for changes which might have occurred in crystal form and aggregation at the different pyrophosphate concentrations. The reproducibility for comparing precipitates of calcium oxalate has been shown previously [17] to be good.

In studies of calcium phosphate crystal formation the amount of precipitate was determined by twice washing the centrifuged deposits with phosphate buffer (0.06 M, 0.5 ml, pH 7), dissolving it in HCl (1 M, 1.5 ml) and finally measuring dissolved calcium by atomic absorption flame spectrophotometer [21]. The reproducibility of this method has also been found to be satisfactory in a study to be published elsewhere. It was assumed that the contribution of calcium oxalate to this phosphate precipitate was negligible and this seems a reasonable assumption for the following reasons. Firstly, the phosphate to oxalate concentration ratio in urine is about 100:1 and the ratio of the size of the precipitates appears to be of the same order. Secondly, attempts to measure calcium oxalate precipitated at pH 5.3 failed because of the imprecision due to so small a mass and at pH 6.8 even less oxalate would be precipitated. Finally, the absence of oxalate crystalluria above pH 6.2 is supported by our previous studies [6] and those of others [14].

Sixty crystal-free samples were obtained from normal subjects. Twenty-four were evaporated at pH 5.3, twenty-four at pH 6.1 (calcium oxalate inhibition tests) and twelve were at 6.8 (calcium phosphate tests).

It was thought desirable to ensure that any changes in crystal formation observed were due to changes in pyrophosphate concentration and not a spurious result of the coil cycling process. Accordingly, a further twenty normal urines were divided into three samples. The first remained in the collecting (Dewar) vessel, the second was passed through a dummy coil, and the third was passed through a pyrophosphatase coil for the same time period. In the latter the fall in pyrophosphate was restored to approximately normal level by addition of 35 μmol/L of sodium pyrophosphate after the cycling process. After evaporation, incubation and estimation of ^{14}C-oxalate precipitated no significant changes in crystal formation were found. Using the semi-quantitative microscope method the changes were small and of doubtful significance.

Pre- and post-coil urinary calcium levels were determined in all samples used in the study and were found to be unchanged. Measurements of glycosoaminoglycan levels were made [19] in ten samples before and after passage through the pyrophosphatase coil and the uro-mucoid concentrations was similarly determined [18] in three instances. Levels of these substances were unaltered by the coil.

Results

The post evaporation urine pyrophosphate concentrations obtained by lowering and raising pyrophosphate levels are shown in Table 1.

The effect of changing the levels of pyrophosphate in normal urine upon calcium oxalate precipitation are shown in Figs. 1 and 2, and the effect upon calcium phosphate in Fig. 3. In these figures each result for high and low pyrophosphate has been expressed as a percentage of the corresponding result for normal pyrophosphate urine. Microscope and isotope results then conformed to approximately normal distribution.

Figure 1 shows the effects of low, normal and high pyrophosphate levels upon the induced calcium oxalate crystalluria at pH 5.3 measured by the microscope and isotope methods. Students "t" test was applied to the normal versus low, normal versus high, and low versus high pyrophosphate groups in each case, but no significant differences appeared. Similarly Fig. 2 shows the extent of calcium oxalate precipitation at pH 6.1. No statistically significant differences were found. Fig. 3 shows the percentage of calcium phosphate precipitated at pH 6.8 in low, normal and raised pyrophosphate urine as determined by atomic absorption flame spectrophotometry. Again, no significant differences emerged. In looking at the crystals under the microscope, no change in size, shape or aggregation of the crystals could be observed.

Discussion

The results show that in respect of either total crystal formation or of crystal growth or of crystal aggregation,

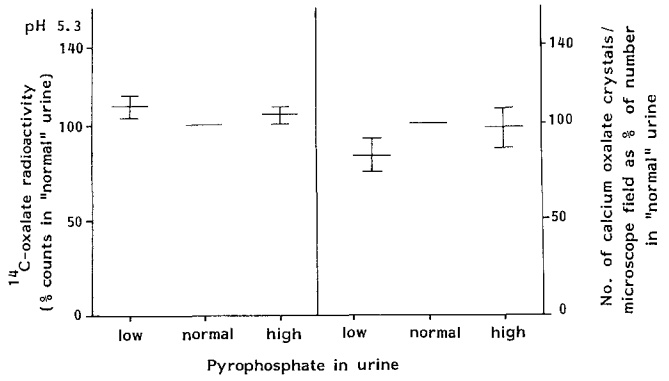

Fig. 1. The effect of changing urinary pyrophosphate upon the calcium oxalate crystals formed after evaporation of urine at pH 5.3 to 1,050 mosmol/L (microscope method) or 1,250 mosmol/L (isotope method). Results (mean and SEM) are shown relative to urine with normal (unchanged) pyrophosphate levels

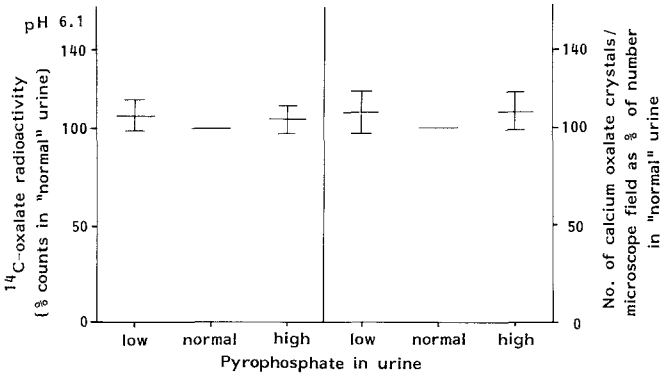

Fig. 2. Same as for Fig. 1 but with urine at pH 6.1

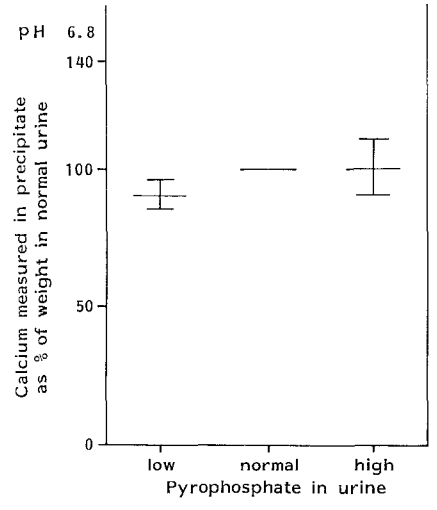

Fig. 3. Effect of changing urine pyrophosphate upon calcium phosphate crystals formed after evaporation at pH 6.8 to 1,250 mosmol/L. Crystals were assessed by chemical method. Results (mean and SEM) are shown relative to urine with normal (unchanged) pyrophosphate levels

pyrophosphate has no measureable crystal inhibitory activity towards calcium oxalate and phosphate in concentrated urine at pH values studies. This may seem surprising in view of the findings of others reviewed above, but two main points should be considered. Firstly, the validity and reliability of the evaporation test for crystal growth inhibitors is supported by tests on magnesium and citrate using the technique. Both materials reduce calcium oxalate crystalluria when present in obtainable urinary concentrations [8, 11], and the inhibitory powers of both substances are well known and seem widely accepted. It therefore seems reasonable to claim that the methods used are sensitive enough to detect changes in crystal formation if they occurred. Secondly, this study is the first to examine the effects upon crystals of the removal of endogenous urinary pyrophosphate from the urine, and also the first to assess inhibition of crystal formation by pyrophosphate in urine samples undergoing concentration in a way analogous to the natural process. Earlier tests of inhibitory activity were performed using either simple aqueous solutions or dilute urine. However, these solutions as previously demonstrated by others [22] did not themselves give identical effects with the same inhibitors and hence the properties of an inhibitor in concentrated urine may well be different again. This possibility is supported by the two separate findings. Firstly calcium oxalate crystals arising by infusion of calcium and oxalate ions into water or dilute urine are not only dissimilar [7, 16] but are quite different from those produced by evaporation of urine. The latter are however, identical with natural urinary crystals [7]. Secondly, urinary uromucoid molecules aggregate as urine osmolarity rises within the physiological range giving rise to an insoluble polymer [9], which promotes calcium oxalate and phosphate crystal formation in concentrated urine whereas the disaggregated molecule present in dilute urine is inactive in this respect [9].

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