Studies of urease-induced crystallisation in undiluted human urine using the Coulter counter technique

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Summary. Urease-induced crystallisation was studied in different human urine samples after urease incubation. The studies were performed using the Coulter counter technique, which enables determination of the number and size of particles in a solution and calculation of the total particle volume. The crystallization took place in three consecutive but overlapping steps: (1) nucleation, (2) growth and (3) aggregation. The maximal number of particles obtained in the different samples varied little, but there was a great variation in particle size and total particle volume. The variation in particle size appeared to be mainly due to differences in particle growth, a factor that might be of importance for stone formation.

Key words: Urease-induced crystallisation – Coulter counter – Undiluted human urine – Magnesium ammonium phosphate – Calcium phosphate

The formation of urinary tract stones is related to crystallisation processes in urine. It has generally been acknowledged that urease-producing microorganisms can induce the crystallisation of both calcium phosphate and magnesium ammonium phosphate (MAP), which can lead to the formation of so-called infection stones [6]. The formation of urease-induced concrements in urine, however, is a complicated process that to some extent remains poorly understood. In some individuals, colonisation of urease-producing microorganisms does not lead to the formation of any concrements at all, whereas in others there is rapid stone formation and progression to large staghorn stones [12]. Recent studies have shown that the urine composition is of paramount importance for urease-induced crystallisation [11]. Human urine appears to exert an inhibitory action on urease-induced crystallisation [5, 7], and proteins in urine, such as albumin, have been shown to influence the process [11]. We do not know, however, how the different phases involved in urease-induced crystallisation (nucleation, crystal growth and aggregation) are influenced by variations in urine com-

position and by different potential inhibitors. Indeed, no studies aimed at elucidating the nature of the different phases of urease-induced crystallisation appear to have been performed.

The use of the Coulter counter to study growth and dissolution rates of sparingly soluble materials was pioneered by Higuchi [9] and Higuchi and Lau [10]. The instrument has since been used, among other purposes, to measure the rates of growth and dissolution of various urinary compounds, including hydroxyapatite and calcium oxalate crystals [13]. Ryall et al. [15, 16] have studied the kinetics of particle aggregation and the factors affecting the aggregation of crystals in calcium oxalate crystallisation. In the present study, the Coulter counter technique was used to study urease-induced crystallisation in synthetic urine and in urine samples from different individuals, the aim being to obtain detailed information on the process, i.e. the order in which the different phases occur and the extent to which they are inter-related.

Subjects and methods

Urine samples

Morning urine specimens were collected without the use of a preservative from three men and two women whose age ranged from 28 to 42 years. None of these subjects had a history of urinary tract infection or stone disease and all had negative urinary cultures. The urine samples were collected in sterile bottles at the laboratory, pH was measured, and the samples were directly centrifuged at 4°C for 15 min at 1,500 g and used for urease incubation within 4 h. Urinary pH was measured with a surface pH electrode (Orion 9–35, Orion Research Inc.).

Synthetic urine

The composition of the synthetic urine used was that previously described by Griffith [6]. It contained 11 solutes and had a pH of 5.7. It was sterilised by Millipore filtration (0.22 µm) and stored in glass bottles at 4°C until used.
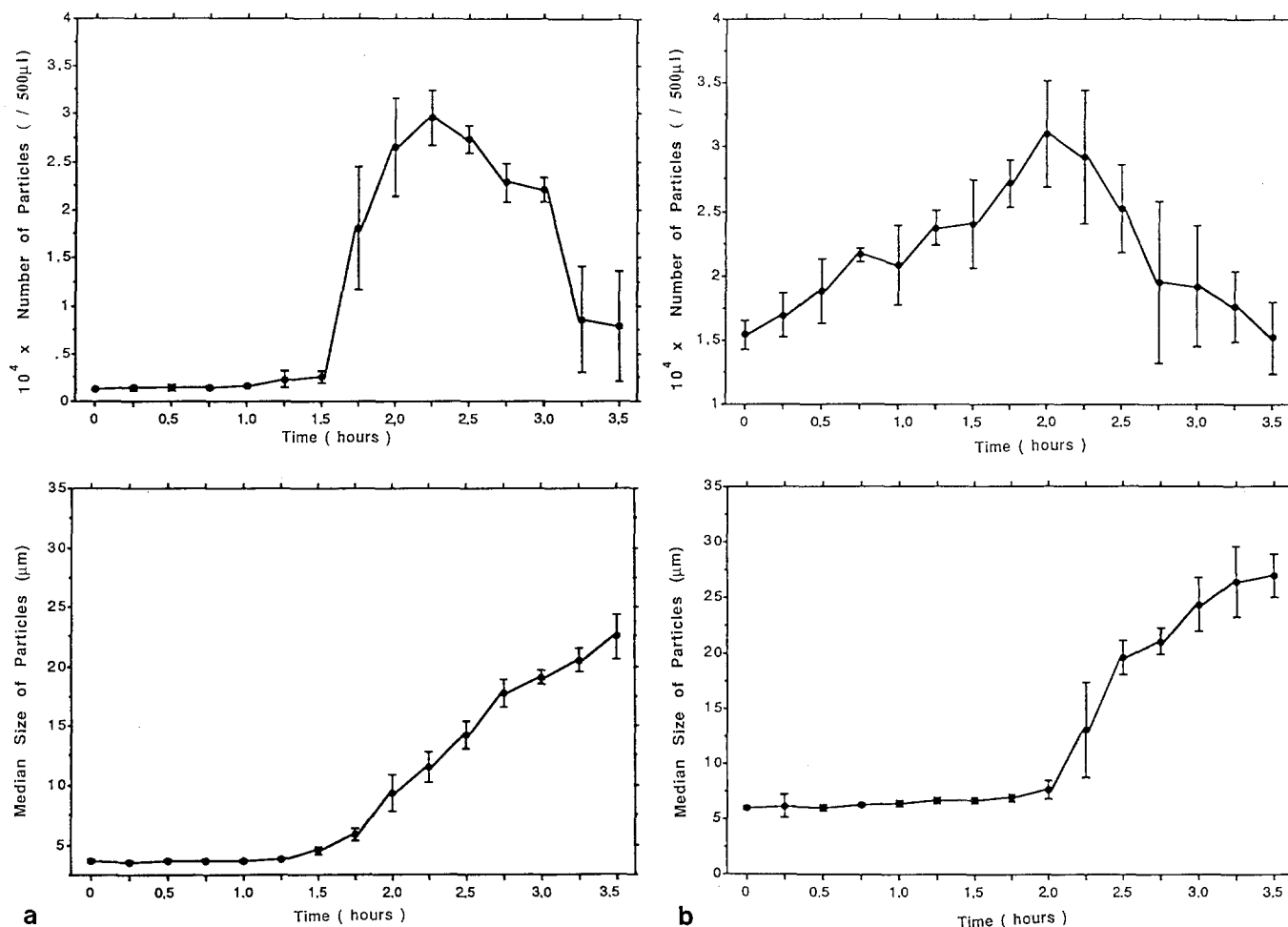
Fig. 1a, b. The change with time in particle number and median size after urease incubation in **a** synthetic urine and **b** a human urine sample. Each value represents the mean of 5 determinations and the bars denote 2 SD

Urease incubation

Human urine samples and synthetic urine in 100-ml portions were incubated with 100 µl urease solution in sterile glass vessels at 37°C for 3.5 h. The urease used was a high-purity preparation of jackbean urease (Sigma Chemical Company, USA) dissolved in 0.01 M TRIS buffer (pH 7), giving an activity of 109.1 µmol NH_3 min^{-1} ml^{-1}. During the incubation, continuous slow stirring was provided by a Teflon-coated stirring bar in each vessel.

In the first experiment, five portions of synthetic urine and 5 portions of one urine sample were incubated simultaneously. This experiment was performed to check the reliability of the Coulter multisizer analyses. In the second experiment, five different urine samples and one portion of synthetic urine were incubated in six glass vessels to study variations between different urine samples and synthetic urine.

Coulter multisizer analysis

Every 15 min during the 3.5 h incubation, portions of urine were sampled and analysed for particle number and particle size using a Coulter multisizer (Coulter Electronics Ltd, England) fitted with a 140-µm orifice tube. The multisizer was calibrated using standard calibration particles measuring 14.2 µm in diameter that were suspended in Isoton (an electrolyte solution). Particles measuring from 2.8 to 84 µm could be registered.

Data treatment

The total particle volume was calculated from particle number and size (diameter in micrometers) by an IMC A/TUR 80 computer using the Coulter Multisizer AccuComp program supplied by Coulter Electronics Ltd. From these basic measurements, the median and mean particle size and the total particle surface area could be calculated. The median is more amenable to mathematical treatment than the mode and was chosen to denote particle size. The results were calculated as the arithmetic mean over the channel span from 001 to 256 using a weighted distribution. In the calculations, it was assumed that (a) all particles were spheres, (b) all particles grew as spheres, and (c) when the particles aggregated, the resultant particles were also spheres [9]. These assumptions are consistent with the results obtained using the Coulter multisizer and the AccuComp program. The data obtained with the Coulter multisizer were further analyzed using a Macintosh SE/30 computer (Apple Computer Inc., USA) supplied with the Cricket Graph programme (Cricket Software, USA), enabling graphic representation of changes in number, size, volume and surface area with time in every sample.

An index of particle growth was calculated from the slope of the linear function that was satisfied by the increase in median particle size with time until the surface area started to decrease. The median particle size was supposed to increase when two consecutive measurements showed increasing values; by analogy, the surface area was expected to decrease when two consecutive measurements showed decreasing values.
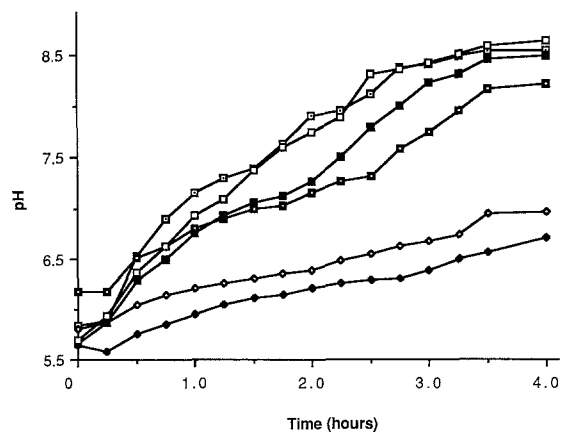
Fig. 2. The pH increase in synthetic urine and five different human urine samples after urease incubation. ▣, Synthetic urine; ◆, man 1; ◻, man 2; ◇, man 3; ■, woman 1; ☐, woman 2

Results

The changes with time in particle number and size in synthetic urine (Fig. 1a) and in urine from man 2 (Fig. 1b) are shown. The coefficient of variation (*CV*) for the determinations of particle number was between 3% and 33% in synthetic urine and between 2% and 22% in

human urine. The corresponding values for determinations of the particle size were between 5% and 25% in synthetic urine and between 1% and 21% in human urine. The CV for pH after 3.5 h urease incubation was low, being 0.2% in synthetic urine and 0.3% in human urine. The growth index in synthetic urine varied between 13.1 and 16.6 (CV, 9.5%). The corresponding range for growth index in the urine from man 2 was 10.7–12.6 (CV, 6.5%). The pH of the five morning urine specimens varied between 5.6 and 6.2 (Fig. 2). On incubation with urease, the pH increased with time; a steady increase was noted in all urine samples. After 3.5 h incubation, pH varied between 6.7 and 8.6.

The results obtained using Coulter counter in the second experiment are presented in Fig. 3 and Table 1. In synthetic urine, the number of particles started to increase after approximately 1.25 h; the increase lasted for 0.5 h and was followed by a continuous decrease. The median particle size began to increase after 1.25 h and kept increasing until the experiment was stopped. Both volume and surface area began to increase rapidly after 1.50 h. After 2.5 h, the volume increase levelled out and remained stable; the increase in surface area also ceased after 2.5 h, followed by a decline. This was interpreted as follows: nucleation accompanied by particle growth started simultaneously. Nucleation ceased quickly but growth continued until the beginning of particle aggregation, which continued throughout the experiment.
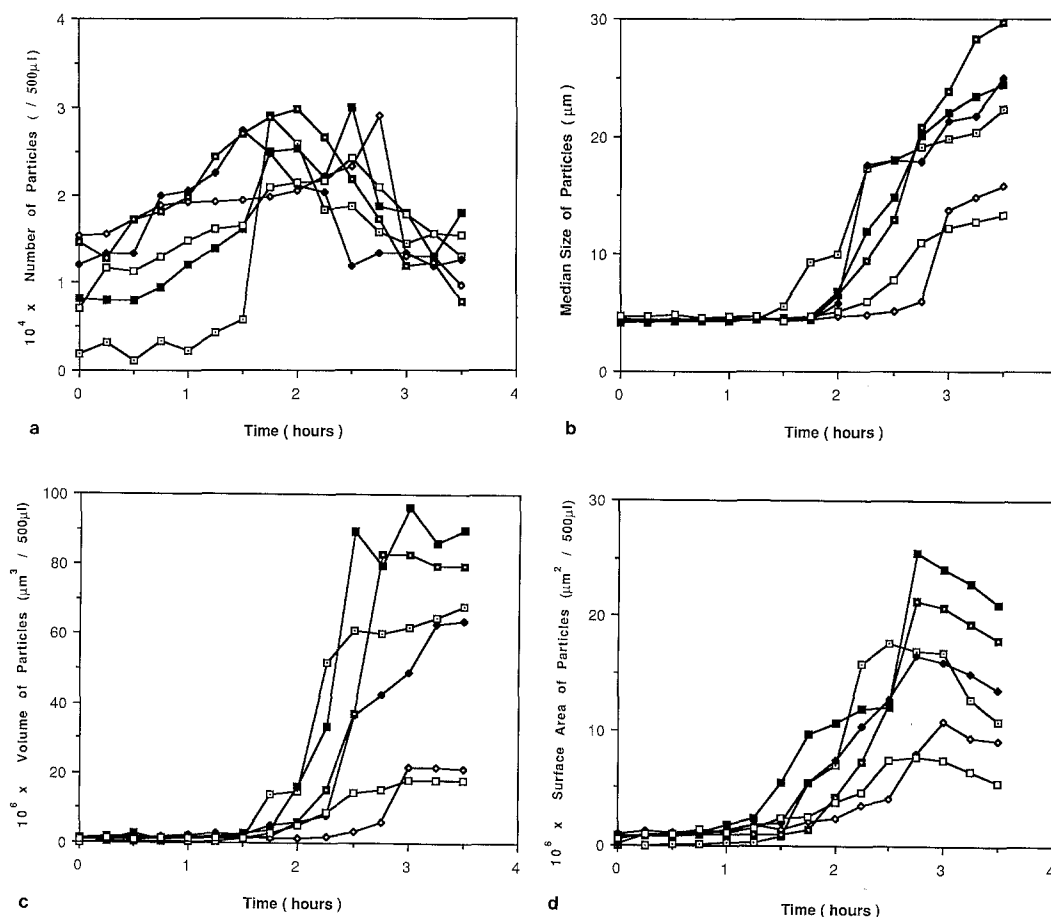

Fig. 3a–d. The change in a total particle number, b median particle size, c total particle volume and d total particle surface area in five human urine samples and synthetic urine after urease incubation. ▣, Synthetic urine; ◆, man 1; ◻, man 2; ◇, man 3; ■, woman 1; ☐, woman 2

Table 1. Urease-induced crystallisation in different urine samples

Parameters analysed after various times of incubation	Urine samples					
	Synthetic urine	Man 1	Man 2	Man 3	Woman 1	Woman 2
Particle number at start ($\times$ 500 μl^{-1})	1,900	12,000	13,800	15,400	8,200	7,100
pH when nucleation started	7.3	5.7	6.2	5.8	6.3	6.3
Nucleation started at (h)	1.25	0.50	0.25	0.25	0.5	0.5
Nucleation lasted for (h)	0.50	1.00	1.75	2.5	2	1.75
Maximum particle number	29,000	27,500	29,800	29,000	25,400	24,200
pH when nucleation stopped	7.7	6.1	7.1	6.6	7.4	7.8
Growth started after (h)	1.25	1.75	1.75	2.25	1.75	1.75
Median particle size when growth started (μm)	4.38	4.39	4.42	4.39	4.71	4.7
pH when growth started	7.3	6.1	7	6.5	7.1	7.6
Particle volume stable after (h)	2.5	3.25	2.75	3	2.75	3
Median particle size at that time	18.2	21.3	20.9	14.9	15	12.2
pH at that time	8	6.3	7.6	6.7	7.7	8.4
Particle volume at that time ($\mu l \times 10^6 \times 500 \mu l^{-1}$)	61	62	82	23	89	17
Growth index	12.6	11.5	10.2	5.8	11.1	4.5
Aggregation started after (h)	2.50	3	2.75	3	2.75	3
Surface area at that time ($\mu m^2 \times 10^6 \times 500 \mu l^{-1}$)	17.7	16	21.3	10.8	25.5	7.4
Surface area after 3.5 h	10.8	13.6	17.9	9.1	21.1	5.5
Median particle size after 3.5 h	22.3	25	29.7	15.9	24.4	13.4
Particle volume after 3.5 h	68	64	79	20	89	18
pH after 3.5 h	8.5	6.5	8.2	6.8	8.5	8.5

In the human urine samples, the particle number started to increase earlier, after 0.25–0.5 h, and this increase lasted between 1 and 2.5 h. It was rather difficult to determine the exact point at which the particle number started to increase, and the rate and pattern of the increase varied among the different samples. Nucleation started at pH levels between 5.6 and 6.4. Although the initial number of particles varied among the different urine samples, the maximal number was almost uniform. The median particle size started to increase after 1.75–2.25 h. The start of this increase could be more precisely defined. It was accompanied by a marked increase in both particle volume and surface area that lasted until 2.75–3 h after the start; thereafter, the particle volume remained more or less stable, whereas the surface area started to decline. At that time, the pH varied between 6.3 and 8.4. The mean particle size after 3.5 h incubation ranged between 13.4 and 29.7 μm. In samples showing a small median particle size, the total particle volume was low.

The crystallisation pattern in human urine was similar to that in synthetic urine as described above. It differed in some important respects, however. In human urine, nucleation started earlier and was less rapid but continued for a longer period than that observed in synthetic urine. In synthetic urine, particle growth and aggregation tended to start earlier and to be more pronounced.

The growth index calculated for the human urine samples varied between 4.5 and 11.5. Two specimens (man 3 and woman 2) had especially low indices and, accordingly, small particles and a low total particle volume. As reflected by the decrease in surface area, crystal aggregation seemed to be rather uniform in the different samples.

Discussion

The Coulter multisizer technique was found to be applicable for studies of urease-induced precipitation in human urine. The precipitation (nucleation) was found to start at various pH values in the different specimens, but the maximal number of particles that formed in the samples studied was rather similar. However, the final median particle size showed a large intersample variation, which resulted in marked differences in the total volume precipitated. The variation in particle size appreared to be mainly related to differences in particle growth.

The rate of the pH increase varied markedly among the different urine samples despite a similar urease inoculate. Previous studies have shown that this variation is due to differences in urinary buffer capacity (mainly urinary phosphate) [2, 3]. Urinary phosphate and pH are strongly influenced by diet and show a marked diurnal variation, exhibiting low pH and low phosphate excretion in the morning [3]. Samples of morning urine were used to obtain the most standardised conditions possible and to reduce the influence of diet and exercise on the excretion of phosphate and on urinary pH. Another way to obtain more standardised conditions is to dilute human urine; a major objection to this is that the effective concentration ranges of different inhibitors are disparate and that diluting the urine may selectively favour or disfavour one or more inhibitors in relation to the others [1, 4, 14]. Therefore, the crystallisation preferably be studied in whole human urine [2, 8, 16].

It appeared that the process of urease-induced precipitation, as for other types of urinary crystallisation, could be broken down into three phase: nucleation, growth and

aggregation [16]. Theoretlically, *nucleation* is manifested as an increase in particle number, volume and surface area; *growth,* as an increase in particle volume, size and surface area, whereby the particle number remins stable; and *aggregation*, as a decrease in particle number and surface area accompanied by an increase in particle size, whereby the particle volume remains unchanged. Nucleation was considered to be the main process as long as the number of particles was increasing steadily and was assumed to have stopped when the number started to fall. When the median size started to increase, growth was considered to be the main process. Growth was thought to have been overtaken by aggregation when simultaneous decreases in particle surface area and number started concomitantly with stabilisation of the particle size. In this experiment, nucleation resulted in only a negligible increase in volume; only when the particle size started to increase was a substantial volume increase noted. When the surface area started to fall, aggregation was considered to be the main process, and it appeared to continue until the experiment was terminated.

However, this process is complex and the different steps are inter-related. In the present study, the nucleation process started in human urine at a pH value between 5.6 and 6.1, then proceeded in a similar way in the different samples, more or less independent of the pH level obtained. We thus verified that once the pH has become high enough to initiate the precipitation of calcium phosphate and MAP, the amount precipitated depends on factors other than the degree of alkalinisation [8]. Nucleation started later and proceeded at a higher rate in synthetic as compared with human urine, probably because the human urine contained more particulate material at the start of the experiment. This material may have constituted nuclei for heterogeneous nucleation that enabled the early initiation of crystallisation. Numerous factors are involved in the formation of infection stones in the urinary tract. It appears reasonable to assume that the volume of particles precipitating would be positively correlated with the risk of formation of such stones. In the present study, the volume precipitated was found to be strongly correlated with particle growth; thus, this phase in the crystallisation process appears to be important. Calculated as described, the growth index could be easily and fairly reliably determined and can thus be assumed to be a useful tool for determination of the rate of crystal growth in different urine specimens. There was a great interindividual variation in growth index. All indices calculated for human urine were lower than that found for synthetic urine, probably due to the different amounts of crystal growth inhibitors present in the human specimens

but not in the synthetic urine. The maximal median particle size, which is also of importance, is influenced by both particle growth and aggregation. However, the method used in this study could not precisely measure the aggregation observed and, thus, could not exactly define its importance.

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