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Crystallisation of Calcium Oxalate Dihydrate in Normal Urine in Presence of Sodium Copper Chlorophyllin

R. Tawashi, M. Cousineau, and G. Denis

Faculty of Pharmacy and Department of Physiology and Service of Nephrology, Hôpital du Sacré-Coeur, University of Montreal, Montreal, Quebec, Canada

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Summary. A method is described for the growth of calcium oxalate dihydrate in normal urine. Soluble chlorophyllin, at a concentration of 20 μg/ml inhibited the crystallisation and the growth kinetics of the dihydrate crystals. The inhibitory capacity of chlorophyllin was compared with previous results. Data obtained suggest that the food and drug colourant chlorophyllin might be useful in the treatment of calcium oxalate stone disease.

Key words: Inhibitors, Calcium oxalate dihydrate, Growth kinetics, Soluble chlorophyllin, Calcium stones.

Introduction

It has been suggested by Berg and co-workers that calcium oxalate dihydrate crystals (weddellite) are most probably the primary product of calcium oxalate crystallisation in urine [1, 2]. Weddellite undergoes phase conversion to calcium oxalate monohydrate if not stabilised by the presence of urinary foreign ions [3]. In our laboratories it was found that calcium oxalate dihydrate is formed in rat kidneys in the first 2 h after the injection of 4 hydroxy-L-proline. This dihydrate undergoes gradual transformation into the more stable calcium oxalate monohydrate [4].

In previous communications we established the powerful inhibiting effect of chlorophyllin on the growth of calcium oxalate monohydrate in aqueous media [5] and in gel systems [6]. Furthermore, soluble chlorophyllin was found to inhibit the growth of calcium oxalate monohydrate in rat kidneys [7]. Soluble chlorophyllin used in these studies was the water soluble sodium copper complex, permitted in many countries, as colourant for food and drugs [8]. The acceptable daily intake is up to 15 mg/kg bwt. [9].

Since calcium oxalate dihydrate is considered to be a primary phase in calcium oxalate stone formation, it would be of interest to develop a reproducible method for the growth of calcium oxalate dihydrate in natural urine and to evaluate the effect of soluble chlorophyllin on the growth kinetics of the dihydrate crystals in the urine environment.

Materials and Methods

Crystals of calcium oxalate dihydrate can be grown by a number of methods but are often contaminated with calcium oxalate monohydrate or are unstable [10]. The crystallisation of the dihydrate by means of the chemical reaction between the oxalate ion and Ca^{++} in simulated urine was described by Gardner and Doremus [11]. The crystals obtained using this technique were contaminated with other hydrates. Better results were achieved in our laboratory when 5% of natural urine was added to simulated urine to stabilise the calcium oxalate dihydrate [12]. In this study further improvement was introduced to the crystallisation process to grow the dihydrate in a reproducible manner using natural urine as the growth medium.

Urine Samples

The urine of normal healthy individuals (20, age 20–25) with no history of stone formation was used. The urine sample (100–150 ml) was taken before lunch. The Ca^{++} concentration (corning calcium analyser Model 940) and pH were determined immediately for each urine sample. The sample was filtered through Millipore filter 0.22 μm. The filtered urine sample (100 ml) was used for each growth experiment, 50 ml used as control and the other 50 ml was used to test the activity of chlorophyllin on the growth process.

Crystal Growth Study

Calcium chloride 0.25 ml (1 M) in bidistilled water was added to 50 ml of the filtered urine. The pH was adjusted to 5.7 and to the system, which was maintained at 37 °C, 2.5 ml of sodium oxalate (0.005 M) was added. The system was kept at 37 °C for 3 h without any agitation. The other 50 ml of the same urine was used to evaluate the effect of chlorophyllin. Soluble chlorophyllin was incorporated to the filtered urine before the Ca^{++} and $oxalate^{--}$ were added. The crystals obtained were separated and characterised by methods described previously [12].

The size distribution of the calcium oxalate crystals obtained in each urine specimen in the presence and in the absence of chlorophyllin was determined using a particle counting technique (Coulter counter Model TA) previously described [13].

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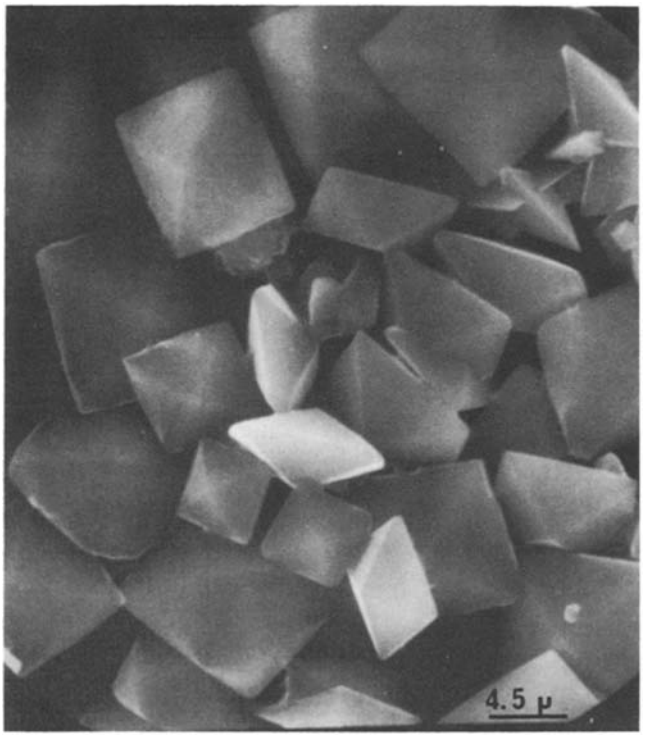

Fig. 1. Scanning electron micrograph of the calcium oxalate dihydrate grown in natural urine (x 2,220)

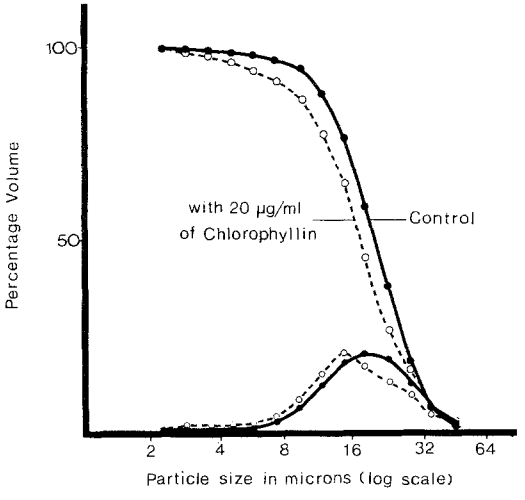

Fig. 2. Size distribution of calcium oxalate dihydrate grown in normal urine

Growth Rate Studies

In kinetic studies, growth was followed up to the moment of agglomeration. The growth process was followed in the presence and in the absence of chlorophyllin using the experimental conditions described above. We monitored the size-number data in a fixed volume (0.05 ml) of the system as a function of time. The volume of calcium oxalate dihydrate precipitated (Vppt) was calculated as a function of time by the method described recently by Markovic [14]. This was done in the presence and absence of soluble chlorophyllin. The change in particle radius in a given period of time was used as a basis for the calculation of the growth rate in cm/s [5]. The growth rates were determined in five urine samples before and after the addition of chlorophyllin.

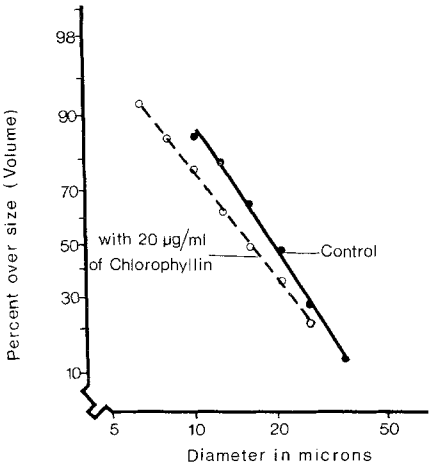

Fig. 3. Linear presentation of the size distribution of calcium oxalate dihydrate in presence and absence of soluble chlorophyllin

Results

Figure 1 shows the calcium oxalate dihydrate obtained in natural urine under the conditions described. The presence of soluble chlorophyllin (20 μg/ml) in the growth media did not change the structural or morphological characteristics of the dihydrate crystals. Figure 2 shows clearly the marked effect of chlorophyllin on the size distribution. The degree of uniformity (n) of size distribution was determined graphically as the slope of the linear relationship between (log log 100/V) vs (log D) (where V is the volume percentage, and D is the diameter in microns) [13]. The value of n provides a measure of the uniformity of the distribution. When all sizes are represented to the same extent $n = 0$ whereas with monodisperse systems, its value is infinity. The median diameter D_{50} was also determined from this plot (Fig. 3).

Table 1 summarises the mean diameter, uniformity factor n and the percentage inhibition in twenty different subjects. In all cases chlorophyllin reduced the mean crystal size, and the inhibition activity ranged from 7.5%–43%.

The effect of chlorophyllin on the uniformity factor n was not significant ($P > 0.05$). This finding agrees with previous data obtained in our laboratory when calcium oxalate was induced in rat kidney by injecting L-hydroxyproline or sodium oxalate [4]. In the experiments soluble chlorophyllin inhibited the deposition and the growth of calcium oxalate, but did not change the distribution characteristics of the crystals, represented by the uniformity factor (n).

The time course of crystal growth in urine is given in Fig. 4. The increase in the crystal volume precipitated over thirty minute periods was determined from the Coulter Counter data. The volume of the precipitated crystals is

Table 1. Effect of soluble chlorophyllin on the size characteristics of calcium oxalate dihydrate grown in normal urine

No.	Control		20 μg/ml chlorophyllin		Inhibition activity	
	D_{50} (μm)	n	D_{50} (μm)	n	ΔD_{50} (μm)	Inhibition [%]
1	16.72	2.95	12.96	3.15	3.76	−22.5
2	17.95	3.82	16.48	3.09	1.47	− 8.2
3	16.45	2.37	9.43	4.13	7.02	−42.7
4	20.05	3.42	15.29	2.95	4.76	−23.7
5	19.40	2.01	13.40	1.62	6.00	−30.9
6	21.56	3.16	19.43	2.55	2.13	− 9.9
7	17.13	2.98	12.94	1.88	4.19	−24.5
8	15.31	2.36	11.50	2.64	3.81	−24.9
9	20.63	2.77	18.79	2.99	1.84	− 8.8
10	16.39	1.94	11.42	2.12	4.97	−30.3
11	21.24	2.51	20.73	2.81	0.51	− 2.4
12	20.78	2.18	17.25	2.13	3.53	−16.9
13	18.38	2.53	11.56	1.65	6.82	−37.1
14	17.38	2.76	16.08	1.86	1.30	− 7.5
15	19.71	2.48	16.11	1.87	3.60	−18.3
16	16.36	2.39	15.06	3.18	1.29	− 7.9
17	18.03	2.64	16.36	2.44	1.67	− 9.3
18	15.14	2.09	12.29	1.92	2.85	−18.8
19	15.10	2.10	9.73	2.24	5.37	−35.6
20	17.32	2.20	10.18	1.85	7.14	−41.2
$\bar{X}$	18.05	2.58	14.35	2.45	3.70	−21.1
S.E.	±0.46	±0.11	±0.74	±0.15	±0,46	±2.8

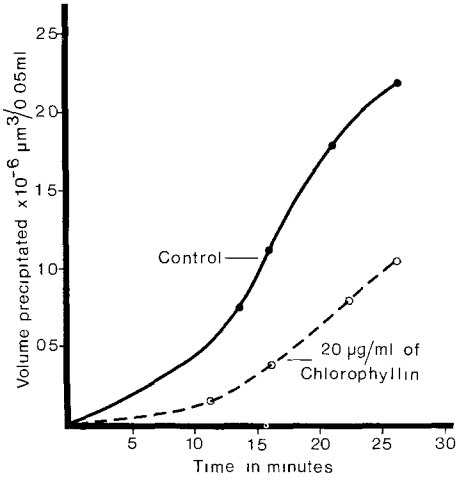

Fig. 4. Effect of soluble chlorophyllin (20 μg/ml) on the growth of calcium oxalate dihydrate in urine

Table 2. Effect of soluble chlorophyllin on the growth rate of calcium oxalate dihydrate in urine
(R_O: Growth rate = $\Delta r/\Delta t$ cm/s, R_C: Growth rate in presence of soluble chlorophyllin, Δr: The change in the crystal radius, Δt: Change in time

	R_O	R_C	$\frac{R_O - R_C}{R_O} \times 100$
1	4.88×10^{-8}	2.85×10^{-8}	41.6%
2	7.73×10^{-8}	6.91×10^{-8}	10.6%
3	7.98×10^{-8}	2.55×10^{-8}	68.0%
4	10.80×10^{-8}	8.02×10^{-8}	25.7%
5	6.73×10^{-8}	3.90×10^{-8}	42.0%
$\bar{X}$	7.62×10^{-8}	4.85×10^{-8}	37.6%
±S.E.	$\pm 0.96 \times 10^{-8}$	$\pm 1.11 \times 10^{-8}$	±9.6%

obtained from the sum of the volumes of all particles detected at the time just preceeding agglomeration as described by Markovic [14]. This plot shows the inhibitory activity of soluble chlorophyllin (20 μg/ml) on the growth process. Increasing the concentration of soluble chlorophyllin above 20 μg/ml did not change growth inhibition significantly.

The growth rate (R) was determined in the absence and in the presence of chlorophyllin (Table 2). In all cases studied there was a marked inhibition on the growth rate of calcium oxalate dihydrate averaging 38%. The influence of soluble chlorophyllin on the growth kinetics of calcium oxalate dihydrate may be attributed to: a) formation of complexes with Ca^{++} which effectively decrease the ($-\Delta G$) for the precipitation or b) absorption of chlorophyllin on the growth sites of the crystal face. At the concentration level of 20 μg/ml, complex formation can be ruled out.

Discussion

If we try to compare the effect of chlorophyllin concentration on the growth of calcium oxalate monohydrate and calcium oxalate dihydrate, we find a marked difference between the concentration required to suppress growth in both cases. In calcium oxalate monohydrate a low concentration of 1 μg/ml produced a remarkable growth retardation, whereas 20 μg/ml was required in the case of the dihydrate. This confirms the observation reported recently by Tomazic and Nancollas on growth of calcium oxalate monohydrate and calcium oxalate dihydrate in the presence of polyphosphate [15]. The pronounced difference in the adsorption behaviour of foreign molecules is probably due to the structural differences between the two calcium oxalate crystals.

The activity of chlorophyllin in this study as inhibitor for the growth of calcium oxalate dihydrate in natural urine supports previous data on calcium oxalate monohydrate and suggests that the food and drug colourant may become a useful medicine for the prevention of calcium stones.

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References

1. Berg W, Schnapp JD, Schneider HJ, Hesse A, Hienzsch E (1976) Crystaloptical and spectroscopical findings with calcium oxalate crystals in urine sediments. A contribution to the genesis of oxalate stones. Eur Urol 2:92
2. Berg W, Hesse A, Schneider HJ (1976) A contribution to the formation of calcium oxalate urinary calculi III. On the role of magnesium in the formation of oxalate calculi. Urol Res 4:161
3. Hienzsch E, Hesse A, Berg W, Roth J (1979) A contribution to the formation mechanism of calcium oxalate urinary calculi IV. Experimental investigation of the intrarenal crystallization of calcium oxalate in rabbit. Urol Res 7:223
4. Tawashi R, Cousineau M, Sharkawi M (1980) Calcium oxalate formation in the kidneys of rats injected with 4 hydroxy-L-proline. Urol Res 8:121
5. Desjardins A, Tawashi R (1978) Growth retardation of calcium oxalate by sodium copper chlorophyllin. Eur Urol 4:294
6. Bisaillon S, Tawashi R (1976) Retardation of growth and dissolution of calcium oxalate monohydrate. J Pharm Sci 65:222
7. Tawashi R, Cousineau M, Sharkawi M (1980) Effect of sodium copper chlorophyllin on the formation of calcium oxalate crystals in rat kidneys. Invest Urol 18:90
8. Harrison JWE, Levin SE, Travin B (1954) The safety and fate of potassium copper chlorophyllin and other copper compounds. J Am Pharm Assoc Sci Ed 43:722
9. Martindale (1977) The extra pharmacopocia. In: Wade A, Reynold JEF (eds) The Pharmaceutical Press, Londen, 27th ed
10. Werness PG, Duckworth SC, Smith LH (1979) Calcium oxalate dihydrate crystal growth. Invest Urol 17:230
11. Gardner GL, Doremus RH (1978) Crystal growth inhibitors in human urine effect of calcium oxalate kinetics. Invest Urol 15:478
12. Tawashi R, Cousineau M (1980) Growth retardation of weddellite by sodium copper chlorophyllin. Invest Urol 18:86
13. Ismail SI, Tawashi R (1980) Size distribution characteristics of mineral phase in renal stones. J Pharm Sci 69:830
14. Markovic M, Komunjer L (1979) A new method to follow crystal growth by Coulter Counter. J Cryst Growth 46:701
15. Tomazic B, Nancollas GH (1980) Crystal growth of calcium hydrates: a comparative kinetics study. J Colloid Interface Sci 75:149

R. Tawashi, Ph.D.
Faculté de Pharmacie
Université de Montréal
C.P. 6128, Succ. "A"
Montréal, Québec
Canada, H3C 3J7