

## Effect of Trace Metals on *In Vitro* Crystallization of Calcium Oxalate in Agarose Gel Media

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**= Abstract =** Trace metals are components of urinary stones, though minute and exist also in normal urine. There have been many studies concerning the role of these materials in crystallization. In this experiment we examined the role of trace metals and the effect of interaction of trace metals and promoters in crystallization of calcium oxalate.

We used the agarose gel model with 3 layered structure and added trace metals in various concentrations into the upper layer of oxalate solution. The seed crystals of monosodium urate, brushite, and hydroxyapatite were used as the promotor and added to the middle layer of agarose gel. The lower layer was the agar containing calcium ions. These test tubes were incubated at 37°C and crystals formed in middle layer were extracted and dried on previously determined time schedule. These crystals were analysed qualitatively and quantitatively.

The X-ray diffractogram of these extracts revealed only whewellite. Iron (III), aluminum(III), copper(II), and zinc(II) were proved to inhibit crystallization of calcium oxalate. And the inhibitory activity was proportional to the concentration of these trace metals. These inhibitory activities were about 40%, 40%, 25%, and 25%, respectively in 0.01 M of trace metals (CaCl<sub>2</sub>: trace metal=10:1 molar ratio). Tin(II) did not inhibit simple crystallization of calcium oxalate, but significantly inhibited the promoting process by 3 seed crystals.

**Key word:** *Agarose gel, Crystallization, Calcium oxalate, Trace metals*

### INTRODUCTION

Trace metals are normally present in human urine and exist in urinary stone, though minute. Trace metals found in amounts of 0.001 per cent or more were iron, copper, zinc, tin, lead, and aluminum (Meyer and Angino 1977). The role of trace metals in urinary stone disease has been under discussion for some time, but their roles remain to be clearly defined. Certain trace metals have shown effect on the crystallization of calcium oxalate and calcium phosphate in *in vitro* experiment. Tin, vanadium, and lead were proved to be effective inhibitors on the crystallization of calcium oxalate (Eusebio and Elliot

1967). There has not been any study concerning the interaction of trace metals and promoters.

In this experiment, using the agarose gel model where the technique is simple and quantitative analysis is possible (Lee and Lee 1987), we tested the role of some trace metals in simple crystallization and the promoting process of calcium oxalate.

### MATERIALS AND METHODS

#### Materials

Reagent grade chemicals were used without further purification. CaCl<sub>2</sub> (Junsei, Japan), K<sub>2</sub>C<sub>2</sub>O<sub>4</sub>·H<sub>2</sub>O (Junsei, Japan), type III agarose (Sigma, U.S.A.) for gel formation, and bactoagar (Difco, U.S.A.) as agar were used. We selected the following 5 trace metals which were found in urinary stone and normal urine, and easily pur-

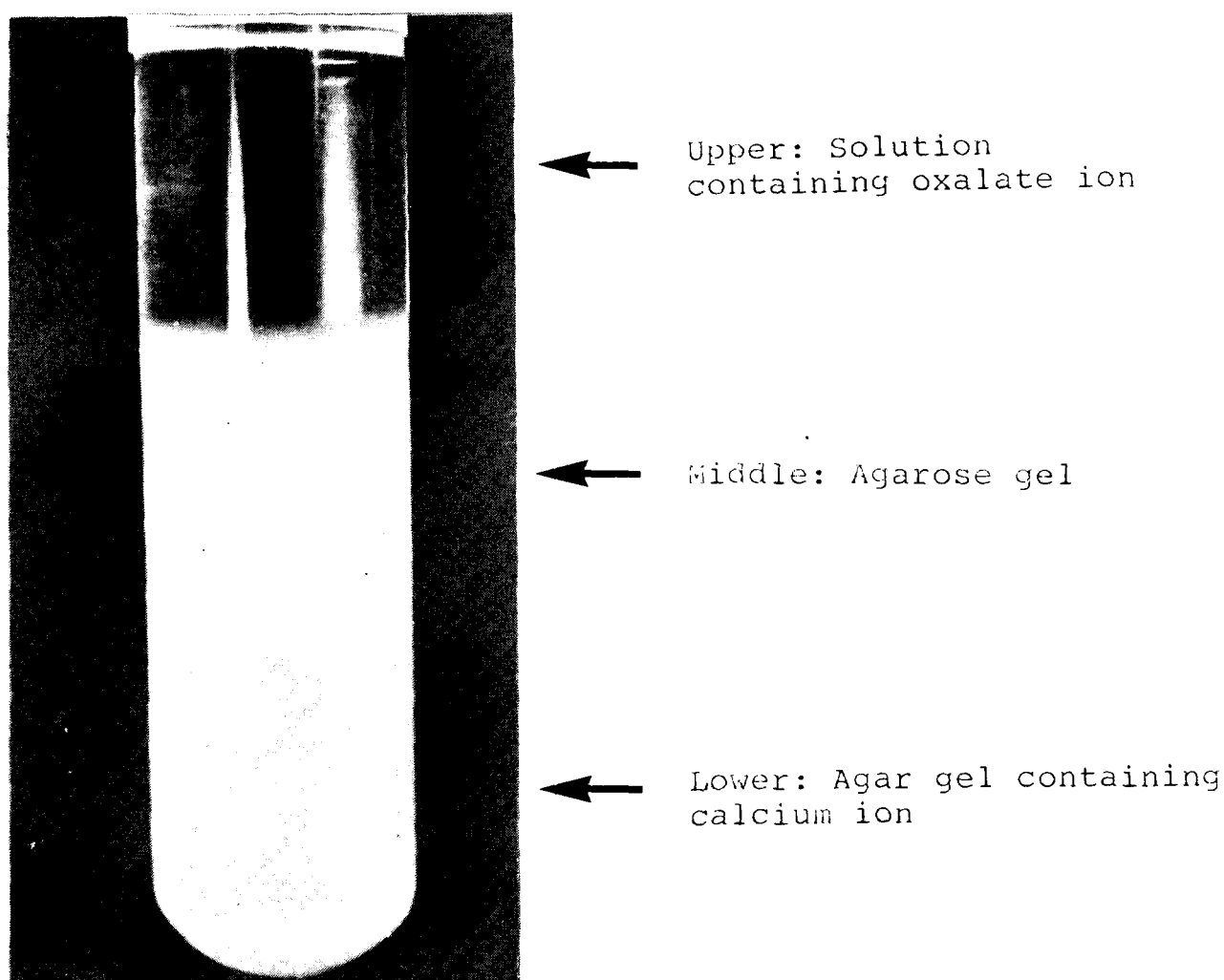


Fig. 1. Three layer structure in test tube using agarose gel as a crystal forming medium.

chasable. They were  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  (Yakuri, Japan),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (Yakuri, Japan),  $\text{ZnCl}_2$  (Duk-san, Korea),  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  (Yakuri, Japan),  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$  (Yakuri, Japan). All solutions were prepared with deionized, tertiary distilled water. As seed crystals, brushite [ $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ , Yakuri, Japan], hydroxyapatite [ $\text{Ca}_5(\text{PO}_4)_3\text{OH}$ , Merck, West Germany], and monosodium urate monohydrate [Sigma, U.S.A.] were used.

pH was measured by ionanalyzer (Orion, U.S.A.) and adjusted by dropping of NaOH,  $\text{CH}_3\text{COOH}$ , HCl. During the reaction the glass tubes were stored in an electric drying oven at  $37 \pm 1^\circ\text{C}$ .

#### 1. Detection of optimal condition

Three layered structure containing gel and solution in 24 mm glass tube was made (Fig. 1). Lower layer was 1% (W/V) agar gel containing 0.05, 0.1, 0.25, 0.5 M  $\text{CaCl}_2$ . Middle layer was 1.5% (W/V) agarose gel. Upper layer was 0.05, 0.1, 0.25, 0.5 M  $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$  solution. pH of gel

and solution was adjusted to 5.5, 6.5, 7.5 and pH of each layer was identical in the same tube. Crystals were to be formed in middle agarose gel layer by diffusion of ions.

Crystals were extracted at 1, 2, 3, 4, 5, 6, 12, 18 hour and in 1, 2, 3, 5, 7 day. Agarose gel layer was isolated and dissolved in distilled water by heat and filtered by  $8 \mu\text{m}$  membrane filtration. Extracts were dried at  $60^\circ\text{C}$ . Crystals were weighed up to 0.1 mg by analytical balance (Sartorius, U.S.A.). These crystals were identified by scanning EM (ISI-SX-30 E type, U.S.A.) and X-ray diffractor (Rigaku, Japan).

Each experiment was run 3 or 4 times: results were acceptable only if replicate values agreed within 5%. Each point in the figure represents the mean of replicate experiments.

#### 2. Promoting experiment of calcium oxalate crystallization

Concentration of  $\text{CaCl}_2$  and  $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$  was 0.1 M. Each seed crystal (10 mg) was added to

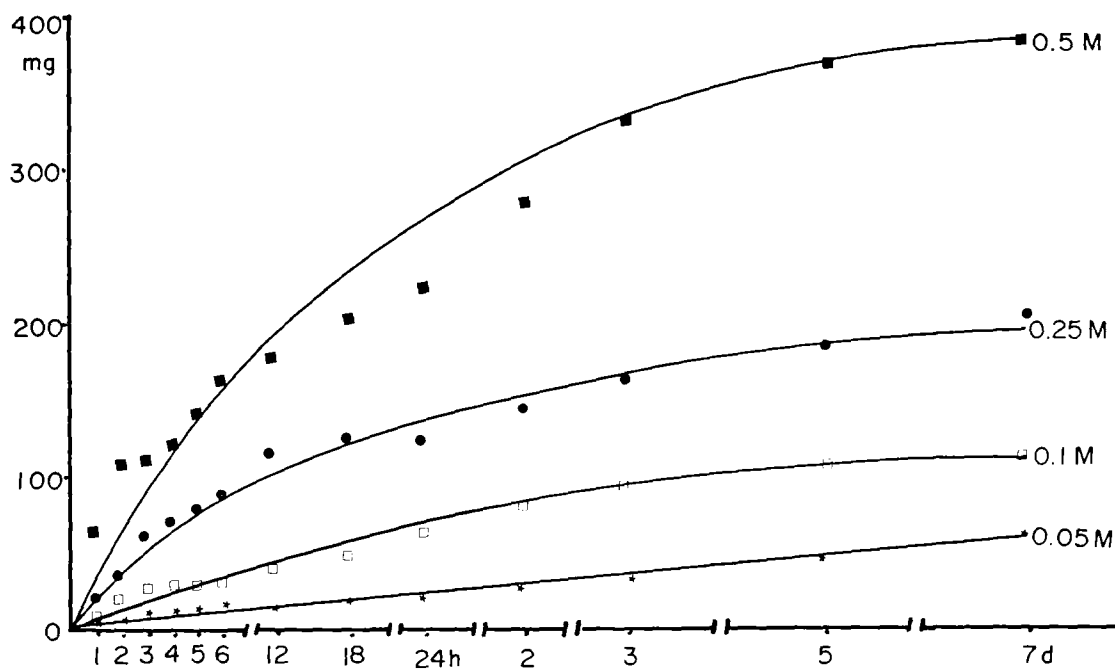


Fig. 2. Weight of calcium oxalate crystals on various concentrations of calcium and oxalate solutions at pH 6.5.

middle agarose layer before gelling. Extracts of 1, 2, 4, 6 hour and 1, 2 day were analysed qualitatively and quantitatively.

### 3. Effect of trace metals on calcium oxalate crystallization

Concentration of  $\text{CaCl}_2$  and  $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$  was 0.1 M. Trace metals were added to upper oxalate solution in 0.0001, 0.001, 0.01, and 0.1 M. Extracts of 2, 4, 6, 12 hour and 1, 2 day were analysed qualitatively and quantitatively.

### 4. Effect of tin on promoting process of calcium oxalate crystallization

Concentration of  $\text{CaCl}_2$  and  $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$  was 0.1 M  $\text{SnCl}_2$  were added to upper layer in the concentration of 0.01 M after each seed crystal (10 mg) was added to middle agarose layer. Extracts of 1, 2, 4, 6 hour and 1, 2 day were analysed qualitatively and quantitatively.

## RESULTS

### 1. Optimal condition

Crystals were seen after 4 hour in the gel as a whitish membrane whose thickness increased to 3 days. No further change was seen after this period. The weight of crystals increased according to the concentration of calcium and oxalate solution and the incubation period, irrespective of the change of pH (Fig. 2). X-ray diffractogram and scanning EM revealed only whewellite. The

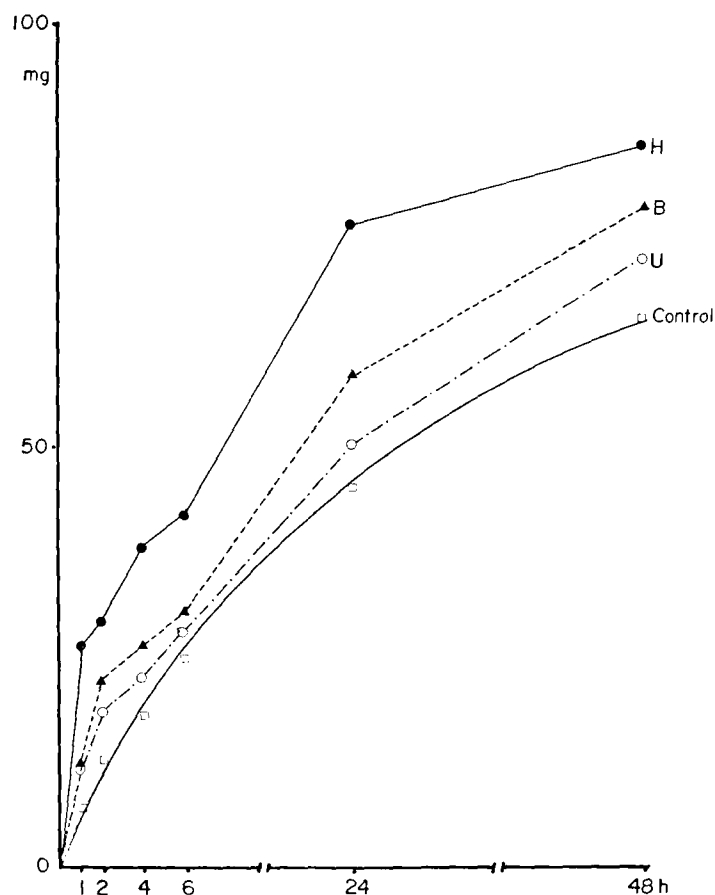


Fig. 3. Promoting effect of calcium oxalate crystallization by 3 seed crystals at pH 5.5 (H: hydroxyapatite, B: brushite, U: monosodium urate).

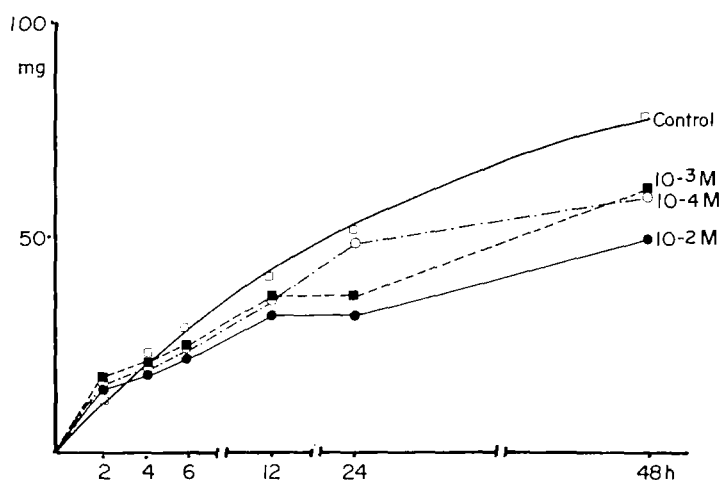


Fig. 4. Effect of Fe(III) on calcium oxalate crystallization at pH 6.5.

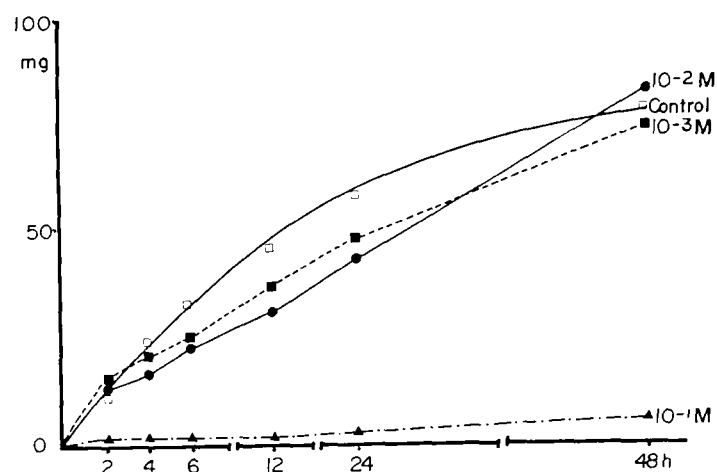


Fig. 5. Effect of Cu(II) on calcium oxalate crystallization at pH 6.5.

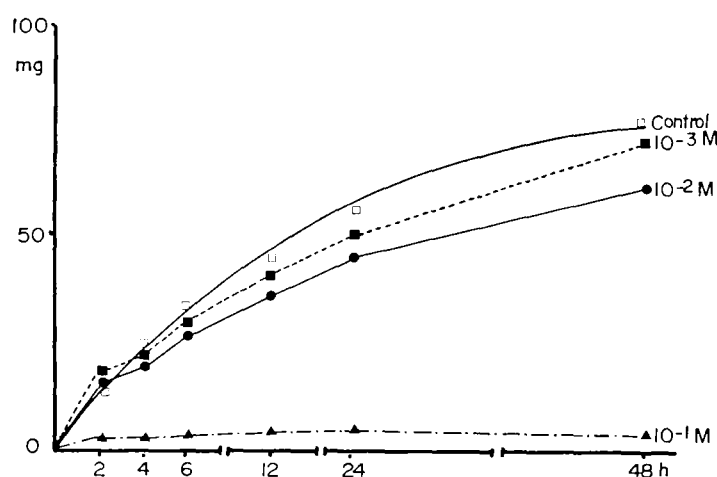


Fig. 6. Effect of Zn(II) on calcium oxalate crystallization at pH 6.5.

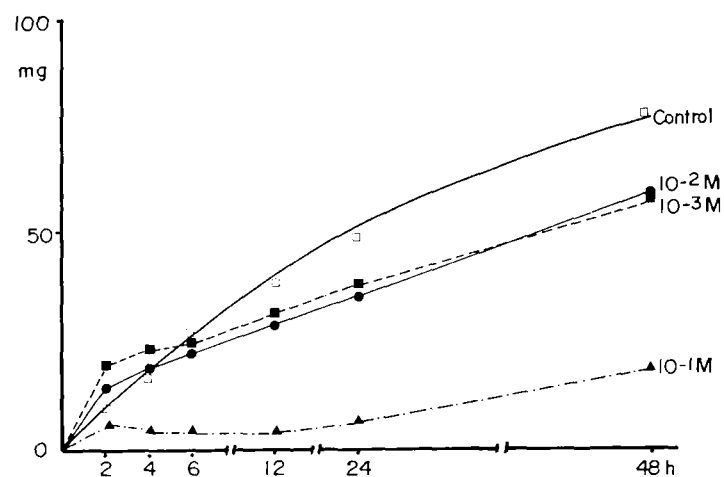


Fig. 7. Effect of Al(III) on calcium oxalate crystallization at pH 6.5.

optimal concentration of calcium and oxalate solution was 0.1 M when 1.5% concentration and 10 ml volume in agarose gel was used.

### 2. Promoting experiment of calcium oxalate crystallization

Crystallization of calcium oxalate was promoted approximately in 135% by seed crystals of hydroxyapatite, 110% by monosodium urate, and 120% by brushite (Fig. 3). X-ray diffractogram revealed only whewellite.

### 3. Effect of trace metals on calcium oxalate crystallization

1)  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ : The weight of crystals decreased according to the increase of concentration of iron. The inhibitory activity was approximately 40% in 0.01 M (Fig. 4).

2)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ : The inhibitory activity was up to 25% in 0.001 and 0.01 M, but about 95% in 0.1 M (Fig. 5).

3)  $\text{ZnCl}_2$ : The inhibitory activity was up to 25% in 0.001 and 0.01 M, but about 75~88% in 0.1 M (Fig. 6).

4)  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ : The inhibitory activity was 24~42% in 0.001 and 0.01 M, and about 88% in 0.1 M (Fig. 7).

5)  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ : The inhibitory activity was not seen in above three concentrations except 15% of inhibitory activity in 12 and 24 hour extracts (Fig. 8). X-ray diffractogram of above extracts revealed only whewellite.

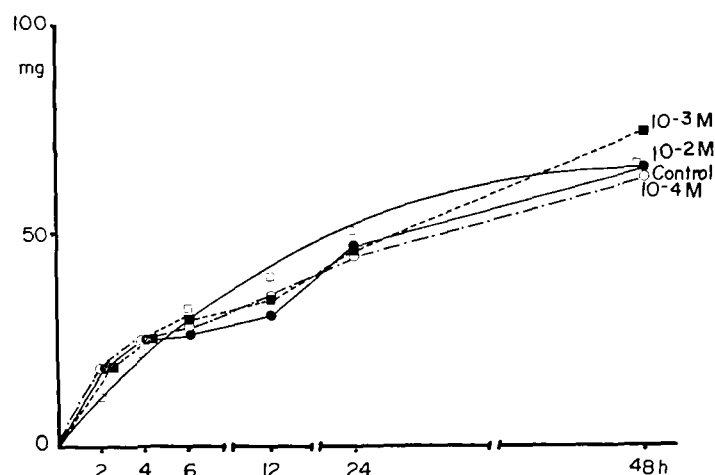


Fig. 8. Effect of Sn(II) on calcium oxalate crystallization at pH 5.5.

#### 4. Effect of tin on promoting process of calcium oxalate crystallization

Tin inhibited the promoting process by the seed crystals of hydroxyapatite, brushite, and monosodium urate in about 30%, 20%, and 45% respectively in 0.01 M concentration of tin. X-ray diffractogram of extracts revealed only whewellite.

### DISCUSSION

Over the last 20 years the presence of trace elements in urinary stone and their influence on stone formation has been studied with increasing intensity corresponding to the advent of improved analytical methods. Nevertheless informations available are mostly inconclusive and controversial. Direct influence of trace elements on stone formation in man is not yet documented (Schneider 1985). If a particular trace metal should have an effect on the crystallization of a urinary stone component it must act at the surface of the crystals since the concentration of trace metals in urine is too small to affect the lattice ions in solution. Metal ions acting in this fashion would likely be trapped and perhaps concentrated within the lattice of crystals (Meyer and Angino 1977).

For calcium phosphate, zinc, cadmium, magnesium, cobalt, tin, copper, aluminum, and its trace metal complexes with iron, chromium, and aluminum inhibited both the mineralization of rachitic rat cartilage and the precipitation of calcium phosphate *in vitro* (Bird and Thomas 1963; Meyer and Angino 1977; Meyer and Thomas

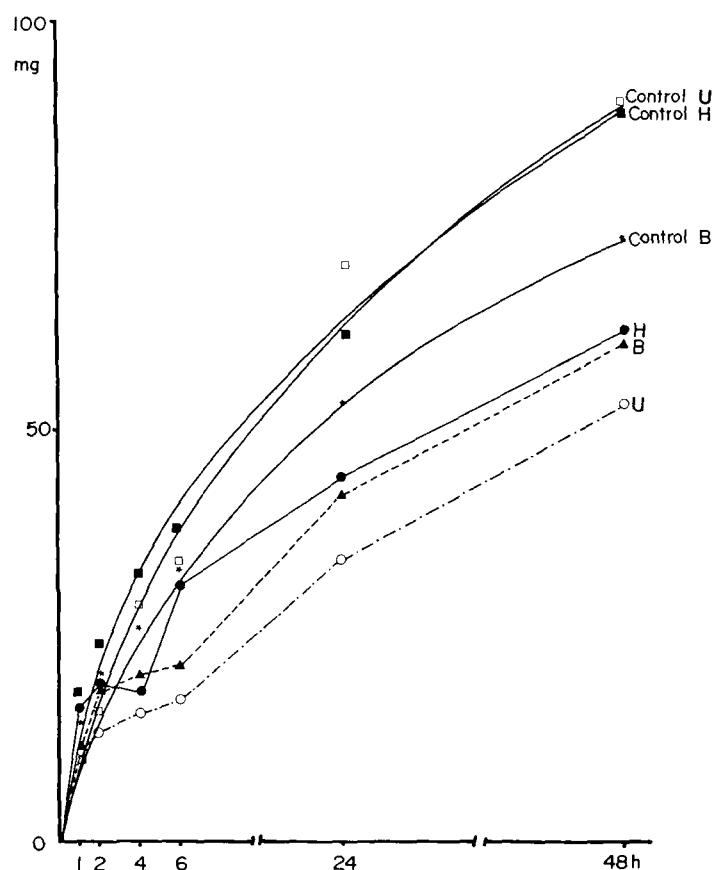


Fig. 9. Inhibitory activity of Sn(II) on the promotion of calcium oxalate crystallization by 3 seed crystals at pH 6.5 (H: hydroxyapatite, B: brushite, U: monosodium urate).

1982). On the other hand tin, vanadium, and lead are known to be effective inhibitors of calcium oxalate crystal growth (Eusebio and Elliot 1967). Experiments performed in nearly physiological condition revealed that copper and lead had a statistically significant inhibitory effect on the crystal growth process, but aluminum, tin, zinc, and iron had no effect at concentrations greater than those found in normal urine, but none of the metals affected the crystal growth of calcium oxalate at concentrations approximating those found in normal urine (Meyer and Angino 1977). However, there are other report that zinc, aluminum, cobalt, nickel, and iron inhibited the precipitation of calcium oxalate *in vitro* (Sutor 1969; Sutor and Wooley 1970; Welshman and McGeown 1972; Meyer and Angino 1977).

Though some calculi seem to consist of pure components, a wide variety of major and trace elements are involved in the composition of calculi (Schneider 1985). Trace metals found in

amounts of 0.001 per cent or more were zinc, iron, aluminum, copper, lead, and tin. Each mean value for trace metal concentration in normal urine was 267, 167, 50, 40, 35 and 17  $\mu\text{g/l}$ , respectively. The computed concentration of trace metals at 0.00001 M was 1,368, 837, 405, 952, 3,108, and 1,785  $\mu\text{g/l}$ , respectively (Meyer and Angino 1977). They thought that the concentration less than 0.00001 M of trace metals was relatively physiologic when concentration of  $\text{CaCl}_2$  was about 0.0001 M (i.e.  $\text{CaCl}_2$ : trace metal molar ratio = 10:1). But this concentration was about 5 times in iron, 25 times in copper, 8 times in aluminum, 5 times in zinc, and 100 times in tin as large as the concentration found in normal urine. We used 0.1 M  $\text{CaCl}_2$  solution and 0.0001~0.01 M solutions of trace metals to keep  $\text{CaCl}_2$ :trace metal molar ratio to be 10 to one.

An inhibitor unit in the calcium oxalate crystal growth system has been defined as that quantity of inhibitor required for a 50 per cent reduction in the rate of crystal growth (Meyer and Smith 1975). In another report, the relative growth rates in the presence of each added compound were calculated as percentages of the average value obtained in the control medium. Values below 50 per cent indicate inhibition and above 150 per cent suggest accelerated growth (Welshman 1972). We regarded the value below 25 per cent compared with the control value as an inhibition in our experiment.

In this experiment, iron, copper, zinc, and aluminum inhibited the crystallization of calcium oxalate in concentration relatively higher than physiologic. The inhibitory activity of iron and aluminum was about 40%, and that of copper and zinc about 25%, in 0.01 M of trace metal. The

inhibitory activity increased in accordance with the increase of concentration of trace metals. But the inhibitory activity of tin was not significant (about 15% in 12 and 24 hour extracts). Tin inhibited the promoting process by the seed crystals of hydroxyapatite, brushite, and monosodium urate in about 30%, 20%, and 45% respectively in 0.01 M concentration of tin. Clinical significance and mechanism of this inhibitory phenomenon need to be further studied.

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= 국문초록 =

## Agarose Gel에서 칼슘수산 결정형성에 대한 미량금속의 영향

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박원희 · 이종욱

미량금속은 비록 미량이지만 요석의 한 성분이고 정상 요내에서도 존재한다. 그래서 이것의 역할에 대한 연구가 많이 있어 왔으나 아직까지도 요석형성에서의 역할에 대한 직접적인 증거는 없다. 그래서 이 실험에서는 칼슘수산 결정형성에서 미량금속의 역할 및 미량금속과 촉진제의 상호작용의 효과에 대하여 알고자 하였다.

유리시험관내에 gel과 용액으로 3층구조를 만들어 실험하였다. 하층에는 칼슘이 함유된 agar gel, 상층에는 수산용액을 만들고, 중층에는 agarose gel을 만들어 이곳으로 각 이온이 확산 침투해서 결정이 형성되게 하였다. 미량금속은 상층에 첨가했고, hydroxyapatite, brushite, monosodium urate의 종자결정은 각각 중층에 투입하였다. 각 시험관은 37°C배양기에 설치한 후 정해진 시간에 따라 결정을 추출했고, 이 추출물을 정성 및 정량적으로 분석했다.

칼슘용액과 미량금속의 농도 비가 10 : 1인 경우 철과 알루미늄은 약 40%, 구리와 아연은 약 25% 정도 억제하였고, 그 억제력은 미량금속의 농도에 비례하였으나, 주석은 칼슘수산 결정형성을 거의 억제하지 못했다. 주석은 상기 세 가지 종자결정에 의한 촉진과정을 monosodium urate에서는 45%, hydroxyapatite는 30%, brushite는 20%정도 억제하였다.