



Liesegang patterns, growth kinetics, inhibition and dissolution of calcium phosphate: A constituent of renal stone

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ABSTRACT

Urinary stone disease has been prevalent in human beings from many centuries. It has affected up to a quarter of population in certain geographic area and hence poses a significant health problem. Approx 85% human stones are calcium stones comprising of oxalate and phosphate, either alone or in combined forms. Though, super saturation of stones forming salts in urine is essential. The stone formation is a multistep process and it includes nucleation, crystal growth, crystal aggregation and crystal recantation. Various substance in the body have an effect on one or more of the these stone forming processes, thereby influencing a person's ability to promote or prevent stone formation. Herein we studied formation of calcium phosphate, which is deposited in many diseases but mechanism of its formation has been speculative. We investigated the precipitation pattern of calcium phosphate, effect of pH and role of bacteria particularly *E.coli* in the precipitation of calcium phosphate, role of inhibitors and promoters in formation of calcium phosphate.

Keywords: Urinary Stones, Urolithiasis, stone formation

INTRODUCTION

The knowledge of urinary stones is important as urolithiasis is a recurrent disease in many people and preventive measures are based on several informations.¹ The struvite stones are usually associated with urinary tract infection with urea splitting organisms such as certain stains of *E.coli*, *Proteous vulgaris*, *P. klebsiella*, *Pseudomonas* by producing NH_3 from urea. The infected urine is therefore alkaline and high in NH_4^+ contents.² In the present investigation we report the precipitation of calcium phosphate which has relevance from the view point of stone formation in kidenys³ and the results are important point from view of understanding importance of relative role of crystal growth and nuclei formation. It is believed that basic crystalline nidus of all renal calculi is a sub microscopic unit of calcium and phosphate.⁴ When a soluble electrolyte CaCl_2 is

placed in contact with second electrolyte ammonium dihydrogen phosphate (ADP) in gel media, both react to form poorly soluble phosphate stone on diffusion. The patterns thus obtained have features quite unusual from the Liesegang phenomenon.⁵⁻¹⁴ Since such precipitation patterns can be used to explore interesting diffusion and transport processes, dependence of reactant concentration, pH tube diameter, additives on precipitation has been investigated. Growth kinetics in presence and in absence of external electric field, bacteria *E. coli* and a nutrient glucose has been investigated. There is considerable interest in the study of dissolution and inhibition of calculi. We have also investigated the influence of certain inhibitors such as α -ketoglutaric acid, ascorbic acid and leucine on the precipitation and dissolution of calcium phosphate and results are included in this report.

EXPERIMENTAL

Materials: Calcium Chloride LR grade (Qualigen) ammonium dihydrogen phosphate AR grade (BDH), agar agar(difcoUSA), ascorbic acid (Merk, Germany), glucose (Qualigen), leucineLR grade (S.D,Fine), acetic acid (Qualigen), ammonia (Qualigen).

Precipitation in Corning glass Tubes:

Calcium phosphate was precipitated in corning glass tubes by the diffusion of CaCl_2 solution into agar-agar gel containing

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ADP. The ADP solution of known concentration was prepared in double distilled water containing 1% agar- agar at 80-90°C. The solution was mixed with the help of a magnetic stirrer. The hot solution was then poured into the pre steamed Corning tube (inner diameter=11.7mm) and cooled at room temperature to solidify. An equal volume of CaCl₂ solution was added to the upper portion of tube which was sealed at the top with a wooden cork. All these experiment were carried out in an air thermostat maintained at the 25±0.1 °C. In order to check reproducibility of the pattern, studies were performed in triplicate. In order to examine the influence of electrolyte concentration, one of the electrolyte CaCl₂ was systematically varied. Experiments were

Table 1. Width of the calcium phosphate precipitate in gel media as a function of distance from the initial junction. Calcium chloride of different concentration were taken in the upper portion of the tube. Condition: (ADP) = 0.01M containing 1% agar agar in the lower portion of tube.

Distance from initial junction	Band location (cm) at different CaCl ₂ concentrations		
	0.1 M	0.3 M	0.5 M
0.5	1.0	1.0	1.0
1.0	1.0	1.0	1.0
1.5	1.0	1.0	1.0
2.5	1.0	1.0	1.0
3.0	1.0	1.0	1.0
3.5	1.0	1.0	1.0
4.0	1.0	1.0	1.0
4.5	1.0	1.0	1.0
5.0	1.0	1.0	1.0
5.5	0.3	1.0	1.0
6.0	0.2	0.9	1.0
6.5	0.2	0.9	1.0
7.0	0.1	0.8	0.8
7.5		0.7	0.8
8.0		0.5	0.5
8.5		0.3	0.4
9.0		0.2	0.3
9.5		0.2	0.2



Figure 1. Precipitation pattern of calcium phosphate in agar agar gel at different conc. of CaCl₂. Conditions: [ADP] = 0.001 containing 1% agar-agar (in lower portion of tube). [CaCl₂]=0.10 m, 0.30m & 0.50m (from left to right) at 37.0±0.1°C.

also carried out in tubes of varying diameters. Data are recorded in table 1 and table 2. Results are shown in figures 1,2,3,4.

Table 2. Values of location of nth band for precipitation pattern of calcium phosphate in tube of different diameters. Conditions: (ADP) = 0.01M containing 1% agar-agar, (CaCl₂) = 0.01M. Tube diameter 3, 4, 5 and 10 mm respectively

Distance from the junction	Band location X _n (cm) in tubes of different diameters			
	3mm	4mm	5mm	10mm
0.0	0.3	0.4	0.5	1.0
0.5	0.25	0.3	0.5	1.0
1.0	0.24	0.3	0.5	1.0
1.5	0.19	0.25	0.5	0.8
2.0	0.15	0.2	0.5	0.7
2.5	0.10	0.1	0.5	0.7
3.0	0.05		0.5	0.5
3.5			0.4	0.3
4.0			0.4	0.3
4.5			0.4	0.3
5.0			0.3	0.2
5.5			0.2	0.2
6.0			0.2	
6.5			0.1	
7.0			0.1	

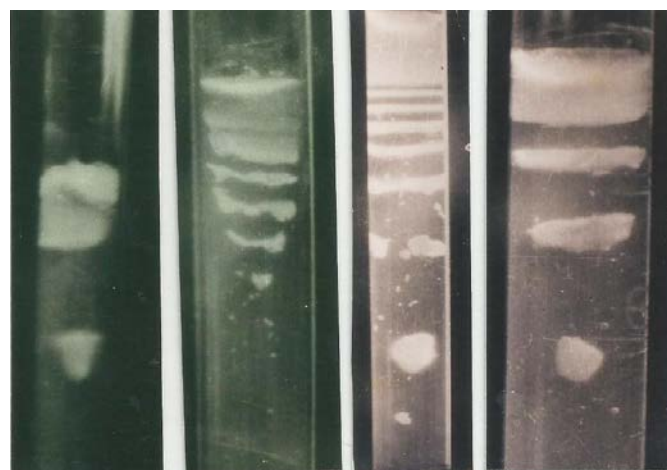


Figure 2. Precipitation pattern of calcium phosphate observed in tubes of different diameters. Conditions: (CaCl₂) = 0.01M, (ADP) = 0.01M containing 1% agar agar, Tube diameter: 3,4,5 and 10 mm respectively from left to right at 37.0± 0. 1°C.

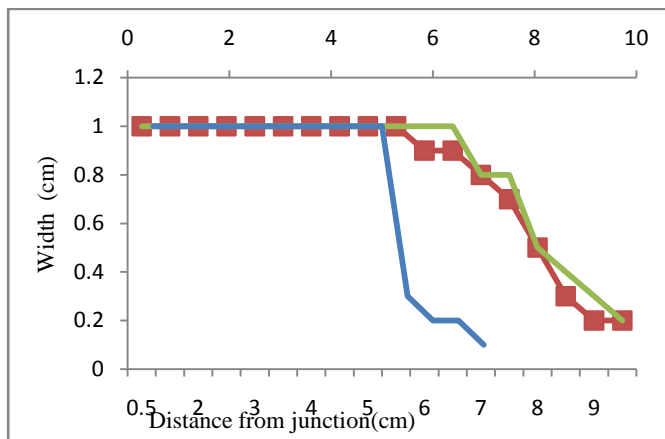


Figure 3. Diameter of bands at various distances from the initial junction from the pattern shown in fig (1)

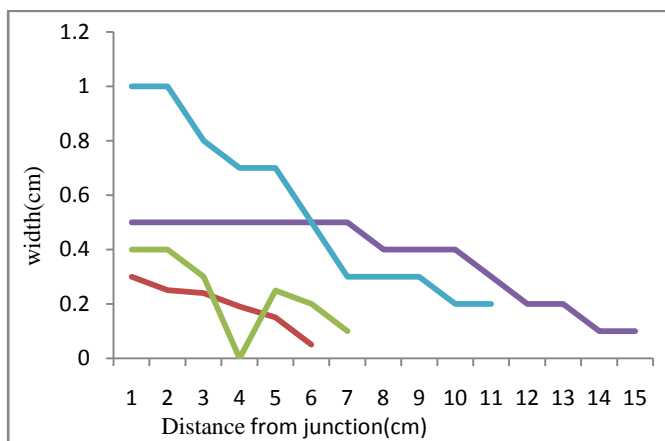


Figure 4. Diameter of the band at various distances from the initial junction for the pattern shown in fig (2)

Effect of pH of the medium

pH of the medium influences the precipitation pattern. For this study, initial pH of the ADP solution containing 1% agar-agar was changed by adding dilute ammonium hydroxide solution or dil CH_3COOH drop by drop and pH was measured with a Toshniwal India digital pH meter. Lower portion of the tube contained agar- agar, ADP and NH_4OH or CH_3COOH . A solution of CaCl_2 was allowed to diffuse from the upper end of the tube. Precipitation patterns thus obtained are shown in fig 5.

pH change during precipitation: To monitor pH changes during precipitation of calcium phosphate, pH electrode was immersed into the gel containing ADP kept in a Corning tube (11.7 mm diameter) to the extent of 2.0 cm from the initial junction employing an experimental set-up as described by Das et al.¹⁶ The experimental set up was kept in an air thermostat maintained at $30.0 \pm 0.1^\circ\text{C}$. Precipitation started as soon as the aqueous CaCl_2 solution was poured into the tube. The progress of the reaction was followed by recording pH of the solution as a function of time. Experiments are also performed in presence of (i) Vit C and (ii) bacteria *E. coli*. Results are recorded in table 3 and 4, and shown in fig 6, 7 respectively.

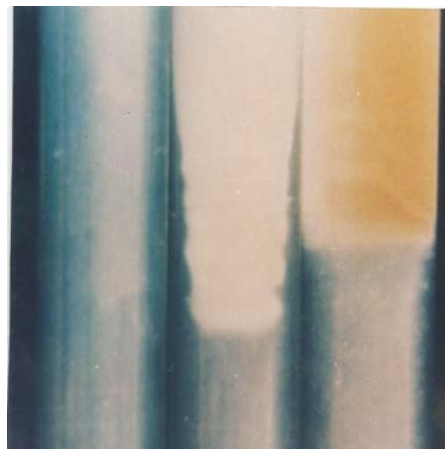


Figure 5. Precipitation patterns of calcium phosphate at different pH of ADP solution tubes are arranged in increasing pH of ADP solution [pH of 0.01M ADP solution were 4.0,6.2, 7.6 (from left to right) pH was changed by the addition of CH_3COOH or NH_4OH [CaCl_2] = 0.01M, ADP = 0.01M containing 1% agar-agar (PH=6.2) at $37 \pm 1^\circ\text{C}$.

Table 3. pH change with time during precipitation of calcium phosphate from CaCl_2 Of different concentrations . Conditions:(ADP) = 0.01M containing 1% agar agar at $30 \pm 0.1^\circ\text{C}$.

Time (min)	(CaCl ₂)			
	0.3M	0.4 M	0.5M	0.7M
	pH			
10	6.93	6.86	6.91	6.84
20	6.93	6.86	6.91	6.84
30	6.93	6.86	6.91	6.84
40	6.93	6.86	6.91	6.84
50	6.93	6.86	6.91	6.84
60	6.93	6.86	6.91	6.84
70	6.93	6.86	6.91	6.84
80	6.93	6.86	6.91	6.84
90	6.93	6.86	6.91	6.84
100	6.93	6.86	6.91	6.84
110	6.93	6.86	6.91	6.84
120	6.93	6.86	6.91	6.84
130	6.93	6.86	6.91	6.84
140	6.92	6.86	6.76	6.53
150	6.92	6.60	5.72	6.24
160	6.92	5.74	5.30	6.02
170	6.37	5.24	4.98	5.90
180	5.87	4.93	4.83	5.66
190	5.56	4.72	4.65	5.13
200	4.86	4.18	4.47	4.75
210	4.56	4.01	4.39	4.48
220	3.92	3.98	4.39	4.30
230	3.90	3.98	4.39	4.30
240	3.79	3.98	4.39	4.30
250	3.79	3.98	4.39	4.30

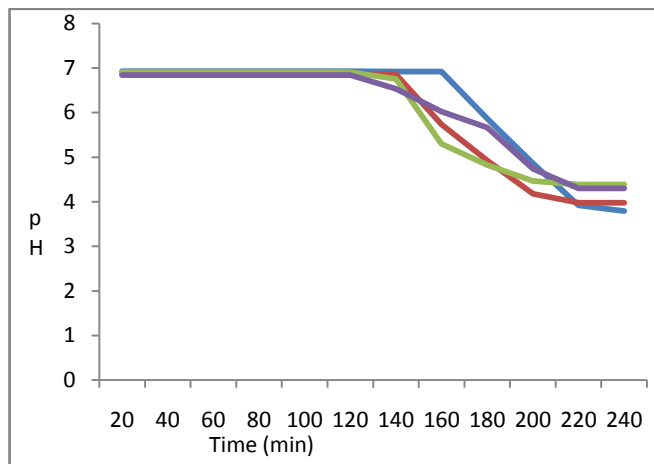


Figure 6. Variation of pH of the ADP solution during precipitation of calcium phosphate Conditions: $[CaCl_2]$ (in the upper portion of the tube) = 0.30,0.40,0.50 & 0.70, $[ADP]$ = 0.01M containing 1% agar-agar at $37.0 \pm 1^{\circ}C$.

Table 4. pH changes with time during precipitation of calcium phosphate in presence of different additives **Conditions:** ($CaCl_2$) = 0.5M, (ADP) = 0.01M containing 1% agar- agar and *E.coli*, 1% agar-agar and portion of tube).(ADP) = 0.01M containing 1% agar-agar, temperature= $30.0 \pm 0.01^{\circ}C$

Time	pH				
	ADP +1% agar agar	ADP+1% agar agar+E coli	ADP+1% agar agar+vit C	CaCl ₂ + <i>E.coli</i>	CaCl ₂ +1% vit C
10	6.91	7.24	5.59	6.30	6.88
20	6.91	7.24	5.59	6.30	6.88
30	6.91	7.24	5.59	6.30	6.88
40	6.91	7.24	5.59	6.30	6.88
50	6.91	7.24	5.59	6.30	6.88
60	6.91	7.24	5.59	6.30	6.88
70	6.91	7.24	5.59	6.30	6.88
80	6.91	7.24	5.59	6.30	6.88
90	6.91	7.24	5.59	6.30	6.88
100	6.91	7.24	5.59	6.30	6.88
110	6.91	7.24	5.59	6.30	6.88
120	6.91	7.24	5.59	6.30	6.88
130	6.36	7.24	5.59	6.30	6.88
140	5.32	7.24	5.59	6.30	6.88
150	5.40	7.24	5.59	6.30	6.28
160	5.08	7.24	5.31	6.30	6.28
170	4.93	7.24	5.13	6.30	6.02
190	6.56	4.31	5.40		
180	6.98	4.80	5.82		
190	6.56	4.31			
200	6.02	4.02	5.33		
210	5.59	3.96	4.60		
240	5.01	3.96	4.60		
250		3.96	4.60		

Experiment were also performed in corning tube containing ADP 0.01M and agar - agar 1% in the lower portion of the tube and $CaCl_2$ 0.01M containing a colony of bacteria in the upper portion of tube to see the effect of bacteria *E. coli* on precipitation patterns. Results are shown in figure 8.

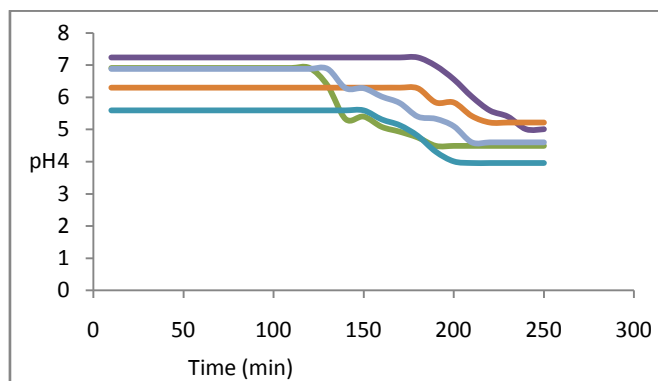


Figure 7. Variation of pH changes with time during precipitation $CaPO_4$ in presence of *E.coli* and Vitamin C. **Conditions:** $[CaCl_2]$ = 0.50M $[ADP]$ = 0.01M containing 1% agar-agar ADP = 0.01M containing 1% agar-agar & *E.coli*, $[ADP]$ = 0.01M containing 1% agar-agar and 1% vitamin C (in the lower pattern of the tube), $[CaCl_2]$ = 0.50M containing vitamin C. $[ADP]$ = 0.01M containing 1% agar-agar in the lower portion of the tube at $30.0 \pm 0.10^{\circ}C$.

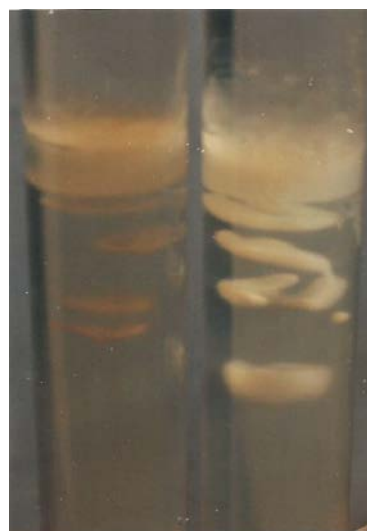


Figure 8. Influence of bacteria on *E. coli* on precipitation pattern of the calcium phosphate. **Conditions:** $[ADP]$ = 0.01M containing 1% agar-agar (in the lower portion of the tube) $[CaCl_2]$ = 0.01M (in the upper portion the tube containing the *E. coli*).

Growth kinetics studies in absence and presence of electric field

An experimental setup (figure 9) with horizontal tube containing ADP and agar-agar was attached to two reservoirs fitted with $CaCl_2$ of higher concentration (0.01M) to study the growth kinetics. Precipitation takes place as soon as $CaCl_2$ comes in contact with the gel containing ADP. The kinetics was

studied by measuring thickness of the precipitate as a function of time with a travelling microscope. CaCl_2 solutions were prepared in distilled water and experiments were performed in presence of electric field in the field intensity range 0.04 V/cm. Results are shown in figure - 10 and recorded in table 5 and 6.

Table 5. Extent of calcium phosphate precipitation from the initial junction in the presence of electric field of different field intensities. **Conditions:** (ADP) = 0.01M containing 1% agar-agar in horizontal tube. Left and Right reservoirs contain CaCl_2 (0.5M) and ADP (0.01M) respectively. Temp. = $30.0 \pm 1^\circ\text{C}$

Time (min)	Distance from Initial junction				
	0.00 V/cm	0.10 V/cm	0.20 V/cm	0.30 V/cm	0.40 V/cm
15	0.360	0.360	0.20	0.350	0.511
30	0.500	0.453	0.460	0.510	0.850
45	0.710	0.600	0.510	0.750	1.251
60	0.810	0.710	0.642	1.012	1.510
75	0.910	0.850	0.810	1.300	1.950
90	1.012	0.910	1.00	1.700	2.410
105	1.110	1.125	1.1602	2.050	2.850
120	1.209	1.128	1.268	2.320	3.125
135	1.259	1.420	1.410	2.620	3.510
150	1.300	1.651	1.641	2.950	3.920
165	1.412	1.790	1.855		
180	1.500	1.902	2.054		
195	1.560		2.202		
210	1.610		2.412		

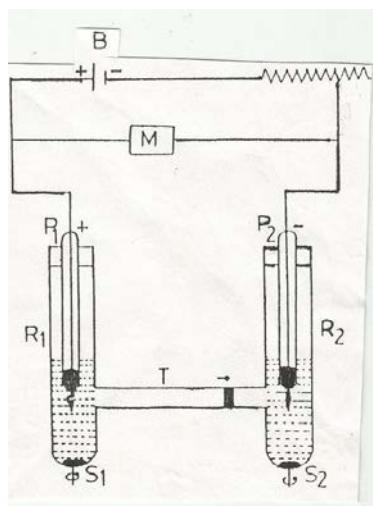


Figure 9. Experimental setup for electric field experiment in batch reactor. P_1 , P_2 and platinum electrodes R_1 , R_2 are reservoirs containing CaCl_2 and ADP solution respectively. B, DC voltage source. M, digital multimeter. T, tubular reactor of the 30.0 cm, S_1 & S_2 are magnetic stirrers.

Table 6. Dependence of field intensity on growth kinetics during the reaction between Calcium chloride and ammonium dihydrogen phosphate in agar-agar gel. **Conditions:** (CaCl_2) = 0.5M, (ADP) = 0.01M containing 1% agar-agar at $30 \pm 1^\circ\text{C}$. Distance between electrodes = 30.0 cm.

0.0	$d^2=mt+c$	0.013	0.109	0.999
0.10	$d=mt+c$	0.010	0.159	0.996
0.20	$d=mt+c$	0.011	0.146	0.994
0.30	$d=mt+c$	0.020	0.101	0.996
0.40	$d=mt+c$	0.026	0.084	0.999

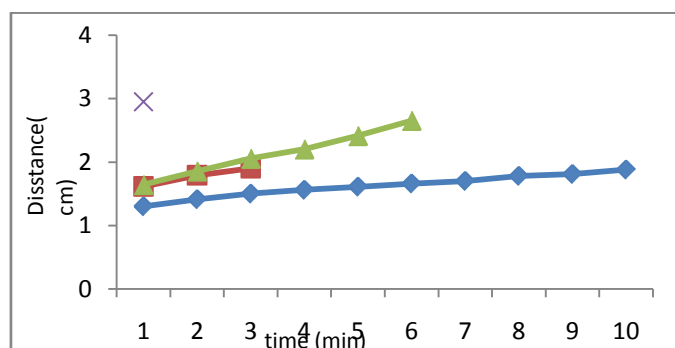


Figure 10. Effect of electric field on growth kinetic. **Conditions:** ADP = 0.01M containing 1% agar-agar in horizontal tube, left and right reservoirs contain [CaCl_2] = 0.50M and [ADP] = 0.01M respectively temp. $30.0 \pm 1^\circ\text{C}$. Field intensity 0.0v/cm (curve 1), 0.10v/cm (curve 2), 0.20v/cm (curve 3), 0.30 v/cm (curve 4), 0.40v/cm (curve 5).

Determination of charge on bacteria *E. coli* :

An electrophoresis experiment was performed in an experimental setup shown in figure 11 to determine the charge on microorganism. It consists of a U tube filled with peptone water, lag phase bacteria grown in peptone water was poured gently as to form a boundary at the surface of pure peptone water. Two platinum electrodes were dipped at the two ends and connected with a DC battery. Direction of the movement of the boundary in presence of electric field was noted.

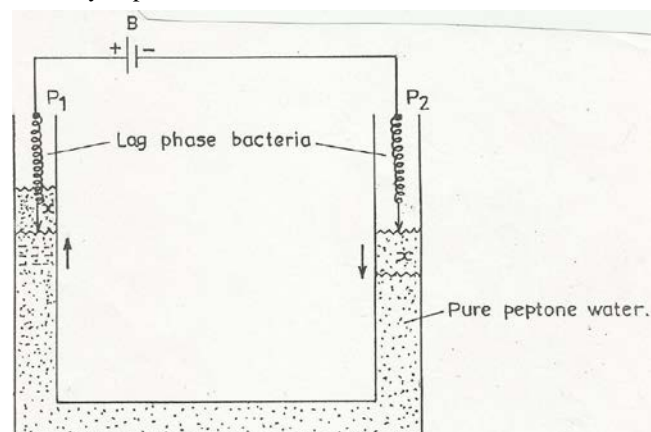


Figure 11. Experimental setup for electrophoresis experiment B (DC battery) P_1 , P_2 platinum electrode

Influence of bacteria on growth kinetics:

Solutions were prepared in (i) distilled water, (ii) water contaminated with bacteria, (iii) contaminated water admixed with 1% glucose to study the influence of bacteria on growth kinetics. Results are recorded in table 7, 8 and shown in figure 12.

Table 7. Growth kinetics data for the precipitation of calcium phosphate. ADP solution was prepared in distilled water, water contaminated with *E. coli* and contaminated water admixed with 1% glucose.

Time	Distance from initial junction		
	Distilled water	Water contaminated with <i>E. coli</i>	Time (min)
15	0.300	0.350	0.260
30	0.500	0.506	0.400
45	0.700	0.610	0.450
60	0.810	0.700	0.550
75	0.910	0.759	0.560
90	1.012	0.800	0.610
105	1.110	0.860	0.650
120	1.209	0.900	0.680
135	1.259	0.940	0.702
150	1.300	0.986	0.730
165	1.412	1.020	0.780
180	1.50	1.060	0.810
195	1.560	1.10	0.850
210	1.610	1.160	0.890
225	1.660	1.200	0.890
240	1.700		
255	1.780		
270	1.810		
285	1.880		
300	1.930		

Table 8. Dependence of field intensity on growth kinetics of the reaction between calcium chloride and ammoniumdihydrogen phosphate in gel medium. **Conditions:** (CaCl_2) = 0.50 M, (ADP) = 0.01 M, containing 1% agar agar at $30.0 \pm 0.1^\circ\text{C}$.

Field intensity V/cm	Experimental condition	Equation obeyed	Slope (m)	Intercept (c)	Correlation Coefficient
0.0	In absence of <i>E. coli</i>	$d^2=mt+c$	0.013	0.109	0.99
	In presence of <i>E. coli</i>	$d^2=mt+c$	0.06	0.650	0.99
	In presence of <i>E. coli</i> + 1% glucose	$d^2=mt+c$	0.003	0.328	0.99
0.1	In absence of <i>E. coli</i>	$d = mt+c$	0.010	0.159	0.99
	In presence of <i>E. coli</i>	$d = mt+c$	0.008	0.149	0.99
	In presence of <i>E. coli</i> + 1% glucose	$d = mt+c$	0.004	0.268	0.99
0.2	In absence of <i>E. coli</i>	$d = mt+c$	0.011	0.002	0.98
	In presence of <i>E. coli</i>	$d = mt+c$	0.010	0.173	0.99
	In presence of <i>E. coli</i> + 1% glucose	$d = mt+c$	0.009	0.029	0.99

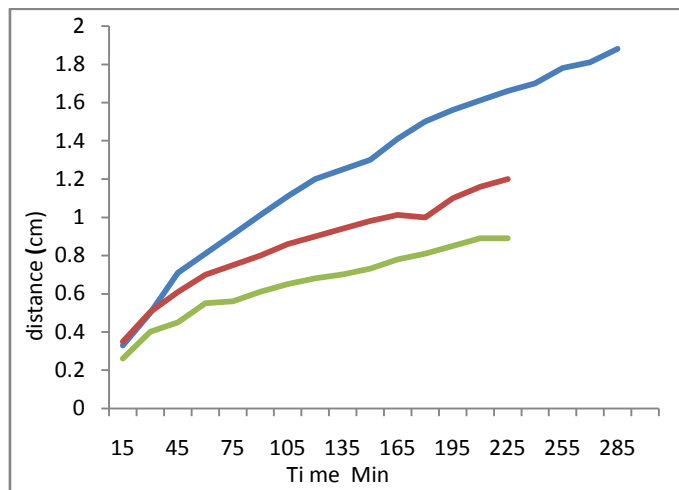


Figure 12. Growth kinetics and precipitation of the calcium phosphate when solutions were prepared in distilled water (curve 1), water contaminated with *E. coli* (curve 2), contaminated water admixed with 1% glucose (curve 3).

Two dimensional Studies:

Experiment were performed in (i) semi batch reactor and (ii) in a DPL continuous flow reactor to obtain two dimensional patterns of calcium phosphate.¹⁶ The bottom of the square petridish (10 cm x 10 cm) of flat surface was covered with ADP containing 1% agar agar to obtain two dimensional precipitation patterns in a semi batch reactor. A circular portion of gel layer (diameter 12 mm) was removed from the centre of the dish and subsequently the empty portion was filled with CaCl_2 solution of relatively higher concentration at every $\frac{1}{2}$ h and the dish was covered with a glass plate to avoid evaporation. Result are recorded in table 9 and shown in fig 13 and 14. In this experiment concentration of CaCl_2 may change due to diffusion and chemical reaction. This difficulty may be overcome by using DPL continuous flow reactor as described earlier.¹⁶ The DPL reactor is open with respect to the flow of one reactant CaCl_2 . Concentration and level of entering reactant in the empty space were always kept constant. CaCl_2 solution was fed at a rate of 2.0 ml /h. Results are shown in figure 13.

Table 9. Growth kinetics data for the precipitation of calcium phosphate in a semi batch reactor. An empirical equation $d^2 = mt+c$ was obeyed (ADP) 0.01

Time (h)	Distance from initial junction					
	(CaCl_2) Concentration (M)					
	0.05	0.10	0.20	0.30	0.40	0.50
0.5	0.725	0.740	0.875	0.877	1.140	1.10
1.0	0.726	0.850	0.895	1.075	1.108	1.30
1.5	0.840	0.850	0.950	1.090	1.125	1.650
2.0	0.910	0.990	1.018	1.195	1.275	1.760
2.5	1.050	1.153	1.083	1.300	1.195	1.825
3.0	1.150	1.173	1.105	1.300	1.275	1.825
3.5	1.180	1.225	1.375	1.850	1.300	1.850

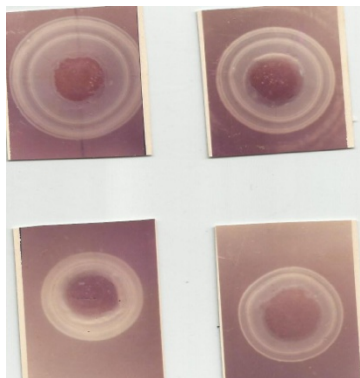


Figure 13. Precipitation of calcium phosphate in two dimensional gel media in a semi batch reactor in which CaCl_2 solution was filled periodically conditions: $[\text{CaCl}_2]$ 0.05M, 0.2M, 0.4M, 0.5M, from {1 TO 4 respectively} in the inner portion of the reactor , $[\text{ADP}] = 0.01\text{M}$ in 1% agar-agar at $37.0 \pm 0.1^\circ\text{C}$.

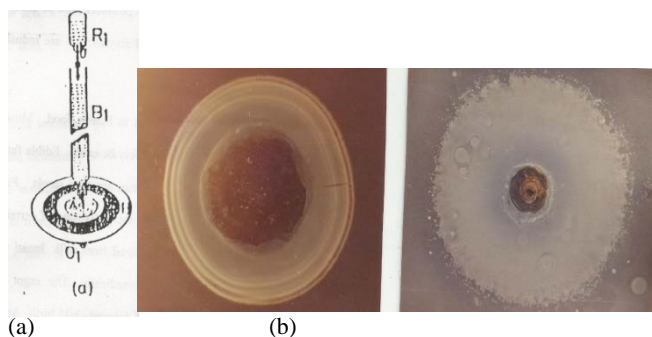


Figure 14. (a) Experimental set up for **Das-Pushkarna-Lall reactor** (b) Two dimensional precipitation pattern.

Experiments on inhibitory effect

The precipitation of calcium phosphate in presence of α -Ketoglutamic acid (normal physiological constituents, leucine,

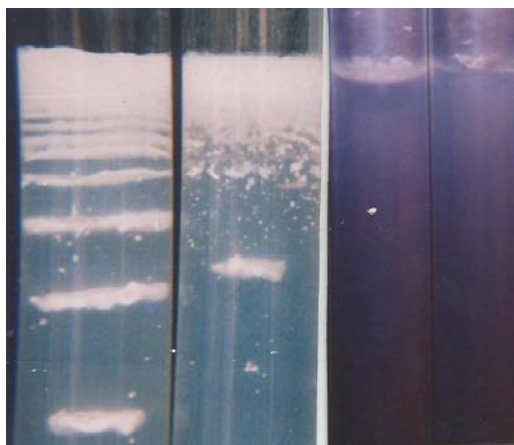


Figure 15. Inhibition of Calcium phosphate precipitation in presence of different additives. Conditions: $[\text{CaCl}_2] = 0.01\text{M}$ in the upper portion of tube, $[\text{ADP}] = 0.01\text{M}$ containing 1% agar agar and additives (ii) leucine 0.001M,

and ascorbic acid as studied in corning glass tubes. The lower end of tube contained ADP, agar agar and an additive, while aqueous CaCl_2 was allowed to diffuse from the upper side. Results are shown in figure 15 and 16. Dependence of α -ketoglutaric acid concentration on the inhibition of calcium phosphate precipitation has also been studied.

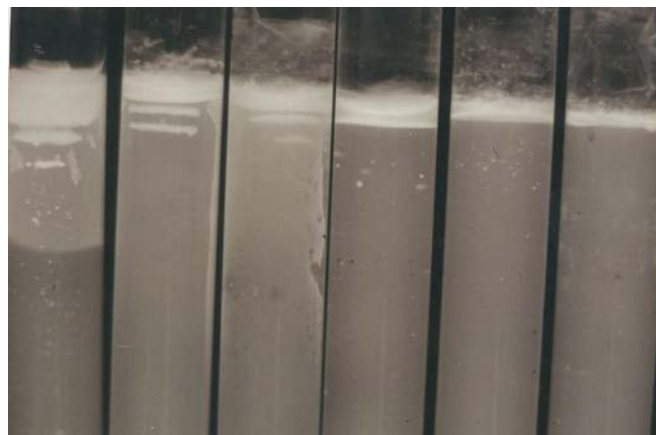


Figure 16. Inhibition of calcium phosphate precipitation in presence of α -ketoglutaric acid of different concentrations. Conditions: $[\text{CaCl}_2] = 0.01\text{M}$ in the lower portion of tube. $[\text{ADP}] = 0.01\text{M}$ containing 1% agar agar. α -ketoglutaric acid 0.2mM (ii) 0.4mM, (iii) 0.6mM (iv) 0.8mM, and 1.0mM in the lower portion of tube.

Experiments on the dissolution:

The experiment on the dissolution of calcium phosphate calculi was performed by taking a known amount of the washed and dried precipitate of calcium phosphate obtained by mixing aqueous solution of CaCl_2 and ADP (0.01M) in equal proportion. Aqueous solution of leucine, α -ketoglutaric acid, ascorbic acid, 1% extract of neem (*Azadirchata indica*) tulsi (*oscumum sanctum*) and bel (*Aglemormelos*) were used as solvents. The solution were filtered through sintered glass crucible and the filtrates were quantitatively analyzed for calcium content by atomic absorption spectroscopy and data are recorded in table 10 and shown in figure 17 and 18

Table 10. Dissolution of Calcium phosphate in presence of different additives and estimation of calcium content in the liquid phase after 24 hour. Conditions: 50 mg of calcium phosphate admixed with 20 ml of additives and left for 24 hour.

Additives	Concentration/M	Ca content in liquid phase (ppm)
Control		0.0
Neem extract	1%	25
Bel extract	1%	16
Ketoglutaric acid	2.0×10^{-4}	54
	4.0×10^{-4}	66
	6.0×10^{-4}	93
	1.0×10^{-3}	115
Ascorbic acid	1.0×10^{-3}	39
Leucine	1.0×10^{-3}	35

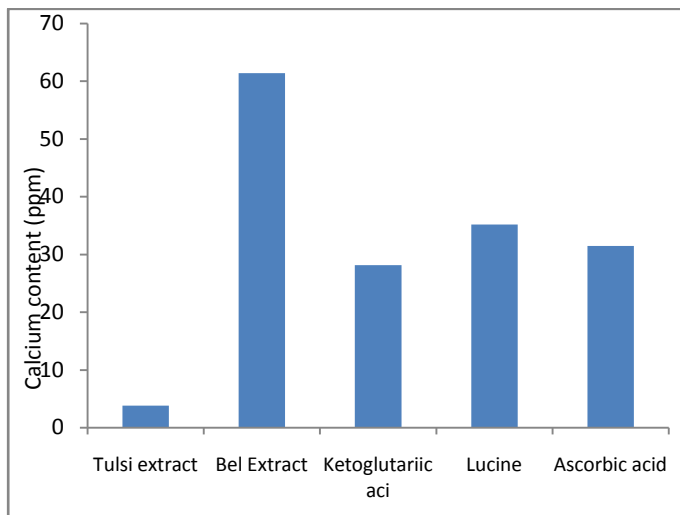


Figure 17. Dissolution of Calcium Phosphate in presence of different additives (i) Tulsi extract, (ii) Bel extract, (iii) α -ketoglutaric acid, (iv) leucine, (v) Ascorbic acid.

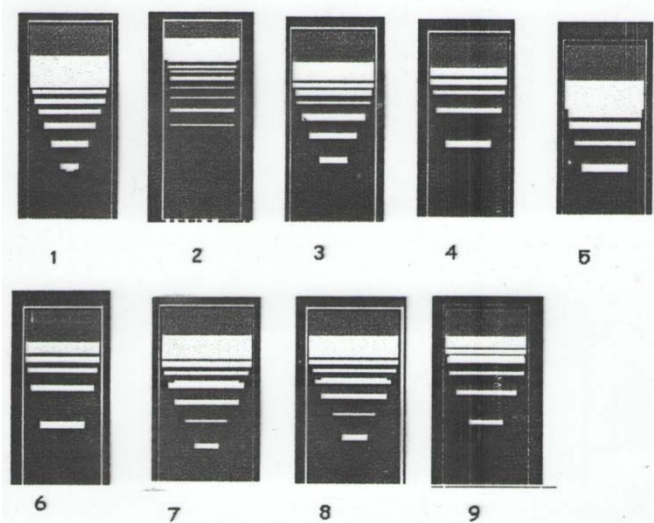


Figure 18: Screen display of the precipitation patterns based on computer programme with $K_s = 200$, $DA = DB = 0.01$, $CA = 10$ and $CB = 3, 4, 5, 6, 7, 8, 9$ and 10 .

RESULTS AND DISCUSSION

Precipitation in corning glass tube

Calcium Chloride diffuses into an aqueous solution of ADP containing agar agar 1% to form bands of calcium phosphate. Figure 1 shows precipitation patterns of calcium phosphate when $CaCl_2$ of different concentration taken in the upper portion of tube. Figure 2 shows precipitation pattern in tubes of different diameters. Initially widths of the bands were the same as that of the tube diameter. Beyond a certain distance, width of the band was found to decrease as shown in figures 1 and 2, and finally it attain a spherical shape.

Normally the rate of nuclei formation is more than the rate of crystal growth. Situation would be different when the rate of crystal growth is greater than the rate of nuclei formation. In the present case, it appears that once a nucleus is formed, it acts as a centre of crystallization and the crystal nuclei grows due to

lateral diffusion of the molecules of the precipitate. This tendency is facilitated and diameter of the precipitate front decreases as band move away from the junction.

The pH of the solution containing ADP and agar agar has marked influence on the precipitation patterns of calcium phosphate. There is no precipitation in acidic medium ($pH=4.0$) due to its dissolution at this condition. At normal condition ($pH=6.22$) precipitation takes place in the form of bands. However on addition of little ammonium hydroxide pH was increased to 7.63 and a transition from banded to continuous precipitation took place. The sequence may be represented as

pH	4.0	6.22	7.23
	No precipitation	band	continuous precipitation

Change in pH with time during the precipitation of calcium phosphate was monitored. Results are shown in figure 5. An experimental setup consisting of a pH electrode immersed into the corning tube containing ADP and agar agar and the $CaCl_2$ solution above the gel was used. As soon as the advancing front approached a region little above the tip of the electrode, pH suddenly decreased and finally attained a steady state. The drop in pH was due to the formation of HCl and found to decrease with increase in $CaCl_2$. Results are recorded in table 3 and shown in figure 6. Vit C has been recommended for the prevention of renal stone formation because it reduces urinary pH with a few exceptions while *E. coli* favour the stone formation due to increase in pH of the medium. Effect of Vit C and *E. coli* was also studied results are shown in figure 7. The trend of variation of pH during precipitation was the same in all the cases. Sharpe transition from one state of higher pH to another state of lower pH value was observed.

Stone formation is favoured in the alkaline medium. Stones are usually associated with urinary tract infection with organism such as *Klebsiella*, *E. coli* and *pseudomonas*. By producing urease these organisms catalyse the formation of ammonia from urea.



The infected urine is, therefore, alkaline and high in NH_4 content. Statistical data show that pH of urine samples of stone patients lies in the range of 8.0-9.0. Figure 8, show comparatively dense precipitation patterns of calcium phosphate in the presence of *E. coli* which is in support of above discussion.

Electric field experiment:

Figure 9 shows an experimental setup to study the influence of electric field on the growth of calcium phosphate in batch reactor. Figure 10 shows the plot of the growth kinetics of precipitation of calcium phosphate from $CaCl_2$ and ADP in gel media. In the absence of electric field Ca^{++} ions diffuses into the gel containing ADP due to concentration gradient and form the precipitate of calcium phosphate at the junction. When an electric field was imposed Ca^{++} ions moves towards the gel with an enhanced velocity. The velocity was found to be proportional

to applied electric field in agreement with the theoretical development of Feeny *et.al.*¹⁸ Results are summarized in tables and results also show a transition from nonlinear to linear growth behaviour by imposing an electric field. It indicates that a different mechanism is operative in presence of electric field.

It has been observed earlier that precipitation patterns of calcium phosphate are greatly influenced in the presence of bacteria which are found to contaminated water. It was though worthwhile to determine the charge on *bacteria E.coli* using an experimental setup shown in figure 11. Results show that *E. coli* carries a negative charge as the boundary moves towards the anode in presence of electric field. Experiments were also carried out by preparing the solution in water contaminated with (i) *E.coli*, and (ii) *E.coli* admixed with 1% glucose. As shown in figure 12, the growth rate was found to decrease in the following sequence.

V pure water > V water contaminated with *E. coli* > V water containing *E. coli* +1% glucose.

Similar trend was also observed in the presence of electric field. Results are summarized in table 04 and 05. The reason for lowering in velocity in presence of negatively charged bacteria *E.coli* may be due to neutralization of charge of the Ca⁺⁺ and aggregation particles.

Precipitation in Petridishes

Results of precipitation patterns of calcium phosphate at different concentrations of semi batch reactor is shown in figure 13a Growth rate and number of bands were found to increase with CaCl₂. The Extent of precipitation as a function of time is recorded in table 06 and shown in figure 13b. In a semi- batch reactor Ca⁺⁺ ion diffuses into the gel containing ADP of relatively low concentration and as a result of diffusion and chemical reaction, precipitation takes place. There is a possibility that Ca⁺⁺ concentration in the empty space may change due to consumption of ions in chemical reaction and precipitation. Experiments were then performed in DPL reactor which can serve as a tool for systematic studies in spatial pattern formation in the same way as the CSTR has served in studies of homogeneous reaction,¹⁹⁻²⁰ In case of flow reactors, the electrolytes inflexed through the reservoir at a definite flow rate to maintain the constant electrolyte concentration at a liquid/gel interface throughout the experiment in the reactor and the system was maintained earlier from equilibrium. As a result of change in flow condition a transition from bands to continuous precipitation takes place as shown in figure 14.

Precipitation and aggregation of calculi in presence of other substances which can act as inhibitors of the crystalline growth are of great importance in view of their potential applications in the urolithisis therapy. There are several constituents of urine which act as inhibitors. These include Mg⁺⁺, citrate and α -ketoglutaric acid. α -Ketoglutaric acid not only inhibits the growth but also increases the solubility of calcium oxalate in various physiological solutions. Figure 15, shows patterns of calcium phosphate in presence of several inhibitors viz leucine, α -ketoglutaric acid and ascorbic acid. Results show that α -Ketoglutaric acid and ascorbic acid are more effective than leucine. Inhibiting capacity of α - ketoglutaric acid at different

concentrations have been studied and results are shown in figure 16. Similar inhibitory capacity of ascorbic at different concentrations has also been observed. Dissolution of calcium phosphate in presence of different additives has been studied. Results are recorded in table 7 and 8. Fig 17 shows dissolution of calcium phosphate at different concentrations of α -ketoglutaric acid which shows a marked increase in dissolution as the concentration of α - ketoglutaric acid increased.

Theoretical development of pattern:

Liesegang rings usually appear when a concentrated salt solution diffuses through an aqueous medium containing a dense matrix such as agar- agar and a second electrolyte. The chemical reaction between the salt lead to slightly soluble product that precipitate in the form of bands parallel to a diffusion front surface. A theoretical analysis of the phenomenon was proposed by Talanquer¹⁵ which explained the parallel bands. He considered two diffusing species A and B that react to form a weakly soluble product AB.



He also considered that the electrolyte move independently of each other according to usual diffusion equation.

$$\frac{\partial C_a}{\partial t} = D_a \frac{\partial^2 C_a}{\partial X^2}$$

$$\frac{\partial C_b}{\partial t} = D_b \frac{\partial^2 C_b}{\partial X^2}$$

Where D_a and D_b are the appropriate diffusion coefficient. The proposed BASIC program could explain only usual Liesegang bands. In our investigation we have observed parallel bands with decreasing diameter as show in figure 1 and 2. Thus the BASIC program proposed by Talanquer was modified. The modified program is enclosed herewith. Results show that number of bands depend upon CA/CB ratio. Number of bands was found to increase with increase in CB at a fixed CA and DA, DB values whereas. diameters of bands are decreased as we move downward with increase in CB values. Theoretical pattern at different electrolyte concentrations are shown in figure 18.

BASIC programme

```

10DIM CA(500),CB(500),CAN(500),CBN(500),EW(100)
20CLS:SCREEN 1
30NO=100
40WINDOW(0,-.05*NO)-(50,1.2*NO)
50 LINE (19.8,0)-(30.2,1.11*NO),,B
60LINE(20,NO+1)-30.2,1.11*NO),2,BF
70 DA=1 : DB=1.
80 KS =200
90 CA(0)=10:CB(0)=3TO 10
100CA(NO+1)=400:CB(NO+1)=0
110 FOR X =1 TO NO: CA(X) = 0:CB(X) =CB(0) : EW (X)=0:NEXT X
120FOR T= 1 TO1500
130FOR X =1 TO NO
140 K=CA(x)* CB(X)
150 CAN(X) = CA(X)+DA*(CA(X+1)+CA(X-1)-2*CB(X))
160 IF K>KS OR CB(X)=0 THEN 190
170 CBN(X) = CB(X)+DB*(CB(X+1)+CA(X-1)-2*CB(X))
180 GOTO 210
190 CBN(X) =:IF X <>0THEN EW (X) =1
200 LINE(20+S,X)-(30-S,X+1),,BF
210 NEXT X : SP +0
220FOR (Y =1TO NO
230 IFEW (Y) = 1 THEN SP =Y: GOTO
240 NEXT Y
250 S=0
260 FOR Z = NO TO1STEP-1

```

```

270CA(Z) = CAN(Z) :CB(Z)=CBN(Z)
280 IF SP= 0 THEN 330
290 IF EW (Z) =0 ANDZ> SP THENS=S+3: GOTO330
300 IFEW (Z)=0 AND Z <=SPTHEN 330
310IF EW (Z) =1 AND S=0 THEN S=0
320LINE (20+S,Z)-(30-S,Z +.5),BF
330 NEXT Z
340 LOCATE 1,1:PRINT T : NEXT T
350 FOR I = 0 TO .5 STEP .01 :CIRCLE (2.5,SP+2.5),I:NEXT I
360 END.

```

Formulation of mechanism:

The formation of Liesegang patterns has been investigated by several workers. The models proposed so far belong to the following categories (a) Sol coagulation model²¹⁻²⁴ (b) competitive particle growth model²⁵ (c) Super saturation model.²⁶ Although, none of these models is able to account for all experimental observation described below.

- (i) Particles are present not only within bands but also in the spaces between them.
- (ii) Spatial inhomogeneities in the distribution of precipitate may arise even in the absence of concentration gradients. Liesegang patterns are only obtained for narrow range of parameters. In particular, it is important that the initial A concentration be significantly larger than the initial B concentration.
- (iii) When the super saturation and /or the difference between initial concentrations of reacting substances are small, irregular patterns are formed.
- (iv) Sometimes complex precipitation pattern are formed such as dislocation or helicoidally bands.²⁷

According to the sol coagulation model, the two reactants A and B diffuses from opposite directions and co exit in the gel until the solubility product reaches a critical value above which the nucleation in occurs and the product AB is formed. Once the nucleation has started a depletion of concentrations of A and B occurs in the surrounding. Hence, at this stage, the formation of precipitate stops nucleation in the neighbourhood. Precipitation also stopped as diffusion continues further. This is followed by the formation of clear spaces. Diffusion of ions persists and after a certain stage, the critical super saturation exceeds and another band is formed.

In a more recent version, the two species A and B react to produce a new species C which also diffuse. When the local concentration of C reaches some threshold value, nucleation occurs. The nucleated particle D at the reaction front deplete their surrounding of the reaction product. As a result the level of super saturation drops and the nucleation processes stops. After some time, the reaction front moves away and the concentration of product at the moving front moves away and the concentration of product at the moving front reaches a large enough value, allowing the nucleation to occur again and separated bands appear. We believe, based on the Prager,²⁶ Smith,²⁸ Dee,²⁹ Le-van and Ross,³⁰⁻³² that the super saturation mechanism based on Ostwald ideas plays a critical role in band formation.

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