Nucleation of Calcium Phosphate from Solutions at Physiological pH

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In a previous paper[1] it was shown that when phase transition from aqueous solutions occurs at metastable equilibrium, the stoichiometry of the nucleating phase can be conveniently evaluated from the precipitation boundary[2]. The method was applied to calcium phosphates nucleating in the pH range 6.3 to 9.1 from solutions containing calcium chloride and sodium phosphate at constant total phosphate concentration 1×10^{-3} M. The calcium phosphate ratio of the nucleating phase was found to be 1.5 and the formation of an amorphous metastable precursor to hydroxyapatite was concluded. Because of its implications on biological calcification it was of interest to reexamine this result restricting the experiments to the physiological pH region (pH 7.25—7.5), but taking into account possible variations of total phosphate and total calcium concentrations. This was done as part of more extensive investigations of the precipitation of calcium phosphates from aqueous solutions and from media containing 0.15 M sodium chloride.

Analytical grade chemicals and tetradistilled water were used and the sodium chloride stock solution was further purified of heavy metals by electrolysis. The experimental procedure was similar to that used in a previous work[1], with some alterations meeting different requirements. Samples were prepared by direct mixing of calcium chloride solutions with solutions of sodium phosphate, which were previously adjusted with sodium hydroxide to pH 7.4. Because of their possible influence on precipitate formation no foreign buffers were added. Since in most samples total phosphate was in excess over calcium chloride, the solutions were self-buffered to a certain extent, but some scattering of the final pH values could not be avoided. Where required, sodium chloride solution was added to the phosphate solution prior to mixing, the final sodium chloride concentration being 0.15 M. In each series of runs the concentration of sodium phosphate was kept constant, while the concentration of calcium chloride was varied in a wide concentration range. All samples were incubated in a 25^0 C and 37^0 C water bath before, during, and after mixing, up to 24 hours. The last clear and first turbid solutions were then detected either visually with the aid of a microscopic lamp or with a Zeiss tyndallometer. The pH was measured with a Radiometer model 26 pH meter.

In order to minimize contamination by microorganisms, most of the experiments were performed under sterile conditions. All glassware was sterilized and solutions were filtered through bacteriological filters prior to pipetting. Pipetting and mixing of the solutions was carried out in the presence of lit gasburners, a procedure which is used in microbiological laboratories. Samples thus treated, when kept in stoppered

test tubes, remained sterile for several weeks and could thus be used for observations through extended periods of time. (Some results of these experiments will be presented in subsequent papers.) The samples where sodium chloride was present were prepared without sterilization. Some microbiological contamination of these samples was observed but comparison of the results showed that this did not appreciably affect the precipitation boundary.

In order to investigate the influence of temperature and of physiological concentrations of sodium chloride on the nucleating properties of calcium phosphates, the precipitation boundary was determined at 25^0 C and 37^0 C in aqueous solution and at 37^0 C in 0.15 M sodium chloride solution. The total calcium concentrations and the pH at which the first precipitate appeared were determined in a wide range of total phospate concentrations.

The phosphate concentration ranges and the average pH for the three sets of experiments were as follows: 1×10^{-3} M — 6×10^{-2} M total phosphate, average pH 7.38 for 37^0 C, aqueous solution; same range of total phosphate, average pH 7.26 for 25^0 C, aqueous solution and 3×10^{-3} M — 5×10^{-1} M total phosphate, average pH 7.46 for 37^0 C, 0.15 M NaCl solution. The data were recalculated by means of a computer program written in FORTRAN II in order to obtain the respective activities of the free Ca^{2+} and $H_2PO_4^-$ ions. The computations were made with the same equations as before except that the full Debye-Hückel equation,

$$\log f_i = - \frac{A z_i^2 \mu^{1/2}}{1 + B \mathring{a}_i \mu^{1/2}} , \qquad (1)$$

was used to calculate activity coefficients. Acid dissociation and complex stability constants for the various phosphate species were taken after Nancollas and coworkers[3]. The constants A and B in equation (1) were taken after Robinson and Stokes[4]

TABLE I$_{a-c}$

Summary of Experimental Results and Recalculated Data in Terms of Ionic Strengths, Concentrations of Free Ca^{2+} and $H_2PO_4^-$ Ions and their Respective Activity Coefficients

a: *37^0 C, without NaCl*

Sample No.	Ca$_{TOT}$ $M \times 10^4$	P$_{TOT}$ $M \times 10^3$	pH	$\mu \times 10^3$ (ionic strength)	$[H_2PO_4^-]$ $M \times 10^4$	$\gamma_{H_2PO_4^-}$	$[Ca^{2+}]$ $M \times 10^5$	$\gamma_{Ca^{2+}}$
1	15	1	7.21	5.734	3.278	0.9210	127.5	0.7288
2	9	1.2	7.24	4.567	4.199	0.9285	71.90	0.7510
3	9	2	7.27	6.053	6.740	0.9192	63.66	0.7233
4	8	1.6	7.24	5.059	5.725	0.9252	59.81	0.7411
5	8	2	7.35	5.964	6.056	0.9197	55.14	0.7248
6	8	2.5	7.35	6.991	7.571	0.9140	51.69	0.7085
7	7	3	7.32	7.828	9.655	0.9098	42.77	0.6965
8	5	4	7.37	9.944	12.10	0.9002	26.92	0.6703
9	5	4	7.41	10.08	11.33	0.8997	26.57	0.6687
10	4	5	7.25	11.61	18.20	0.8937	20.64	0.6528
11	4	8	7.45	20.00	20.18	0.8679	16.15	0.5882
12	3	10	7.38	24.40	27.93	0.8574	11.39	0.5638
13	3	10	7.47	25.20	23.85	0.8556	11.02	0.5598
14	3	12	7.46	30.26	28.40	0.8454	10.34	0.5372
15	3	20	7.47	51.33	42.64	0.8135	8.610	0.4719
16	3	30	7.44	77.35	62.35	0.7865	7.627	0.4227
17	3	45	7.57	121.59	65.90	0.7550	6.451	0.3716
18	3	200	7.50	555.97	218.7	0.6459	4.520	0.2360
19	2.5	60	7.46	159.47	101.6	0.7355	5.159	0.3429

b: 37° C, 0.15 M NaCl

Sample No.	Ca$_{TOT}$ $M \times 10^4$	P$_{TOT}$ $M \times 10^3$	pH	$\mu \times 10^1$ (ionic strength)	[H$_2$PO$_4^-$] $M \times 10^4$	$\gamma_{H_2PO_4^-}$	[Ca^{2+}] $M \times 10^5$	$\gamma_{Ca^{2+}}$
1	15	3	7.24	1.611	7.076	0.7348	129.6	0.3419
2	10	5	7.40	1.651	9.008	0.7330	77.52	0.3394
3	6	8	7.40	1.720	14.58	0.7300	41.03	0.3353
4	6	10	7.45	1.774	16.47	0.7278	37.88	0.3322
5	5	12	7.52	1.844	17.88	0.7250	28.66	0.3284
6	5	20	7.50	2.044	29.04	0.7175	23.76	0.3183
7	3	30	7.54	2.322	39.10	0.7082	11.68	0.3063
8	3	50	7.54	2.876	61.37	0.6926	9.198	0.2871
9	3	100	7.53	4.275	111.3	0.6642	6.773	0.2549
10	3	200	7.41	7.008	244.7	0.6302	5.348	0.2208
11	3	500	7.55	15.78	356.5	0.5797	3.657	0.1779

c: 25° C, without NaCl

Sample No.	Ca$_{TOT}$ $M \times 10^4$	P$_{TOT}$ $M \times 10^3$	pH	$\mu \times 10^3$ (ionic strength)	[H$_2$PO$_4^-$] $M \times 10^4$	$\gamma_{H_2PO_4^-}$	[Ca^{2+}] $M \times 10^5$	$\gamma_{Ca^{2+}}$
1	30	1	7.06	9.941	3.670	0.9025	273.1	0.6765
2	18	2	7.04	8.002	8.648	0.9110	146.1	0.7001
3	10	2.5	7.26	7.339	8.823	0.9142	71.73	0.7092
4	8	3	7.23	78.79	11.32	0.9116	54.57	0.7017
5	8	4	7.32	10.25	13.11	0.9012	49.07	0.6732
6	7	4	7.25	98.48	14.72	0.9029	43.55	0.6776
7	6	5	7.25	11.87	18.41	0.8951	34.68	0.6567
8	6	6	7.15	13.47	25.19	0.8895	33.83	0.6422
9	6	8	7.40	19.75	22.46	0.8713	28.29	0.5969
10	6	10	7.25	23.20	35.00	0.8631	27.52	0.5773
11	5	30	7.38	75.35	72.26	0.7924	15.18	0.4337
12	4	20	7.44	50.56	46.72	0.8182	13.54	0.4816
13	3	60	7.37	154.48	126.6	0.7427	7.466	0.3540

for 38° C and 25° C respectively. The ionic size parameters å were taken from Kiellands data[5], except for the parameters for the complex CaH$_2$PO$_4^+$ and CaPO$_4^-$ which are not available. These values were approximated by å = 4.5 since according to Kielland[5] about 40% from a group of 130 ions were found to have an ionic size parameter å between 4 and 5.

In absence of *a priori* knowledge of the ionic strengths of the solutions, approximate concentrations of H$_2$PO$_4^-$, HPO$_4^{=}$, Ca^{2+}, CaHPO$_4$, CaH$_2$PO$_4^+$ and CaPO$_4^-$ ions were found and from these data approximate ionic strengths were calculated. The process was repeated until the ionic strength from one cycle to the next changed by less than 0.00001.

In Tables I$_{a-c}$ the experimental data, the calculated ionic strengths, concentrations of free Ca^{2+} and H$_2$PO$_4^-$ ions and the respective activity coefficients are listed. In figure 1 the results are represented in terms of the following equation[1]:

$$\log a_{Ca^{++}} a_{OH^-}^{-2} = -(m/n) \log a_{H^+} a_{H_2PO_4^-} + \text{constant} \qquad (2)$$

where the slope of the straight line, — (m/n), gives the phosphate/calcium ratio of the nucleating species and the constant is related to the solubility product and the nucleation characteristics (supersaturation and interfacial energy) of the particular system. By the method of least squares the data were fitted to straight lines with slopes and respective standard deviations as follows:

I. (37° C, aqueous solution) b = — 1.045, s_b = 0.037,

II. (37° C, 0.15 M NaCl solution), b = — 0.957, s_b = 0.030,

III. (25° C, aqueous solution) b = — 1.024, s_b = 0.055.

The difference between straight lines I and II is not significant, as tested by the Student's test at 95% confidence level. Thus, a common straight line (IV)
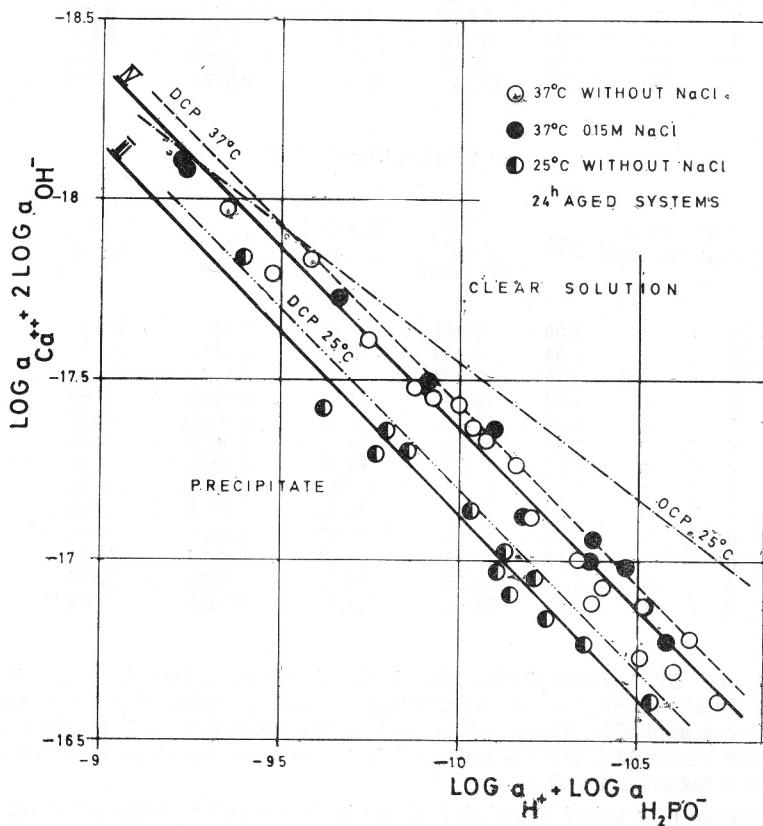
Fig. 1. Critical ion activities at the 24 hours precipitation boundary, recalculated and plotted in accordance with equation (2). By heavy lines III and IV the regression lines through data obtained at 25° C and through all data obtained at 37° C (with and without 0.15 M NaCl) are represented. Dashed lines OCP 25° C, DCP 37° C and DCP 25° C are solubility curves for octacalcium phosphate and dicalcium phosphate dihydrate at 37° C and 25° C, calculated from the respective solubility products.[6-8]

was fitted through all data obtained at 37° C and the slope and its standard deviation b = — 1.006 and s_b = 0.025 respectively have been obtained. In figure 1 the regression lines III and IV are plotted with full lines, while by dashed lines the equilibrium solubility curves for octacalcium phosphate (OCP, $Ca_4HPO_4(PO_4)_2$)[6] and dicalcium phosphate dihydrate (DCP, $CaHPO_4$) for 37° C[7]

and 25^0 C[8], as calculated from the respective solubility products[6-8], are given for comparison. The results indicate that the processes leading to the formation of the initial solid phase are reproducible in the investigated concentration range and are not affected by temperature changes from room to body temperature or by the addition of physiological concentrations of sodium chloride. Interpretation of the data in terms of the concept of precipitation boundary[1,2] (equation 2) leads to the conclusion that the Ca/P ratio of the nucleating phase is 1 : 1, a result which is in disagreement with the previously found Ca/P ratio of 1.5. Since the two sets of data have been obtained in two complementary but different concentration regions, the stoichiometry of the nucleating phase must not necessarily be identical, but the obtained result was nevertheless surprising. Further experiments have thus been carried out and have shown that the slope of the precipitation boundary changes with time. At this point some discussion on the applicability of the precipitation boundary for determinations of the stoichiometry of the nucleating phase seems appropriate.

With this, as with other methods it is impossible to differentiate between nucleation and subsequent processes (crystal growth, agglomeration *etc.*) which all together result in the formation of the first visible precipitate. The slope (—m/n) in equation (2) should nevertheless be independent of time (metastable equilibrium is assumed) if the rate of all mentioned processes depends on the supersaturation and on parameters which are characteristic of a given system, as is generally assumed for the nucleation rate. The observation that the slope of the precipitation boundary changes with time indicates that in the investigated system the concentrational relations of the precipitating components might also influence the rate and/or mechanism of either one or all of these processes. Systematic experiments, following the kinetics of slope changes might yield a better explanation of these phenomena. In this connection it is of interest to mention recent experiments on the precipitation of barium sulphate[10], which indicate that at given supersaturations the number and shape of precipitated particles also depends on the concentrational relations of the precipitating components.

Another possible source of error is the fact that the resolution of subsequent changes in the slope of the precipitation boundary (corresponding to subsequent changes in the stoichiometry of the nucleating phase) is limited by experimental error, which with the experimental method employed amounts to about 3—5%. Thus seemingly straight lines could be summarizing a set of phenomena which are more or less continuously changing over a wide range of concentrations.

However, despite these limitations, the evaluation of the precipitation boundary is a useful tool, which in combination with studies of the solid phase can help to clarify the picture of complicated precipitation processes.

In conclusion it may be said that precipitate formation in the three--component system calcium chloride-phosphoric acid-sodium hydroxide is a result of a number of complicated mechanisms and that the influence of the concentrational relations of all three precipitation components has to be considered.

More systematic experiments are conducted in this laboratory with the aim of getting a clear picture of the various phases of precipitate formation in the physiological concentration range.

REFERENCES

1. A. G. W a l t o n, W. J. B o d i n, H. F ü r e d i, and A. S c h w a r t z, *Can. J. Chem.* **45** (1967) 2695.
2. H. F ü r e d i, *Complex Precipitation Systems,* Chapter VI of *The Formation and Properties of Precipitates* by A. G. W a l t o n, Interscience Publishers, John Wiley and Sons, 1967.
3. A. C h u g t a i, R. M a r s h a l l, and G. H. N a n c o l l a s, *J. Phys. Chem.* **72** (1968) 208.
4. R. A. R o b i n s o n and R. H. S t o k e s, *Electrolyte Solutions,* Appendix 9.1, Butterworths Sci. Publ., London 1955.
5. J. K i e l l a n d, *J. Am. Chem. Soc.* **59** (1937) 1675.
6. E. C. M o r e n o, W. E. B r o w n, and G. O s b o r n, *Proc. Soil. Sci. Soc. Amer.* **24** (1960) 99, after L. G. S i l l é n, »*Stability Constants of Metal — Ion Complexes*«, The Chemical Society, Burlington House W. 1, London 1964.
7. N. B j e r r u m, **19**th *Scandinavian Scientist's Meeting* **1936**, Helsingfors, p. 334; in *Niels Bjerrum, Selected papers,* Munksgaard, Copenhagen, 1949, p. 245, after L. G. S i l l é n, *Stability Constants of Metal-Ion Complexes,* The Chemical Society, Burlington House W. 1, London 1964.
8. Y. K a u k o and S. E y u b i, *Tekn. Fören Finland Förhandl,* 1955, 263; *Chem.— Ztg.* **80** (1956) 130; *Österr. Chem. Z.* **58** (1957) 259; *Finska Kemistsamfundets Medd.* **69** (1960) 104, after L. G. S i l l é n, *Stability Constants of Metal-Ion Complexes,* The Chemical Society, Burlington House W. 1, London 1964.
9. E. C. M o r e n o, W. E. B r o w n, and G. O s b o r n, *Proc. Soil. Sci. Soc. Amer.* **24** (1960) 94.
10. J. J. P e t r e s, Gj. D e ž e l i ć, and B. T e ž a k, *Croat. Chem. Acta* **40** (1968) 213.

IZVOD

Nukleacija kalcium-fosfata iz otopina fiziološkog pH

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Određene su granice taloženja kalcium- fosfata iz vodenih otopina na 25⁰C (srednji $pH = 7,26$) i 37⁰ C (srednji $pH = 7,38$), te iz otopine 0,15 M natrium klorida na 37⁰ C (srednji $pH = 7,46$). Iz eksperimentalnih podataka izračunati su aktiviteti slobodnih Ca^{2+} i $H_2PO_4^-$ iona na granici taloženja i prikazani u smislu jednadžbe[1]:

$$\log a_{Ca^{++}} a_{OH}^{-2} = -(m/n) \log a_{H^+} a_{H_2PO_4^-} + \text{konstanta}$$

Nagib pravca opisanog ovom jednadžbom — (m/n) daje odnos fosfata prema kalciumu u metastabilnoj krutoj fazi koja se inicijalno formira prilikom taloženja. Statističkom obradom podataka dobiveni su pravci sa slijedećim nagibima i pripadnim standardnim devijacijama:

I. (vodena otopina, 37⁰ C) $b = -1.045$, $s_b = 0.037$
II. (0.15 M NaCl, 37⁰ C) $b = -0.957$, $s_b = 0.030$
III. (vodena otopina, 25⁰ C) $b = -1.024$, $s_b = 0.055$ i
IV. (I + II) $b = -1.006$, $s_b = 0.025$.

Prema tome se može zaključiti, da se prilikom taloženja pod ovim uvjetima inicijalno formira dikalcium-fosfat ($CaHPO_4$), koji u daljnjim fazama taloženja prelazi u bazičnije kalcium-fosfate.