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Factors Affecting the Precipitation of Calcium Phosphate *in vitro*

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The precipitation of calcium phosphate *in vitro* was studied in order to define the processes of homogeneous and heterogeneous nucleation and to study the effect of certain inhibitors of crystallization on these processes. It was shown that the solubility-determining surface phase of the calcium phosphate formed by homogeneous nucleation had a Ca/P molar ratio close to that of octacalcium phosphate (OCP) and that the most constant formation product in solution at the onset of spontaneous precipitation was that of OCP. Known inhibitors of crystallization had no effect on the formation product of homogeneous nucleation. Below the formation product of spontaneous precipitation nucleation was essentially heterogeneous and was markedly influenced by adding nucleating material to the system, by increasing the time of incubation, and by adding inhibitors of crystallization containing the grouping P-X-P (where X = O, C, or N). The level of supersaturation at the point of maximum inhibition was close to the formation product of spontaneous precipitation of calcium phospate. The implications of these observations are discussed in relation to the precipitation of calcium salts in urine.

Key words: Crystallization — Calcium — Phosphate — Pyrophosphate — Phosphonates.

La précipitation du phosphate de calcium *in vitro* est étudiée dans le but de définir les processus de nucléation homogène et hétérogène et de tester l'effet de certains inhibiteurs de cristallisation dans ces processus. La phase de surface, déterminant la solubilité du phosphate de calcium, formé par nucléation homogène, a un rapport molaire Ca/P proche de celui du phosphate octocalcique (OCP) et le produit le plus constant formé, en solution au début de la précipitation spontanée, est l'OCP. Des inhibiteurs connus de cristallisation n'ont aucun effet sur le produit de formation de la nucléation homogène. Au dessous du produit de formation de précipitation spontanée, la nucléation est essentiellement hétérogène et est fortement influencée par adjonction de substances facilitant la nucléation, par augmentation du temps d'incubation et par adjonction d'inhibiteurs de cristallisation contenant le groupement P-X-P (ou X = O, C ou N). Le taux de supersaturation au point d'inhibition maximum est proche du produit de formation de la précipitation spontanée du phosphate de calcium. Les conséquences de ces observations sont envisagées en fonction de la précipitation de sels calciques dans l'urine.

Die Fällung von Calciumphosphat *in vitro* wurde studiert, um die Prozesse der homogenen und der heterogenen Nukleation zu bestimmen und um die Wirkung bestimmter Kristallisationshemmer auf diese Prozesse zu untersuchen. Es wurde nachgewiesen, daß die Löslichkeits-bestimmende Oberflächenphase des Calciumphosphats, welches durch homogene Nukleation gebildet wurde, ein molares Ca/P-Verhältnis aufwies, das demjenigen von Octocalciumphosphat (OCP) sehr ähnlich war und daß das konstanteste Bildungsprodukt in der Lösung zu Beginn der spontanen Fällung dasjenige von OCP war. Bekannte Kristallisationshemmer hatten keine Wirkung auf das Bildungsprodukt homogener Nukleation. Unterhalb dem Bildungsprodukt der spontanen Fällung war die Nukleation vorwiegend heterogen und wurde

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stark beeinflußt durch: Zufügen von nukleierendem Material zum System; Erhöhung der Inkubationszeit; und Zufügen von Kristallisationshemmern, welche die P-X-P-Gruppierung enthielten (wobei X = O, C oder N). Die Höhe der Übersättigung bei maximaler Hemmung war dem Bildungsprodukt der spontanen Fällung von Calciumphosphat sehr ähnlich. Die Bedeutung dieser Beobachtungen wird in bezug auf die Fällung von Calciumsalzen im Urin diskutiert.

Introduction

During the last 20 years much has been written about the precipitation of "calcium phosphates" from solutions in the physiological range of pH [3, 4, 6, 14, 17, 22–24, 38, 41]. Although there is no concensus as to the precise stoichiometry of the salt first precipitated from solution, it is clear that whatever its stoichiometry it is not the most stable salt under the experimental conditions employed and probably undergoes a spontaneous conversion to a more stable form [4, 6, 13, 16, 23]. The initial solid phase has been claimed to have the stoichiometry of dicalcium phosphate (DCP) [17, 23, 38], octacalcium phosphate (OCP) [4, 18, 20], and tricalcium phosphate (TCP) [25, 41]. More recently it has been suggested that calcium phosphate precipitates in an "amorphous" form without any fixed stoichiometry and that a gradual recrystallization takes place to hydroxyapatite, which is thermodynamically the most stable salt under physiological conditions [5, 6].

Apart from the problem of the stoichiometry of the nucleating species there are many other interesting aspects of the crystallization of calcium phosphates from inorganic solutions. In particular the precise role played by inhibitors of crystallization in systems involving calcium phosphate and the conditions under which these inhibitors act are only loosely understood. Many such inhibitors, particularly compounds containing a P-X-P grouping (where X = O, N or C) have been identified and have been shown to affect the precipitation [9, 10, 29], maturation [11, 15, 29], growth [12] and dissolution [29, 35] of calcium phosphate *in vitro*.

The objects of this paper are to define some of the factors involved in the crystallization of calcium phosphate in simple inorganic solutions; to determine the formation product of calcium phosphate under various conditions of pH and concentrations of calcium and phosphate; and to define the conditions under which the crystallization of calcium phosphate is influenced by inhibitors of crystallisation containing the grouping P-X-P (where X = O, N or C).

Materials and Methods

Spontaneous Precipitation of Calcium Phosphate at High Levels of Supersaturation

A series of experiments was carried out to determine the formation product of spontaneous precipitation (upper limit of metastability) of calcium phosphate at 25°. The method of study was a development of the precipitation technique of Nordin [25], involving titration with alkali over a period of 20 min of solutions containing various concentrations of calcium (0.4–4.0 mmoles/l) and phosphate (0.8–10.0 mmoles/l) in physiological saline weakly buffered at pH 6 with sodium cacodylate (1 mmole/l). The pH of the solution was monitored continuously and the onset of precipitation detected by light-scattering using a modified nephelometer coupled to a sensitive galvanometer (Evans Electroselenium Ltd., Halstead, Essex). The technique is described in detail elsewhere [30]. All solutions used in these experiments were filtered through a Millipore membrane (0.45 μm pore size) before use.

From the concentrations of calcium and phosphate and the pH of the solution at the point of onset of precipitation the activity products of dicalcium phosphate ($(a_{Ca^{2+}}) \times (a_{HPO_4^{2-}})$), octacalcium phosphate ($(a_{Ca^{2+}})^4 \times (a_{H^+}) \times (a_{PO_4^{3-}})^3$), tricalcium phosphate ($(a_{Ca^{2+}})^3 \times (a_{PO_4^{3-}})^2$) and hydroxyapatite ($(a_{Ca^{2+}})^5 \times (a_{OH^-}) \times (a_{PO_4^{3-}})^3$) were calculated using a computer program [26, 28]. The activity products of calcium hydroxide ($(a_{Ca^{2+}}) \times (a_{OH^-})^2$) and phosphoric acid ($(a_{H^+})^3 \times (a_{PO_4^{3-}})$) were also calculated for each solution at the point of onset of precipitation and the regression coefficient of the line obtained by plotting $-\log_{10}$ ($(a_{H^+})^3 \times (a_{PO_4^{3-}})$) against $-\log_{10}$ ($(a_{Ca^{2+}}) \times (a_{OH^-})^2$) determined. The modulus of the regression coefficient is equal to the calcium/phosphorus molar ratio of the salt governing the nucleation process at the point of observation [2, 17, 20].

Similar precipitation studies were carried out in solutions of calcium phosphate to test the effect of an inhibitor of crystallization on the formation product of spontaneous precipitation of calcium phosphate. Sodium pyrophosphate was added to solutions containing calcium chloride (3.2 mmoles/l), sodium phosphate (2.4 mmoles/l), potassium chloride (130 mmoles/l) and weakly buffered at pH 6 with sodium barbital (5 mmoles/l), to give pyrophosphate concentrations between 1 and 10 μmoles/l which is the range over which pyrophosphate has previously been found to be active [7]. The formation product of spontaneous precipitation was measured as described above.

A third series of experiments was carried out to measure the formation product of calcium phosphate in solutions containing heterogeneous nucleating material in the form of crystallites of calcium oxalate. Calcium oxalate crystallites were prepared by quickly mixing equal volumes of solutions of calcium chloride (1.25 mmoles/l) and sodium oxalate (1.25 mmoles/l) and diluting the resultant crystal suspension ten times with sodium barbital buffer (5 mmoles/l) at pH 6. Increasing volumes of this stock suspension were added to solutions containing calcium chloride (3.2 mmoles/l), sodium phosphate (2.4 mmoles/l), potassium chloride (130 mmoles/l) and buffered at pH 6 with sodium barbital (5 mmoles/l), and the formation product measured as described above.

Precipitation of Calcium Phosphate at Levels of Supersaturation below the Point of Spontaneous Precipitation

The effect of time of incubation on the formation product of calcium phosphate was tested using the incubation technique devised by Solomons and Neuman [37] and used extensively by Fleisch and his associates [7, 10, 29]. With this technique the formation products of calcium phosphate solutions of various concentrations were determined at times of incubation extending from 1—320 h.

The effect of increasing concentrations of various inhibitors [sodium pyrophosphate (PP_i), sodium imidodiphosphate (IDP), sodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) and sodium dichloromethylene diphosphonate (Cl_2MDP)] on the formation products of calcium phosphate after 72 h incubation were calculated in terms of OCP from the data of Fleisch and his coworkers ([7, 10, 29], and H. Fleisch, personal communication) using a computer program [26, 28].

Nucleation Induced by Crystals of Hydroxyapatite at low Levels of Supersaturation

Precipitation of calcium phosphate induced by seed crystals of hydroxyapatite was measured in solutions of low supersaturation with respect to calcium phosphate using the particle-sizing and counting technique previously described for the study of inhibitors on the growth and aggregation of calcium oxalate crystals *in vitro* [32]. Particle size distribution between 4 and 30 μm were measured using a Model B Coulter Counter with a Model M Volume Converter attachment [27]. Hydroxyapatite crystals (Merck, A.G., Darmstadt, Germany) were suspended in a solution containing calcium chloride (2 mmoles/l), sodium phosphate (3 mmoles/l), sodium chloride (150 mmoles/l) and buffered at pH 7.4 with sodium barbital (20 mmoles/l). This solution was metastable with respect to OCP ($-\log_{10}$ activity product = 45.3) i.e. not sufficiently supersaturated to cause spontaneous precipitation of new crystals in the absence of seed crystals within the time period of the experiment. Induced nucleation of crystals of calcium phosphate was measured in the absence and presence of various concentrations of EHDP (0.01, 0.1, 1.0, 10 and 100 μmoles/l).

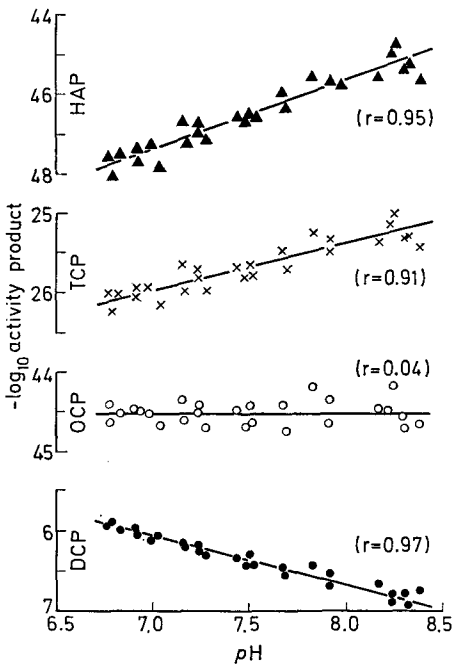

Fig. 1

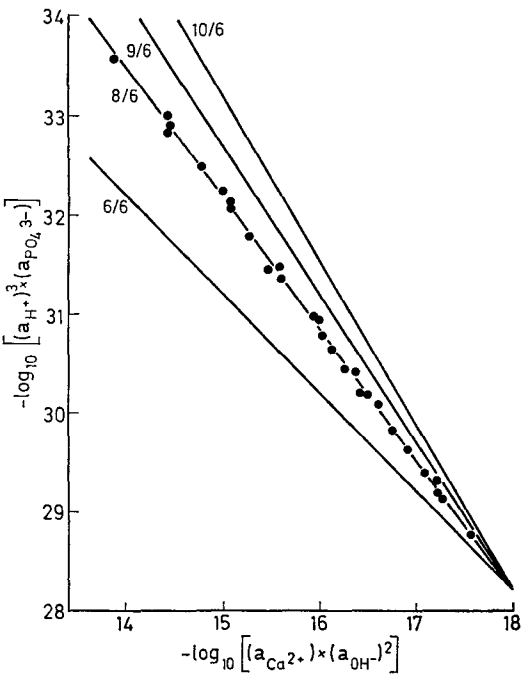

Fig. 2

Fig. 1. The relationships between the activity products ($-\log_{10}$) of dicalcium phosphate (*DCP*), octacalcium phosphate (*OCP*), tricalcium phosphate (*TCP*) and hydroxyapatite (*HAP*) and the pH at the onset of spontaneous precipitation in metastable solutions of calcium phosphate

Fig. 2. The relationship between the activity products ($-\log_{10}$) of phosphoric acid and calcium hydroxide at the onset of spontaneous precipitation in metastable solutions of calcium phosphate. Also shown are lines whose slopes correspond to the Ca/P molar ratios of DCP (6/6), OCP (8/6), TCP (9/6), and HAP (10/6)

Results

Spontaneous Precipitation of Calcium Phosphate at High Levels of Supersaturation

The results of 28 experiments to measure the formation product of spontaneous precipitation of calcium phosphate at various initial concentrations of calcium and phosphate are shown in Fig. 1. The formation products are expressed in terms of $-\log_{10}$ of the activity products of dicalcium phosphate (DCP), octacalcium phosphate (OCP), tricalcium phosphate (TCP) and hydroxyapatite (HAP) and are shown in relation to the pH of the solution at the point of onset of precipitation. The most constant product over the pH range studied is that of OCP, suggesting that at the point when precipitation is first detected by light-scattering the solubility-determining surface phase has the stoichiometry of octacalcium phosphate. The mean ($\pm$ 1 S.E.M.) formation product in terms of OCP is 44.50 ± 0.03. This compares with a solubility product of 47.63, calculated from the data of Moreno *et al.* [21] using the computer program described above.

Table 1. The effect of pyrophosphate (PP_i) on the formation product of spontaneous precipitation of calcium phosphate

Concentration of PP_i (μmoles/l)	n	$-\log_{10}$ (OCP formation product) (mean ± 1 S.E.M.)
0	28	44.50 ± 0.03
1	4	44.54 ± 0.04
3	4	44.55 ± 0.04
10	4	44.53 ± 0.03
100	2	47.20 ± 0.10

The relationship between the activity products of phosphoric acid and calcium hydroxide (expressed as $-\log_{10}$) at the onset of precipitation is shown in Fig. 2. The modulus of the regression coefficient is 1.323 or 7.94/6 corresponding closely to the Ca/P molar ratio of OCP. This confirms that at the point when precipitation is detected by light-scattering the nucleating phase has the stoichiometry of OCP. All subsequent measurements of formation products have therefore been expressed in terms of OCP.

The results of the experiments to study the effects of various concentrations of pyrophosphate on the formation product of spontaneous precipitation are given in Table 1. This shows that in the concentration range 1–10 μmoles/l pyrophosphate has no significant effect on the spontaneous precipitation of calcium phosphate at high levels of supersaturation. At higher concentrations of pyrophosphate (100 μmoles/l) precipitation of calcium phosphate was *enhanced*, probably because of prior precipitation of the insoluble calcium pyrophosphate.

The values of the OCP formation products measured in the presence of increasing amounts of two separate suspensions of calcium oxalate crystals are given in Fig. 3. One suspension was allowed to age for 2–3 h before use, the other was a freshly prepared suspension of crystals. Fig. 3 shows that the calcium oxalate crystals act as heterogeneous nuclei to lower the formation product of OCP at high levels of supersaturation and that the crystals from the freshly prepared suspension are more active than those from the aged suspension. The formation product (FP) of spontaneous precipitation in the absence of heterogeneous nuclei and the solubility product (SP) of OCP are shown for comparison.

Precipitation of Calcium Phosphate at Levels of Supersaturation below the Point of Spontaneous Precipitation

The effect of the time of incubation on the formation product of calcium phosphate is shown in Fig. 4. This shows that the longer the time of incubation the lower the level of supersaturation required to cause precipitation within that time. As the incubation time is decreased to 1 h, however, the formation product reaches a maximum value close to the formation product of spontaneous precipitation (FP) already described. Also shown in Fig. 4 is the formation product observed at 72 h by Fleisch and Bisaz [7] recalculated in terms of OCP using the computer program [26, 28].

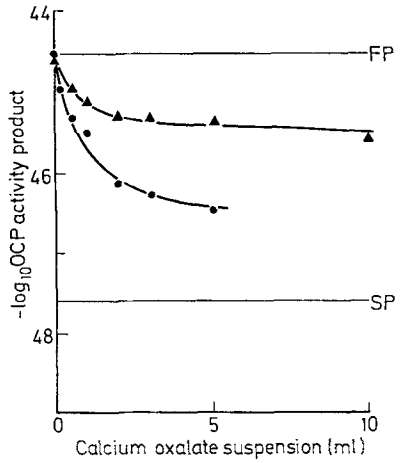

Fig. 3

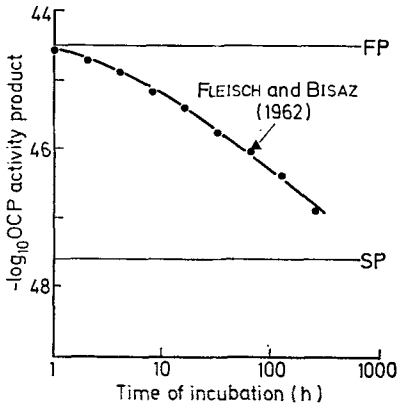

Fig. 4

Fig. 3. The effect of adding increasing amounts of an aged suspension (▲) and a fresh suspension (●) of calcium oxalate crystals on the formation product of calcium phosphate. The aged suspension was allowed to mature for 2–3 h before use. The results are shown in relation to the formation produced of spontaneous precipitation (*FP*) and the solubility product (*SP*) of *OCP*

Fig. 4. The effects of time of incubation on the formation product of calcium phosphate. Also shown is the formation product at 72 h calculated from the data of Fleisch and Bisaz (1962)

The effects of various concentrations of PP_i, IDP, EHDP and Cl_2MDP on the 72-h formation product calculated in terms of OCP are presented in Fig. 5. The data are taken from the work of Fleisch and his coworkers [11, 15, 29] and are shown in relation to the formation product of spontaneous precipitation (FP) and the solubility product (SP) of OCP. Fig. 5 shows that all of the compounds studied inhibited the precipitation of calcium phosphate i.e. increased the level of supersaturation required to cause calcium phosphate to precipitate within 72 h. Moreover there appears to be a maximum concentration for each inhibitor above which no further increase in inhibitory activity occurs. The level of supersaturation at this point is very close to the formation product of spontaneous precipitation of calcium phosphate.

Heterogeneous Nucleation Induced by Crystals of Hydroxyapatite at low Levels of Supersaturation

The increase in the volume of calcium phosphate crystals and aggregates in the standard metastable solution of calcium phosphate ([EHDP] = 0) after adding nucleating crystals of hydroxyapatite is shown in Fig. 6 in relation to the time of incubation. This shows that appreciable induced nucleation and growth of new crystals took place within the 4-h incubation period.

Also shown in Fig. 6 are the effects of adding various concentrations of EHDP to the metastable solution prior to adding the nucleating crystals of hydroxy-

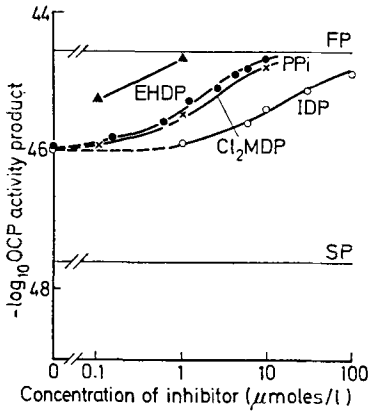

Fig. 5

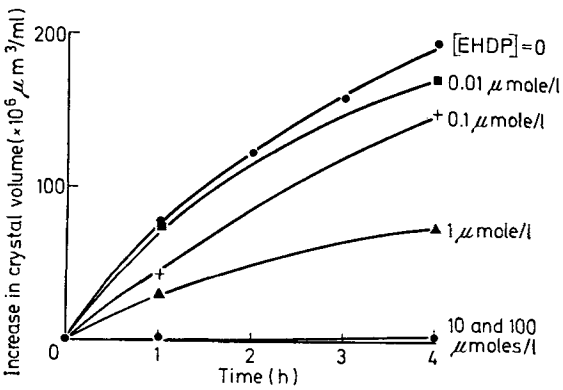

Fig. 6

Fig. 5. The effect of various concentrations of pyrophosphate (PP_i), imidodiphosphate (IDP), ethane-1-hydroxy-1,1-diphosphonate ($EHDP$) and dichloromethylene diphosphonate (Cl_2MDP) on the 72-h formation product of calcium phosphate (taken from the data in references [7, 10, 29]

Fig. 6. The increase in the volume of calcium phosphate crystals over 4 h following the addition of hydroxyapatite crystals to a metastable solution of calcium phosphate in the absence and presence of various concentrations of EHDP

apatite. At concentrations of 10 and 100 μmoles/l EHDP completely inhibited induced nucleation of new crystals of calcium phosphate. At lower concentrations the inhibitory effect of EHDP decreased until at 0.01 μmoles/l the induced nucleation was almost the same as that in the absence of EHDP.

Discussion

The nature and stoichiometry of the salt first precipitated from a given supersaturated solution of calcium phosphate has remained a problem to chemists and biologists for many years. Much of the confusion has arisen because of an incomplete understanding of the many variables involved in the processes of nucleation, growth and maturation which lead to the formation of a crystal of "calcium phosphate". Most workers are agreed, however, that the salt first precipitated is not the most stable one under the experimental conditions employed and that a conversion to a more stable form subsequently takes place [4, 6, 13, 16, 23]. While the system is in this intermediate state of instability the Ca/P ratio of the growing embryo may be changing rapidly [16]. Moreover, the composition of the solubility-determining surface phase of the embryo may be quite different from that of its interior [23]. Thus any measurements made will depend firstly, on the stage of crystallinity reached by the growing embryo at the time of observation and secondly, on whether measurements are made on the composition of the whole embryo or of its surface, or of the solution phase in contact with the surface.

From this study it would appear that during the rapid growth phase of the spontaneous precipitation of calcium phosphate in the pH range 6.7–8.4 the solubility-determining surface phase has the stoichiometry of octacalcium phosphate. It does not necessarily follow, however, that the original critical embryo also had the stoichiometry of OCP since subsequent hydrolysis and maturation of the critical embryo may have proceeded some way before the embryo was large enough to be detected by the light-scattering technique employed in this study. It would be necessary to make measurements at shorter times using a more sensitive technique to establish the nature and composition of the critical nucleus. Conversely, at longer time periods the surface stoichiometry may change even further and this may explain why observations made at 24 h have suggested a higher solubility-determining Ca/P ratio of 9/6 [41].

The constancy of the OCP activity product at the onset of spontaneous precipitation of calcium phosphate over a wide range of pH values and concentrations of calcium and phosphate suggests that there is under the conditions of the study a "constant" upper limit of metastability for solutions containing calcium and phosphate at and above which spontaneous precipitation of calcium phosphate will occur. This constant we have defined as the formation product of spontaneous precipitation and may be thought of as the point at which homogeneous nucleation commences in the system. Between the formation and solubility products lies the metastable zone where nucleation is predominantly heterogeneous [40]. Thus the observed formation product of a metastable solution of calcium phosphate may be lowered from the value corresponding to the commencement of homogeneous nucleation by adding heterogeneous nuclei in the form of crystals of calcium oxalate (Fig. 3). The latter would be expected to nucleate growth of calcium phosphate by epitaxy [19]. Similarly it has been shown that addition of hydroxyapatite crystals or collagen to metastable solutions of calcium phosphate will lower the 1 day and 10 day formation products [9, 12].

Formation products lower than that of spontaneous precipitation are also observed when metastable solutions of calcium phosphate are incubated for time periods longer than 20 min (Fig. 4). At supersaturation levels less than the formation product of spontaneous precipitation, nucleation is predominantly heterogeneous [40] and probably takes place on active sites on the walls of the vessel. The longer incubation times are necessary to allow the slower process of heterogeneous nucleation to take place.

Turning to the effect of inhibitors of crystallisation on the nucleation of calcium phosphate precipitation it is clear that pyrophosphate and its structurally related analogues inhibit all the processes of crystallisation which take place within the metastable region of supersaturation viz. heterogeneous nucleation on glass (Fig. 5), on Formvar grids [36] and on sheep bone collagen [1]; induced nucleation (Fig. 6); and crystal growth [12]. The amount of inhibition produced by each inhibitor increased according to the concentration of inhibitor added but there appears to be a maximum concentration of inhibitor above which no further increase in activity is observed. In the case of heterogeneous nucleation, maximum inhibition is reached when the supersaturation is close to the formation product of spontaneous precipitation. At supersaturations at or higher than the formation product of spontaneous precipitation, however, inhibitors have no effect on the

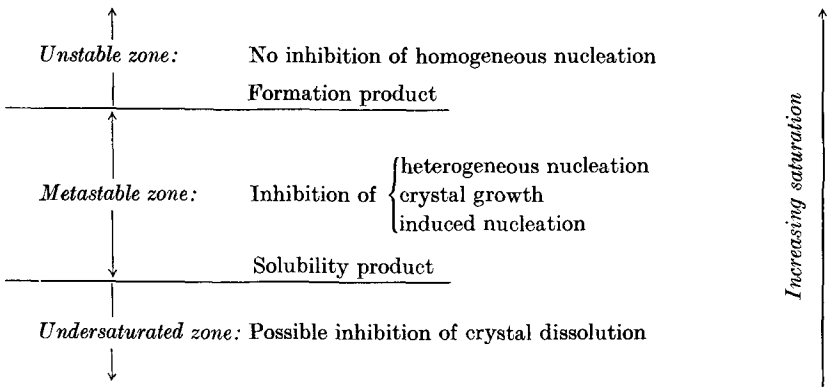

Fig. 7. A summary of the effects of inhibitors on the crystallisation of calcium phosphate at different levels of saturation

process of nucleation of calcium phosphate from solution (Table 1). This is confirmed by the titration studies of Francis and others [15, 16, 29] who showed that the precipitation of calcium phosphate occurs in two stages from solutions which exceed the formation product of spontaneous precipitation. Firstly there is spontaneous precipitation of amorphous or microcrystalline calcium phosphate and this is followed by conversion to crystalline hydroxyapatite. Pyrophosphate [13], EHDP [15], and IDP [29] have all been shown to have no effect on the first step, i.e. homogeneous nucleation, but they markedly inhibit the conversion to crystalline hydroxyapatite. The mechanism by which these inhibitors block the process of maturation probably involves adsorption of the inhibitor on to the surface of the microcrystalline calcium phosphate thereby increasing the activation energy barrier to the recrystallisation processes involved in the conversion to crystalline hydroxyapatite [39]. Similarly adsorption of the inhibitor on to active nucleation and growth sites is probably the important first stage in preventing heterogeneous nucleation, induced nucleation, growth and aggregation of crystals and may also account for the inhibitory effect of pyrophosphate [35], EHDP [35] and IDP [29] on the kinetics of dissolution of hydroxyapatite crystals coated with these ions. In the case of homogeneous nucleation, however, the rate of nucleation is so fast [40] that a large and expanding surface with a high surface energy is being formed in a short space of time. It is possible that either there is insufficient inhibitor to cover the whole surface, or the rate of adsorption is too slow, or the energy of the highly supersaturated system is greater than the activation energy required to overcome the adsorption barrier set up by the inhibitor.

A summary of the effects of inhibitors on the crystallisation of calcium phosphate and of the regions of saturation over which they operate is shown in Fig. 7.

The above observations are relevant to the precipitation of calcium salts in biological fluids, particularly in urine. Since urine can cover a wide range of saturation levels from undersaturation to oversaturation in the unstable zone [33] and since urine is known to contain inhibitors such as pyrophosphate [8], then the relationship between the degree of saturation, the concentration of inhibitors, the time period during which urine remains in the kidney, and

the presence or absence of heterogeneous nucleating material will determine whether crystal nucleation and growth will take place in any given urine. Since the time during which urine is in contact with the kidney is small then in the absence of heterogeneous nuclei urine containing no inhibitors at all could theoretically survive at supersaturations up to the formation product of spontaneous precipitation without precipitation taking place. In fact fresh urines in the metastable region from stone-formers and normal subjects have been shown to contain no crystals of calcium phosphate [28]. However, calcium phosphate crystalluria was observed in all urines which exceeded the formation product of spontaneous precipitation and the amount of crystalluria increased in relation to the degree of oversaturation [28]. Thus the inhibitors present in urine have no effect on the formation product of spontaneous precipitation [31], as was found for pyrophosphate in simple inorganic solutions (Table 1). The importance, if any, of the inhibitors in urine must therefore lie in their ability to block heterogeneous nucleation, for instance on crystals of other salts, cell debris or renal epithelium, or to modify the growth and aggregation of crystals produced by homogeneous nucleation. This would prevent the formation of crystals and aggregates large enough to become trapped at some narrow part of the urinary tract within the time period that the urine is in contact with it. There is now evidence that urine does indeed contain inhibitor(s) of the growth and aggregation of calcium oxalate [32] and of hydroxyapatite (Robertson: in preparation) crystals *in vitro*, and that urines from recurrent stone-formers are less able to inhibit the growth and aggregation of calcium oxalate crystals *in vitro* than urines from normal subjects [32]. It has also been found that recurrent stone-formers pass larger crystals and aggregates of calcium oxalate [32, 34] and of calcium phosphate (Robertson; in preparation) in their urines than do their controls. These observations strongly suggest that urines of recurrent stone-formers may be deficient in some inhibitor which in normal urine modifies the growth and aggregation of calcium salts and thereby allows small particles to be passed harmlessly when the supersaturation of urine exceeds the formation product of spontaneous precipitation. Work is at present under way to identify the inhibitor(s) responsible for the difference in inhibitory activity between urines from recurrent stone-formers and normal subjects.

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