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Threefold Effect of an Organic Molecule in Biomineralization*

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Glutamic acid either in the monomeric or the polymeric form, even at very low concentration has a strong effect on the crystallization of calcium oxalate. It was shown to retard precipitation, to enhance nucleation and serve as a mediator starting epitaxial growth on foreign seed crystals.

The interaction between glutamic acid and calcium oxalate is due to dimensional compatibility and formation of electrostatic and hydrogen bonds between the functional groups of the organic acid and ions of the inorganic salt. The outcoming effect or combined effects depend upon the concentration ratio of the constituents and the physical conditions in the system.

GENERAL

Organic molecules are intimately involved in formation of mineral phases in living organisms. Some of the molecules serve as initiators and templates for the growth of vertebrate skeleton bones, and mollusk shells. Other are known as inhibitors of growth in kidney and gall stones. The distinction between inhibitors and promoters is not clear cut in any complex biological environment. For instance the amino acids which were shown to be effective retardants and inhibitors to calcium oxalate and phosphate precipitations are nevertheless abundantly found in kidney stones and therefore may be considered as initiators. These findings started the prolonged fundamental controversy between the scientists favoring the concept of the organic matrix acting as an initiator to kidney stones formation and those who believe in the spontaneous formation of the mineral phase and subsequent adsorption and coverage by the organic matter.

The organic molecules which interact with inorganic crystalline phase are characterized by having active functional groups, periodically spaced. The most effective ones are those in which the distances between the, say, acidic groups match the distances between the cationic sites on one of the crystallographic faces of the inorganic crystal. A graphic example of the effectiveness of structural compatibility was presented in a simulation of sea water desalination. Whereas polyvinyl sulfonate retarded the formation

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of calcium sulfate hemihydrate scale, it was hardly effective toward calcium sulfate dihydrate. On the other hand, the glutamic acid retarded the formation of the dihydrate but not of the hemihydrate.[1] Recently this approach has been put to advantage for finding the best inhibitors for the various industrial scales by computer modelling.[2]

In the kidney stone inhibitor/promoter controversy the nucleating and inhibiting agents were not exactly identified due to the wide range of possible candidates.

However *in vitro* studies are concerned with comparatively simple systems of mineral salt/organic molecule couples. Thus it was found that a specific macromolecule which was found to retard precipitation may act also as a nucleating agent. Polyvinyl sulfonate which modifies the crystal habit of calcium sulfate hemihydrate and inhibits its deposition, acts as a nucleating agent for strontium sulfate. This could be deduced from the number and sizes of the crystallites precipitated with varying amounts of the organic additive, under controlled conditions.[3]

In this lecture the interaction between glutamic acid, in its monomeric and polymeric forms, and between calcium oxalate was presented. The aim of this presentation was to demonstrate that glutamic acid may act either as nucleator-initiator of calcium oxalate precipitation or as retardant-inhibitor or finally as a mediator in epitaxial growth. Whichever of these functions is dominant depends upon the characteristic features of the investigated system. It seems that ionized glutamic acid present in a system in which calcium crystals are being precipitated will associate with the forming phase changing the kinetics and the dynamics of the process, in whatever capacity is expedient.

INITIATOR

The functional and aesthetically pleasing design of mollusk shells could not have been accomplished if the composing calcium carbonate crystallites, which build up the structure, were not characterized by uniform shapes and sizes and special orientation of the crystallographic axes. The precise allignment of the crystallites and their consistently modified habits indicate that they were formed under controlled conditions. The concept of an array of organic matrix macromolecules serving as templates was proposed to explain this phenomenon.[4] Many of these compounds may be specifically synthesized for this purpose.[5]

The initiating-nucleating capacity of polyglutamic acid with respect to calcium oxalate was demonstrated through *in vitro* study, using MSMPR continuous crystallizer.[6] In this experimental set-up, clear unseeded solutions of calcium chloride and sodium oxalate are simultaneously fed into the crystallizer and kept there with stirring for predetermined periods. The crystals in the outflow slurry are analyzed for crystal size distribution. The computational processing according the »Population Balance«[7] yields the nucleation and growth rates. The median or average size is measured directly. The total product may be calculated from the above data and the supersaturation in the outflow stream may be calculated from the overall mass balance. The

additives, in very low concentrations, are added with the oxalate stream. The experiments, with no additives are fairly reproducible.

Figure 1. shows the effect of polylysine, heparin and polyglutamic acid on the total mass (in mg/l) of calcium oxalate produced in the continuous MSMPR crystallizer, at steady state.[6] The mass produced with polylysine is not different from that produced in the control experiment. The effect of heparin, though measurable is not strong. The decrease caused by polyglutamic acid is drastic. This result helps to grade the organic molecules according to their activity toward calcium oxalate crystallization: only polyglutamic acid has periodically spaced carboxylic groups, thus having the best affinity to the mineral phase. The prominent feature that could be revealed only by the determination of nucleation rates is shown in Figure 2. The addition of 2 ppm polyglutamic acid increased the nucleation rate by a factor of 9. This enhancement of nucleation can be explained only by the nucleation of calcium oxalate on the polymeric molecules of glutamic acid.

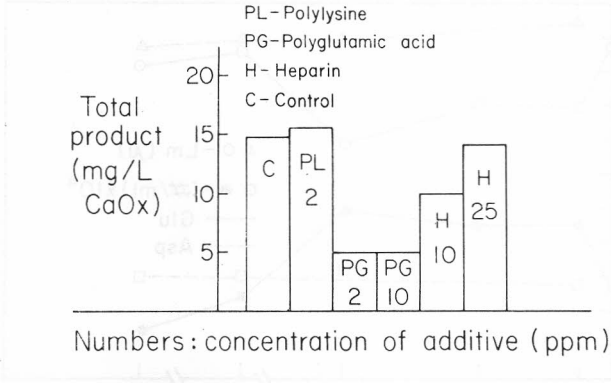

Figure 1. Total product in MSMPR crystallizer in the presence of additives.

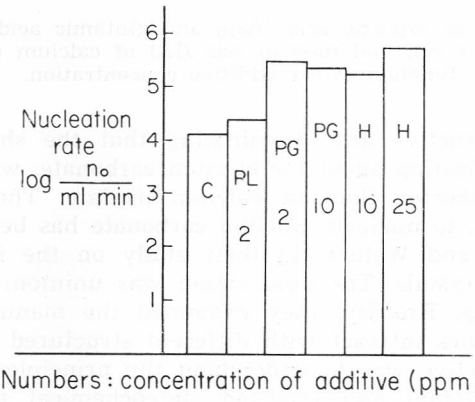

Figure 2. Nucleation rates of calcium oxalate in MSMPR crystallizer in the presence of additives. Designation as in Figure 1.

The number of crystallites per unit volume may also serve as an indicator to the number of nucleation events. Figure 3. shows the variation of the numbers of calcium oxalate crystals per ml in batch crystallization, with the admixture of monoglutamic acid. In this case the number of particles was directly counted. The numbers obtained with 2.5 to 20 ppm are larger by a factor of about 2.5 than those in the control experiments.[8] In this case also the increase of the particles numbers indicates heterogeneous nucleation in which the matching glutamic acid molecules serve as microsubstrates. The other bicarboxylic acid i. e. aspartic acid does not cause any increase in the particles numbers (Figure 3). Glutamic and aspartic acids have identical functional groups. The distance between the carboxylic groups in glutamic acid is larger than in aspartic acid. After calcium ions are attracted to the ionized carboxyls rendering the associate positively charged, there is space enough to accommodate an oxalate ion which may also form a hydrogen bond with the amino group, thus stabilizing the associate and starting a nucleus.

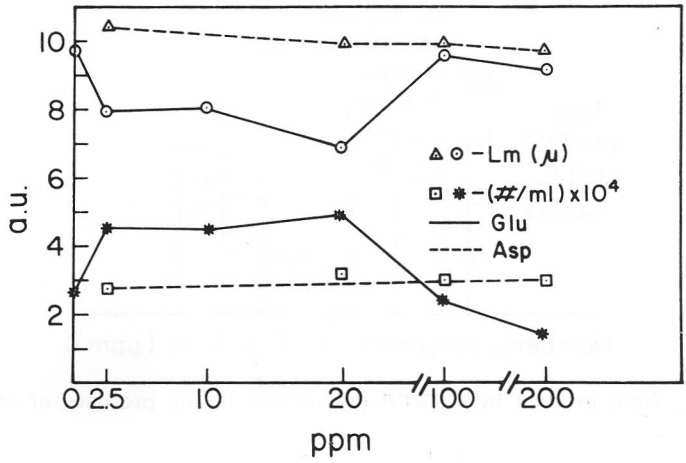

Figure 3. The effects of aspartic acid (Asp) and glutamic acid (Glu) monomers on the number density (#/ml) and median size (L_m) of calcium oxalate crystals as a function of the additive concentration.

It is very instructive and illuminating that the shorter aspartic acid may serve as a nucleating agent for calcium carbonate, where the intercationic distances are shorter than in calcium oxalate. The ability of acidic, aspartic rich protein to nucleate calcium carbonate has been recently demonstrated by Addadi and Weiner[9] in their study on the interaction between acid proteins and crystals. The observation was unintentional and therefore the more convincing. Broadly, they examined the manner in which acidic matrix macromolecules interact with different structured surfaces of various crystals. Their objective was to understand the principles that govern these interactions. They found and reported stereochemical properties that appeared to be an essential requirement for interaction to occur, namely that the carboxylate groups are oriented perpendicularly to the face they are

absorbing upon thus optimally completing the coordination polyhedron around the calcium ion.[9]

Calcite crystals grown without any additive develop the cleavage rhombohedral habit. Crystals grown in the presence of small amounts of protein still had rhombohedral morphology, but in addition developed very small (001) faces (Figure 4.). It was observed that thus affected crystals were actually attached to the bottom of the vial glass, with the small (001) face at the contact. As the relatively large calcite crystals were balanced precariously on a small inclined contact plane this phenomenon could not have been missed.

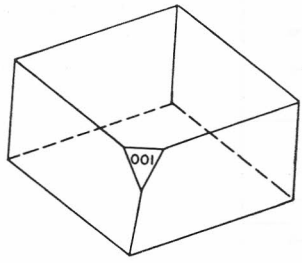

Figure 4. Rhombohedral crystal of calcite in which one of the (001) was developed. The small triangular (001) face was attached to the vial surface (Ref. 9).

The presence of the aspartic-rich protein on the (001) face was shown by indirect immunofluorescence staining. These experiments showed that the calcite crystals developed one of their (001) faces as a result of being nucleated on acidic proteins that had been previously adsorbed onto the container surface.[9]

It seems that the adsorption of a macromolecule on a solid surface and the resulting immobilization does not impair its capacity as an initiator and probably even enhances its nucleating effectiveness. The interaction between the organic initiator and the inorganic phase depends on fulfillment of several stereochemical requirements. The most important conditions for the existence of a specific interaction between the initiator and the mineral phase is the good correspondence of the distances between the functional groups of the nucleating agent and the cationic interdistances in the inorganic crystal. Thus glutamic acid enhanced the nucleation of calcium oxalate and the shorter aspartic acid was shown to act as an initiator for the growth of calcium carbonate crystals in which the distances between calcium ions are shorter than in calcium oxalate.

The specific involvement of glutamic acid in all the aspects of calcium oxalate crystallization is shown in Figure 5. All the singularly irregular modification modes of supersaturation levels, median sizes, growth and nucleation rates as the functions of glutamic acid concentrations are shown on the almost devoid of fluctuations background of aspartic acid.[10]

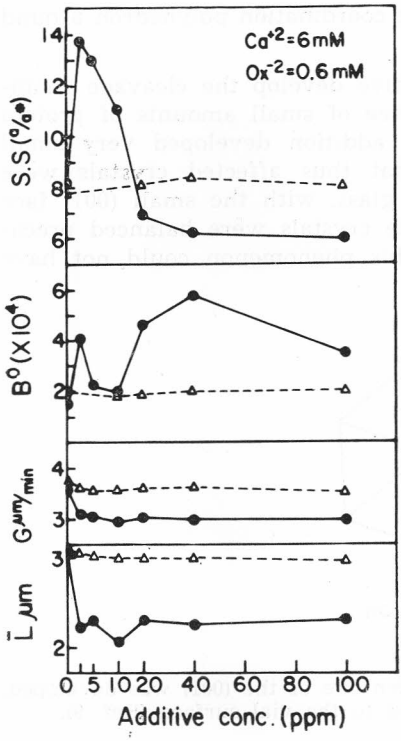

Figure 5. Parameters in MSMPR crystallizer, affected by additives. SS — supersaturation, B° — nucleation rate, G — growth rate, L — average size, Solid line — glutamic acid, Dashed line — aspartic acid.

The experimental system employed was the continuous MSMPR crystallizer.[10] In this set-up the organic molecule has unrestricted freedom of motion in an environment in which extremely fast and irreversible changes occur, due to the birth of solid particles in the whole bulk of the solution. This is quite different from the slow nucleation on immobilized large macromolecules adsorbed on the vials, as described in the calcite growth experiments, in which the number of mature crystals and correlated nucleation events for the whole volume was less than one hundred per experiment. In the steady state system with continuous nucleation events growth, agglomeration, expulsion of the crystallites with the outflow stream the organic molecules can and evidently do take part in each aspect of the process. They adsorb on already formed embryos and crystals, either desorbing or staying incorporated in the expelled crystals. Glutamic acid reduced the growth rate, the median size and affected the supersaturation in an irregular way (Figure 5.). Nevertheless, it markedly enhances the nucletation rate. Aspartic acid does not affect the supersaturation and the nucleation refuting the possibility that the mere presence of charged molecules can change the character of the process of solid phase formation. It is evident that besides all other interactions, glutamic acid molecules serve as nucleating catalysts i. e. initiators for calcium oxalate nucleation.

RETARDANT

The direct and convincing experiment evidence of retarding or inhibitory effect of a substance exerted on a precipitation process is to measure

RETARDANT

the ion activity in a solution from which a sparingly soluble salts of these ions is precipitating in the additive presence and to compare it to the undisturbed precipitation without the additive. Such comparison[11] is offered in Figure 6. The lower curve describes the decrease of calcium ion activity vs.

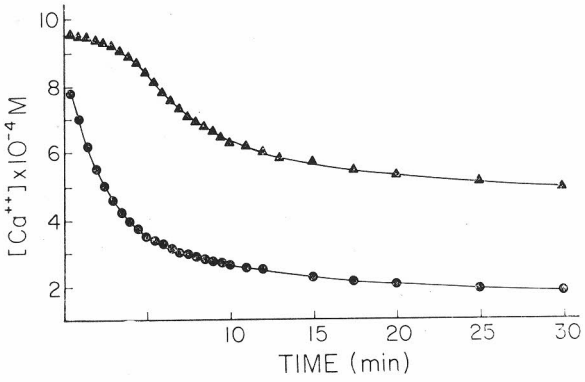

Figure 6. The decrease in the calcium ion concentration versus time in a system containing no inhibitor (●) and 100 ppm polyglutamic acid (▲).

time in a solution containing no inhibitor, as determined by a calcium selective electrode. The precipitation was effected by very rapid mixing of a solution having the composition of mimic urine and containing calcium ions with appropriate solution of oxalate ions. When 100 ppm of polyglutamic acid were present the curve of decrease was the upper one, unequivocally showing the inhibitory or retarding effect on growth, most probably through adsorption on the active growth sites of the calcium oxalate crystals.

The exclusive functioning as a retardant seems to be contradictory to the former strong advocating in favor of the nucleating function. Actually the external display of the function that glutamic or polyglutamic acid assumes in calcium oxalate crystallization depends on the concentrations of the materials. In Figure 7. we can discern that at the concentration of 100—200 ppm glutamic acid retards the precipitation of calcium oxalate, whereas at the low concentration of 2.5—5 ppm it accelerates the precipitation. Though acceleration explicitly implies heterogeneous nucleation, it does not mean that part of the molecules cannot act as retardants. It would rather indicate that after a certain part of the molecules was used up as templates the remaining free molecules adsorb on the nuclei and crystallites thus slowing

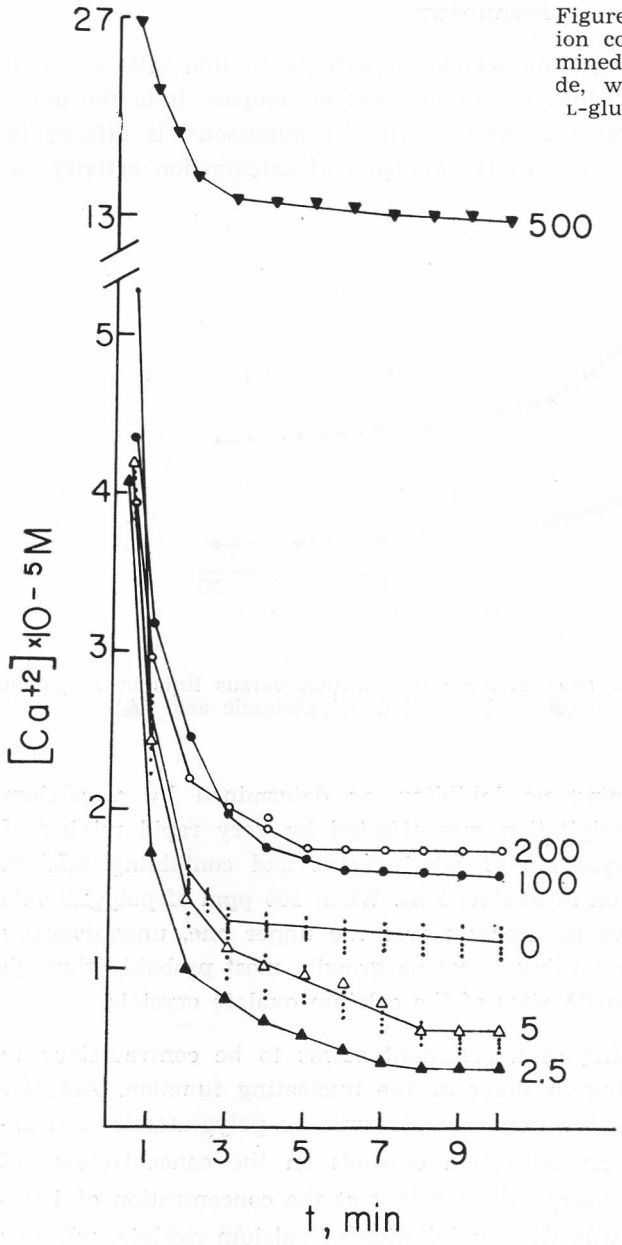

Figure 7. The decrease in calcium ion concentration with time, determined by calcium selective electrode, with variable concentration of L-glutamic acid monomer (ppm).

down the transfer of calcium ions from the solution to the solid phase. The switching between functions is implied by the measurements and comparison between the effects of 2.5 and 5 ppm. Evidently at 5 ppm concentration more molecules »escape« the nucleating function and thus are able to adsorb on growth sites, retarding ion transfer more effectively than at 2.5 ppm con-

centration. At 100 ppm the retarding effect is dominating reaching, at these experimental conditions about its maximum performance, as at 200 ppm it is not perceivably enhanced.[12]

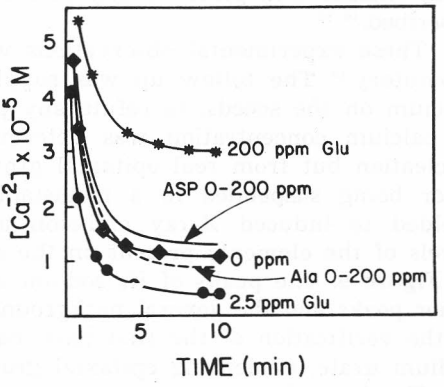

Figure 8. The effect of alanine (Ala), aspartic acid (ASP) and glutamic acid (Glu) on calcium oxalate precipitation as a function of the additive concentration.

Figure 8. shows that the monocarboxylic alaine and the short aspartic acid do not show marked retardation, the curves of calcium decrease in the precipitating systems lying close to the blank.

MEDIATOR

The most convincing way to show the multifunctionality of glutamic acid molecule is to find a clear cut evidence of its acting in an additional function. This has been accomplished by showing that glutamic acid may act as a mediator, allowing calcium oxalate to grow epitaxially on seeds of uric acid.[13]

Epitaxy consists of the growth of one crystal upon another with a near geometrical fit between the respective networks which are in contact. This involves interactions which must satisfy simultaneously the bonding requirements of the two different crystal structures. It is interesting to note that the nature of the chemical bonds in the bulks of the epitaxially grown participants may be quite different: metallic crystals grow on ionic ones and ionic crystals grow on organic ones which are held by a network of hydrogen bonds. The concept of epitaxis was used to explain the mechanism of formation of multicomponent renal calculi such as calcium oxalate stones on nidi of uric acid.[14,15]

However, *in vitro* experiments performed with appropriate care and capability[16–18] failed to demonstrate that calcium oxalate grows on uric acid seeds. The studies were conducted using metastable solutions of calcium oxalate and introducing seed crystals of uric acid and sodium urate. The metastable solutions without seeds did not nucleate spontaneously and thus for a prolonged period of about an hour no decrease in calcium ion concentration was detected. On the introduction of sodium urate seeds a measurable decrease in calcium ions concentration was encountered, indicating probable deposition of calcium ions on the seeds of sodium urate. This finding though interesting, was quite irrelevant wilth respect to calcium oxalate/uric acid stone formation. First, no sodium urate crystals have been ever found in

fresh warm human urine and extremely rarely in human kidney stones. Second, as uric acid seeds did not effect decrease in calcium ion concentration in metastable solutions *in vitro* most certainly no epitaxial growth of calcium oxalate took place on uric acid seeds under the experimental conditions described.[16-18]

These experimental observations were verified in a recent study in our laboratory.[13] The follow up was supplemented by checking the presence of calcium on the seeeds, to refute any possible criticism that when a decrease in calcium concentration was determined it did not result from separate nucleation but from real epitaxial growth on sodium urate seeds. The seeds, after being suspended in a metastable solution were separated, dried and yielded to induced X-ray emission analysis. The spectrum of the energy levels of the elements present on the surface of sodium urate seeds is shown in Figure 9. The peaks of K_α sodium and calcium are clearly delineated. The other peaks and the general background are emitted by the gold coating. This is the verification of the first part: calcium ions were deposited on seeds of sodium urate, evidencing epitaxial growth of calcium oxalate, upon them.

To show absence may require more convincing proof than to show presence. Therefore uric acid seeds were coated by carbon which has less interferring background radiation than gold. Figure 10. shows that even under

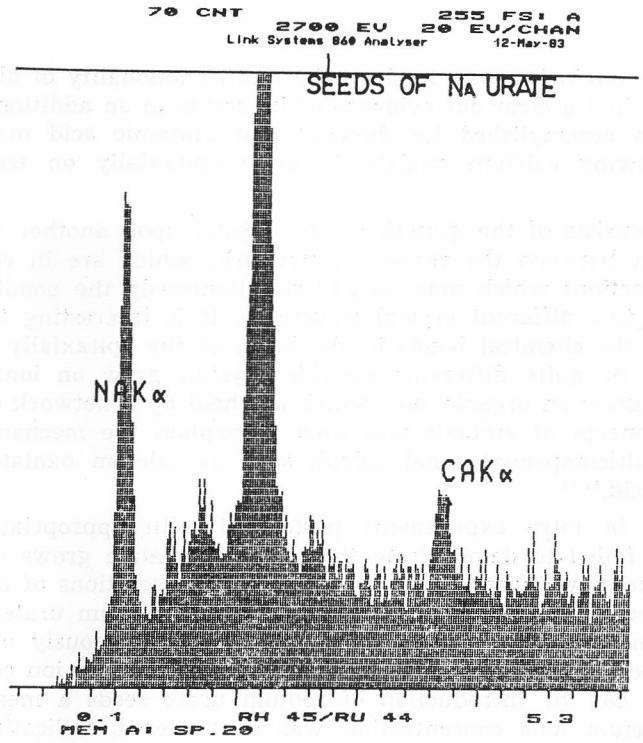

Figure 9. Energy spectrum of x-ray emission from gold coated surface of sodium urate seed contacted with a metastable calcium oxalate solution. Presence of a Ca peak.

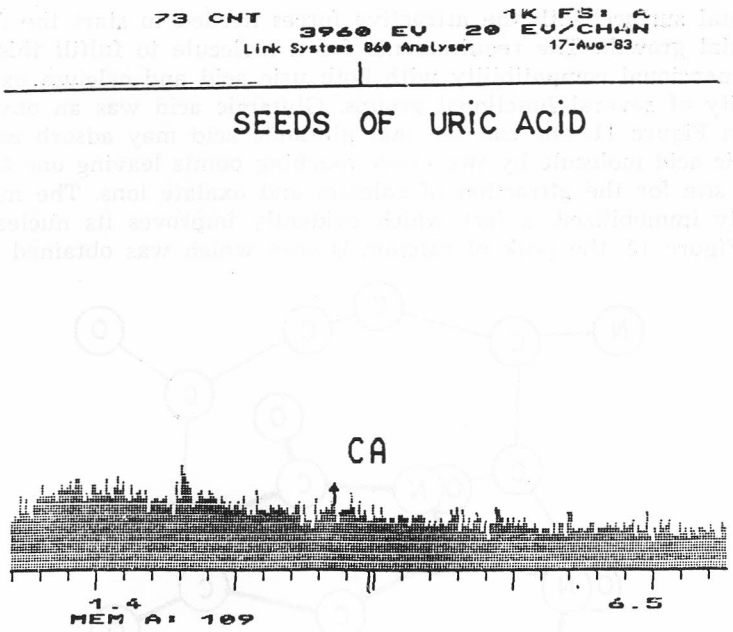

Figure 10. X-ray emission from the carbon coated surface of uric acid seeds contacted with a metastable solution of calcium oxalate. Absence of Ca peak.

these conditions no calcium was detected, in agreement with former conclusions[16-18] that uric acid does not induce growth of calcium oxalate.

When experimental simulation of a physiological system show results entirely contrary to objective existence, it may pay to examine whether the simulation is too far removed from reality. All the simulation systems[13,16-18] were extremely pure in comparison with the physiological medium. It was possible to examine likely candidates for serving as a mediator for starting the deposition of calcium oxalate on uric acid seeds. This was a situation which invited formation of a speculation or a hypothesis concerning the nature of epitaxial growth.

Epitaxy has been carefully phrased in terms of the geometrical fit of the contact surfaces,[14] the bond requirements[15] with fine details of preservation of the sphere of coordination for cationic species in the case of biomineralization.[9,15] These considerations cannot explain the extremely different potentials of uric acid and of sodium urate toward epitaxial growth of calcium oxalate upon them, as both comply with the theoretical requirements. If there is any slight difference, on purely theoretical ground, uric acid should perform even better than sodium urate. A presumption was formulated in our study that the electrostatic charges of urate and sodium ions attract calcium and oxalate ions from the solution. Because of the structural compatibility the attracted ions form a coherent layer of calcium oxalate. On the other hand there are not strong enough alternate negative and positive charges on the crystal faces of uric acid.

If the lack of the attractive forces inhibits the realization of the potential for epitaxial growth, it should be possible to equip artificially the uric

acid crystal surface with the attractive forces needed to start the first layer in epitaxial growth. The requirements for a molecule to fulfill this function were: dimensional compatibility with both uric acid and calcium oxalate and availability of several functional groups. Glutamic acid was an obvious candidate. In Figure 11. one can see that glutamic acid may adsorb easily on a single uric acid molecule by two *O—N* touching points leaving one *O* site and one NH_2 site for the attraction of calcium and oxalate ions. The molecule is practically immobilized, a fact which evidently improves its nucleating ability. In Figure 12. the peak of calcium is seen which was obtained by X-ray

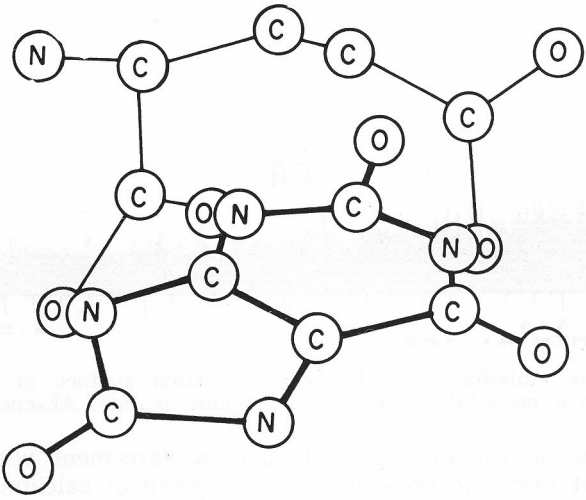

Figure 11. A model of glutamic acid molecule adsorbed on uric acid molecule.

emission from surface of an uric acid seed contacted with metastable calcium oxalate solution in the presence of 4 ppm glutamic acid.

Appropriate decrease of calcium ions concentration was measured in the »corrected« simulation system i. e. uric acid seeds in a metastable solution of calcium oxalate also containing traces of glutamic acid. The beauty of this »corrected« system is that it is much closer to the true rich and complex physiological system than the »pure« two component abstraction[13,16–18] which could not render results imitating reality.

The molecular model in Figure 11 may suggest that a molecule shorter than glutamic acid may also adsorb on uric acid. Following this hint aspartic acid was tried as the mediating agent. An effect of decrease in calcium concentration was observed, though weaker than in the case of glutamic acid. It should be noted that free aspartic acid did not affect the nucleation rate (Figure 5.). Thus it seems that the immobilization resulting from adsorption, improves the chances for the heterogeneous nucleation occurrence.

SUMMARY

Glutamic acid was shown to retard calcium oxalate precipitation as evidenced by the slowdown of calcium ions transfer from solution onto the crystals[8,11,12] by the decrease of the rate growth[10] and by the reduction of

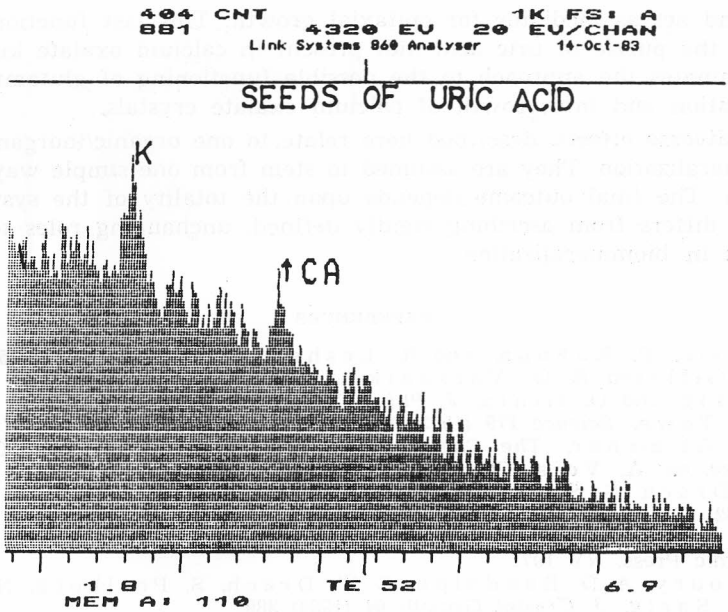

Figure 12. X-ray emission from uric acid seeds contacted with a metastable calcium oxalate solution in the presence of 4 ppm glutamic acid. Presence of a Ca peak.

the precipitated mass at steady state crystallization.[6] Another quite different function evidenced by various effects was enhancement of nucleation rate[6,10] and increase of the number of particles in a precipitate.[8] Aspartic acid was indifferent toward calcium oxalate as a promoter but showed excellent nucleating capacity toward calcite sustaining the theory of the structurally compatible amino acid serving as a template for the crystallization of the matching mineral. Calcium oxalate is unsuitable for such direct demonstration; as yet no crystals of the necessary size and habit have been grown in laboratory. Glutamic acid was shown to act as mediator necessary to induce epitaxial growth of calcium oxalate on seeds of uric acid. Though calcium oxalate and uric acid have a number of matching faces, the attempts to induce epitaxial growth in laboratory simulation failed[16–18] until traces of glutamic acid were added.[13,19] Glutamic acid serves as a glue between the organic and inorganic crystals, evidently adsorbing on the first and serving as a template for the crystallization of the second.

The interaction between glutamic acid in all the three functions described involves attraction between the negative carboxylic groups and calcium ions i. e. existence of an electrostatic bond and formation of a hydrogen bond between the amino group and the oxalate ion augmented by the attraction between them. The function performed by the organic molecule depends upon the conditions in the system. If the ions are free in solution glutamic acid will act as initiator of nucleation of the mineral phase, if solid particles are present it will adsorb on sterically suitable faces, retarding their growth and modifying habit. In more complex environment containing seeds of a different material it may adsorb through different dimensionally compatible

groups and act as mediator for epitaxial growth. This last function, besides resolving the puzzle of uric acid nidi present in calcium oxalate kidney stones, illuminates the approach to the possible functioning of glutamic acid in agglomeration and intergrowth of calcium oxalate crystals.

The diverse effects, described here relate to one organic/inorganic couple in biomineralization. They are assumed to stem from one simple way of bond formation. The final outcome depends upon the totality of the system. This approach differs from ascribing rigidly defined, unchanging roles to organic molecules in biomineralization.

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SAŽETAK

Trostruki utjecaj organskih molekula u biomineralizaciji

S. Sarig

Bez obzira na to nalazi li se u obliku monomera ili polimera, pa i pri vrlo niskim koncentracijama, glutaminska kiselina ima jak utjecaj na kristalizaciju kalcij-oksalata. Pokazano je da ona usporava taloženje, ubrzava nukleaciju, a služi i kao posrednik pri epitaksijskom rastu izazvanomu prisutnošću stranih kristala (sjemena).

Interakcije glutaminske kiseline i kalcij-oksalata posljedica su kompatibilnosti dimenzija, te nastajanja elektrostatskih i vodikovih veza među funkcionalnim skupinama organske kiseline i iona anorganskih soli. Konačni efekt ili kombinirani efekti ovise o koncentracijskom omjeru konstituenata i fizikalnim uvjetima u sustavu.