Effect of Amino Acids, Magnesium Ions and Hydroxyapatite on the Formation of Oxalate Nephroliths

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Abstract

Processes of crystallization of calcium oxalate monohydrate from aqueous solutions and the effect of amino acids on these processes were investigated. It is shown that amino acids inhibit the growth of crystals of the compounds under investigation; the inhibiting action of an amino acid depends on its structure and increases with an increase in its concentration. The inhibiting effect on the crystallization of calcium oxalate monohydrate is also produced by magnesium ions in the concentration corresponding to the physiological solution (urine). It is demonstrated that the presence of hydroxyapatite crystals in solution initiates the crystallization of calcium oxalate monohydrate.

Keywords: nephroliths, calcium oxalate monohydrate, urinary components, dispersion analysis

INTRODUCTION

Investigation of the processes of crystallization of low-soluble compounds is important in connection with the problem of pathogenic mineral formation in living organisms. It has been established by present that pathogenic minerals may be formed in many human tissues and organs [1].

Among pathogenic formations, the most widespread calculi are those of the urinary system [1–6]. Analysis of the available data shows that the major part of nephroliths in the citizens of different regions is composed of calcium oxalates [5], represented by whewellite ($CaC_2O_4 \cdot H_2O$) and weddellite ($CaC_2O_4 \cdot 2H_2O$) with the predominance of the former. To understand the mechanisms of lithogenesis, it is necessary to know the regularities of crystallization of calcium oxalate, first of all whewellite.

For the investigation of the crystallization of calcium oxalate monohydrate, it is important to establish the effect of organic and inorganic admixtures – inalienable constituents of the physiological solution – on the formation of this compound [9].

The goal of the investigation was to study the processes involved in the crystallization of calcium oxalate monohydrate in the presence of amino acids and manganese ions in model solutions close in composition to the physiological liquids.

One of the information-rich methods of studying the crystallization of substances is dispersion analysis allowing one to obtain the size distribution of crystals, which id directly connected with the parameters determining the mechanisms of nucleation and growth of crystals [7, 8].

On the basis of the results obtained, it is possible to reveal the factors that significantly affect the character of the processes leading to the formation of pathogenic minerals, and to predict the features of the system's behaviour during variation of one or another parameter of the medium. This will allow more efficient elaboration of the measures of prophylactics, treatment and prevention of relapses of nephrolithiasis.

EXPERIMENTAL

The conditions for the experiments on the crystallization of calcium oxalate monohydrate

were chosen on the basis of the properties of the natural crystal-forming medium (urine) [3, 6].

Supersaturation with respect to uevellite was achieved by mixing the initial solutions of readily soluble compounds at a stoichiometric ratio according to the reaction

$CaCl_2 + (NH_4)_2C_2O_4 \rightarrow CaC_2O_4\downarrow + 2NH_4Cl$

Mixing was performed in a crystallizing vessel. The rates of the reactions of this kind are usually high, which causes sharp supersaturation of the solution and the appearance of fine crystalline precipitate. The X-ray phase analysis of the crystallization products showed that only calcium oxalate monohydrate was obtained in all the experiments.

Crystallization process was studied mainly at one value of supersaturation of the solution of calcium oxalate: $\gamma = C_0/C_s = 7$ (C_0 is the concentration of calcium and oxalate ions in the supersaturated solution, C_s is the solubility of calcium oxalate monohydrate, which is equal to $0.7 \cdot 10^{-4}$ mol/L). This value of γ was chosen because it is this supersaturation value that characterizes biological media, including the urine of a healthy average statistical person [11]. To obtain more complete information, additional experiments were made for γ = 12, 15, 20, 25.

Crystallization process was studied both in the solutions of pure calcium oxalate and in its solutions with the addition of magnesium ions and amino acids (asparaginic $HOOCCH(NH_2)CH_2COOH$, glutamic $HOOCCH(NH_2)CH_2CH_2COOH$, alanine $CH_3CH(NH_2)COOH$, glycine $CH_2(NH_2)COOH$ and proline $-CH(COOH)CH_2CH_2CH_2)-NH$) within the concentration range $10^{-6}-10^{-2}$ mol/L. These compounds were chosen because a definite set of amino acids is incorporated into the composition of nephroliths according to the data of analysis [12]; glutamic acid and glycine dominate. It is necessary to stress that the chosen amino acids take part in the majority of metabolic biochemical processes in human organism.

Variance analysis of the synthesized solid products was carried out using the diffraction analyse of particle size Shimadzu SALD-2101 (Laser Diffraction Particle Size Analyser) in Institute of Hydrocarbons Processing, SB RAS (Omsk). The advantages of this method include the following characteristics: simplicity of preparation and measuring procedure; the possibility to carry out measurements under the constant ionic force and pH of solution; the possibility to record the curves of the size distribution of crystals containing the information about the parameters of nucleation and crystal growth processes, the possibility to study crystallization dynamics. Using the software of SALD-2101 instrument, we may give a structural geometric representation of the primary numeric and graphical data as the distribution of light intensity depending on the particle size of the sample under investigation. This allows us to obtain the differential curves of the volume distribution of particles over their sizes and the volume mean sizes. To obtain the reliable results, the analysis of the samples was carried out 4-5 times; he relative standard deviation was $S_r = 0.04-0.05$.

Dispersion analysis of the product was performed at the stage of depletion of supersaturation with respect to calcium oxalate monohydrate; the duration of this stage was determined by means of conductometric analysis to be 40 min [13].

RESULTS AND DISCUSSION

The curves of the size distribution of calcium oxalate monohydrate crystals are shown in Fig. 1. One can see that the distribution obtained without introducing any additives is characterized by large dispersion and strong asymmetry. It is known that the following stages proceed sequentially during crystallization: the formation of the solid phase nuclei (nucleation), crystal growth, aggregation of the formed crystals, and further growth of the aggregates; secondary nucleation is also possible. As a result of these processes, crystal growth occurs simultaneously on the crystal centres of different age and size, which is the reason of the polymodal character of the size distribution curves [14].

In the case if amino acids participate in crystallization, the average size of the formed crystals of calcium oxalate monohydrate decreases, and the distribution becomes much more narrow (dispersion decreases). This effect is exhibited in the clearest manner for high amino acid concentrations (see Fig. 1). An increase in the concentration of asparaginic and glutamic amino acids to 10^{-2} mol/L leads to com-

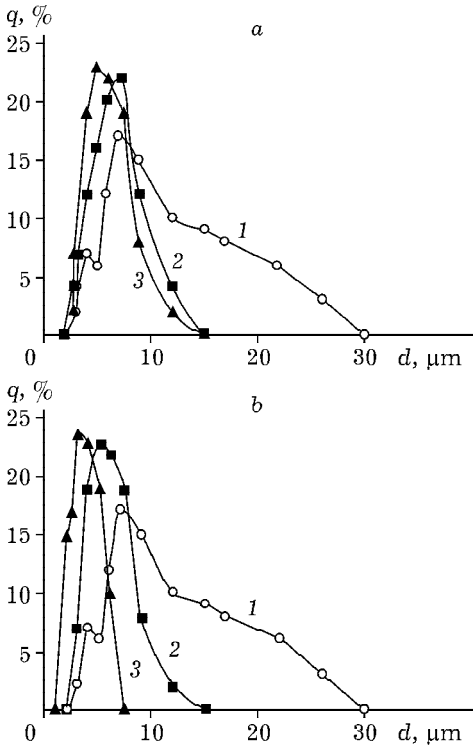

Fig. 1. Size distribution of calcium oxalate monohydrate crystals ($\gamma = 7$): *a* – crystallization in twice distilled water (*1*), in the solutions with $C_{glu} = 10^{-6}$ (*2*) and 10^{-3} mol/L (3); *b* – crystallization in twice distilled water (*1*), in the solutions with $C_{asp} = 10^{-5}$ mol/L (*2*) and $C_{glu} = 10^{-5}$ mol/L (*3*).

plete braking of crystallization at $\gamma = 7$, so a decrease in the average size of the crystals may be explained by an increase in the solubility of calcium oxalate and corresponding decrease in supersaturation. However, calculations involving the instability constants of the complexes Ca–Asp and Ca–Glu [15] show that for the concentrations of amino acids equal to 10^{-5} and 10^{-6} mol/L an increase in solubility is negligible. For the maximal concentration 10^{-2} mol/L used in the experiments, the solubility increases only by a factor of 1.2, which is incomparable with the supersaturation value used, which is $\gamma = 7$. So, changes in solubility cannot serve as the reason of such a sharp change in the character of crystallization of calcium oxalate under the action of amino acids.

Therefore, asparaginic and glutamic amino acids have an inhibiting action on the growth of calcium oxalate crystals; glutamic acid has a stronger inhibiting action (see Fig. 1, *b*).

Experiments at higher supersaturation values showed that crystallization of calcium oxalate monohydrate in the solution of glutamic acid with the concentration $C = 10^{-2}$ mol/L occurs only at $\gamma \geq 10$. This again demonstrates a strong concentration dependence of the inhibiting action of amino acids.

To study the effect of the structure of an amino acid on the degree of braking the growth of the crystals of calcium oxalate monohydrate, we performed experiments on the crystallization of calcium oxalate in the presence of alanine, proline and glycine (Table 1). One can see that the degree of inhibition of crystal growth increases with an increase in the concentration of amino acid and depends on the structure of the acid.

The inhibiting action of amino acids is connected with their adsorption on the active centres of the surface of the formed crystals. According to the data shown in Table 1, the maximal inhibiting action is exhibited by glutamic acid. An increase in the length of the hydrocarbon substituent by one CH_2 group causes an increase in the surface activity by a factor of about 2–3 [15]. In addition, the inhibiting

TABLE 1

Average size of calcium oxalate crystals with amino acid additives

Amino acid	Size, μm	
	Concentration, mol/L	
	10^{-5}	10^{-2}
Glutamic acid	3.88±0.09	<0.03
Asparagic acid	5.08±0.06	<0.03
Glycine	6.11±0.07	3.95±0.05
Alanine	5.52±0.04	4.32±0.06
Proline	5.30±0.07	4.73±0.04

Note. The size of calcium oxalate crystals without additives is (10.31±0.07) μm.

action increases with an increase in the number of carboxylic groups in the acid. It is known that amino acids exist in four forms in aqueous solutions: a conjugated acid, a conjugated base, a neutral molecule, and a bipolar form (zwitter ions); they are able to get sorbed either on positive or negative centres of the crystal surface. It should also be noted that within a broad pH range zwitter ions give different conformation, and for some of these forms the interaction of amino groups and carboxylic groups with the crystal surface is more favourable form the point of view of energy [10, 17].

It may be assumed that the interaction between the positively charged surface of calcium oxalate and the amino acid in the most probable conformation occurs. The authors of [10] assume that at the initial stage the interaction of the uncharged amino acid with the positively charged surface of calcium oxalate crystal occurs, and this may lead to binding the carboxyl group with calcium ion and, as a consequence, to deprotonation of the second carboxylic group and its interaction with another calcium ion. Additional stabilization of the formed surface compounds may be due to the ability of amino acids with two or more carboxylic groups to form polydentate chelate complexes with calcium ions. So, the internal complexation, protonated state of the amino acid and stereochemical factors play an essential part in the adsorption of amino acids on the surface of calcium oxalate crystals.

Amino acids able to get strongly adsorbed on the surface of calcium oxalate monohydrate crystals inhibit the crystallization of calcium oxalate itself. Amino acids may be arranged in the following sequence according to the increasing inhibiting action of the crystallization of calcium oxalate: glycine < alanine < proline < asparaginic acid < glutamic acid.

A strong affinity of dicarboxy groups to calcium oxalate monohydrate is the evidence that the proteins rich in these amino acids will play the functional part in the pathogenic formation of nephroliths; their role is double. On the one hand, poisoning the growth of calcium oxalate monohydrate crystals due to adsorption on its surface, proteins should suppress lithogenesis. On the other hand, proteins adsorbing calcium ions from solution may serve as nucleation centres and thus stimulate lithogenesis.

Analysis of the literature data shows that the crystallization of calcium oxalate is affected, in addition to amino acids, by the presence of magnesium ions in solution [18]. Experiments performed by us in order to study the role of magnesium in the crystallization of calcium oxalate monohydrate actually revealed strong deceleration of crystallization in the presence of magnesium ions. After adding magnesium ions ($C = 10^{-3}$ mol/L) to the solution ($\gamma = 7$), the average size of calcium oxalate crystals decreases by a factor of 2.5, that is, the growth of crystals slows down very strongly. An increase in the concentration of magnesium ions to 10^{-2} mol/L (this value corresponds to the physiological concentration) leads to the situation when the crystallization of calcium oxalate at this supersaturation level does not occur at all. The formation of calcium oxalate monohydrate at the physiological concentration of magnesium ions in solution starts only when supersaturation increases to $\gamma = 20$.

It is necessary to stress that the presence of magnesium ions in solution has a substantial inhibiting action on the crystallization of calcium oxalate monohydrate.

In addition to soluble admixtures, the crystallization of calcium oxalate may be affected also by solid particles of other phases that are present in the physiological solution and take part in lithogenesis. According to the data of electron microscopy and local microprobe analysis, almost all the monomineral oxalate calculi contain an admixture of poorly crystallized apatite [5].

We performed experiments to study the crystallization of calcium oxalate monohydrate in the presence of hydroxyapatite $Ca_5(PO_4)_3OH$. It was established (see Table 1) that the crystallization of calcium oxalate monohydrate does not occur at $\gamma = 7$ in the solution of glutamic acid with the concentration 10^{-2} mol/L. However, according to the data of dispersion analysis, after the introduction of hydroxyapatite crystals (d_{av}= (5.29±0.06) μm) crystallization of calcium oxalate monohydrate occurs; the average size of the particles of the solid phase increases to (9.14±0.29) μm. Hydroxy-

apatite is almost insoluble, so an increase in particle size is connected with the formation of growth of the crystals of calcium oxalate monohydrate.

So, hydroxyapatite crystals in solution initiate the crystallization of calcium oxalate monohydrate. This fact confirms the assumption that hydroxyapatite crystals may act as the nuclei of heterogeneous nucleation of calcium oxalate monohydrate [13]. It is known [10, 20] that mixed nuclei (calcium phosphates + organic matrix) are distinguished in oxalate calculi. Therefore, it may be assumed that the formation and growth of the crystals of calcium oxalate monohydrate occur in the presence of hydroxyapatite crystals even in the case of the high concentrations of amino acids.

CONCLUSIONS

1. It was established that amino acids inhibit the growth of calcium oxalate monohydrate crystals decreasing the average size of the crystals. he inhibiting effect of an amino acid depends on its structure and increases with an increase in the number of carboxylic groups in the acid and with an increase in its concentration.

2. It was discovered that magnesium ions at the concentration corresponding to the physiological solution (urine) also have an inhibiting effect on the crystallization of calcium oxalate by increasing the critical supersaturation of the start of crystallization.

3. It was discovered that the presence of hydroxyapatite crystals in solution initiates the crystallization of calcium oxalate.

The results obtained may become the basis for further research aimed at the establishment of the role of proteins and hydroxyapatite in the formation of nephroliths and potential development of synthetic peptides for use in therapy of nephrolithiasis.

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