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Solubility, inhibited growth and dissolution kinetics of calcium oxalate crystals in solutions, containing hippuric acid

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Summary. An analysis of crystal growth and dissolution of slightly soluble salts in physiological solutions in the presence of complexing ions was carried out, simulating conditions typical in human urine. It was found that hippuric acid, a normal physiological constituent of urine, acts at increased concentrations as a dissolving agent with respect to calcium oxalate (CaOx) and CaOx calculi. The kinetics of dissolution of crystalline CaOx calculi in physiological solutions containing hippuric acid at different concentrations were studied, using the change in the Archimedean weight of samples immersed in the solution. Analysis of the experimental results enabled the determination of the increased solubility of CaOx in the presence of hippuric acid and the quantitative characterization of this substance as a new and promising agent for dissolving CaOx calculi in human urine. The possible effect of hippuric acid as a natural regulator of CaOx supersaturation and crtystallization in human and mammalian urine is also discussed.

Key words: Solubility – Urinary calculi – Hippuric acid – Kinetics of dissolution – Calcium oxalate calculi

Formation and growth of renal calculi has been the subject of many investigations; a summary and critical appraisal of their results may be found in a series of monographs [10, 13] and review articles [9, 14]. To date the efforts of most investigarots have been concentrated on the primary physicochemical events in stone formation in human urine. Of even greater practical importance could be the study of the dissolution of calculi already formed in the urinary tract. As yet there have been positive results in this respect only for urate concrements [11]. Their solubility increases dramatically as a result of a change in the pH of the solution, and this is a way of controlling the supersaturation in urine or of changing it to undersaturation.

The solubility of one kind of renal stones – those formed from the various hydrates of calcium oxalate (CaOx) – has been the subject of many investigations. Unfortunately, the alteration of pH within physiologically tolerable limits changes the solubility of CaOx only slightly [9]. Dissolution of CaOx calculi with complexon III (Na-EDTA, well known from analytical chemistry) is possible in clinical practice only by the difficult technique of direct instrumental haemolysis [16] (i.e. by the direct introduction of Na-EDTA into the urinary tract).

It has been evident since Hammarsten's classic studies [9] (see also [14]) that many ions, such as Mg^{2+}, citrate or HPO_4^{2-}, which are normally present in urine increase the solubility of CaOx in aqueous solutions by forming complexes with either the Ca^{2+} or the $C_2O_4^{2-}$ ions. However, the oral administration of these complexing agents does not lead to encouraging clinical results, as they are metabolized in the organism.

The possible inhibiting influence of various oxy-acids [7] or amino acids [6] on the CaOx growth process in urine has also often been discussed in the literature. According to these investigations, amino acids have a measurable inhibiting effect on the growth of CaOx crystals even when present in minimal concentrations corresponding to their physiological norm in human urine.

In the present study the effect of a simple amino acid not used in previous investigations – the so-called hippuric acid ($C_6H_5CONHCH_2COOH$) – on the formation, inhibition of growth and especially the dissolution of CaOx crystals and calculi is investigated in detail. Hippuric acid is present in human urine in considerable concentrations (according to [4, 8] the excretion is about 0.5–2.5 g/l, corresponding at normal diuresis to a concentration of 2–10 mmol/l), surpassing the urinary concentration of all other amino acids. It is also known that hippuric acid is present in enhanced concentration in the urine of herbivorous mammals; for example, in the urine of the horse (from whence derives its name) it reaches concentrations up to 15 g/l.

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Hippuric acid is a weak complexing agent with respect to calcium; according to Davies [4] it forms a relatively unstable chelate complex with Ca^{2+} in pure water. We found [8], however, that in physiological solutions resembling artificial human urine in their composition, the complexing effect of hippuric acid with respect to Ca^{2+} is unexpectedly increased to up to 100 times its complexing ability in pure water. The possible clinical importance of these results is all the more abvious when the simple possibility of increasing the hippuric acid concentraton in urinex is taken into account (see Discussion).

These preliminary results and the possible biological significance of hippuric acid provided the impetus for a thorough examination of the kinetics of CaOx concrement dissolution in physiological solutions containing various concentrations of hippuric acid. In a second series fo experiments the inhibiting effect of differing concentrations of hippuric acid on the formation of CaOx precipitates was investigated.

Materials and methods

Basic theoretical considerations

Human urine is a complicated physiological solution. In developing the necessary formalism for analysing the kinetics of growth and dissolution of CaOx crystals in such a solution, the following have to be taken into account:

1. The concentration of CaOx in any aqueous solution is so small that it can be considered as an ideal solution. Thus the supersaturation $\Delta\mu$ at a temperature T can be expressed as [8]:

$$\Delta\mu^{+} = kT \cdot \ln (C^*/C_0) \tag{1}$$

where C^* and C_0 denote the actual and equilibrium concentrations of CaOx. For the process of dissolution ($\Delta\mu^{-} < 0$) we have to write

$$\Delta\mu^{-} = kT \cdot \ln (C_0/C^*) \tag{1a}$$

2. In calculating the solubility S and the supersaturation of CaOx in urine according to Eq. 1, we have to take into consideration also that:

a) CaOx falls into the group of strong electrolytes.

b) The ionic strength ψ of urine (and thus the ionic activity coefficient γ of CaOx in it) is determined by ions (Na^{+}, K^{+}, Cl^{-}, etc.) the concentrations of which are one or two orders of magnitude higher than those of Ca^{2+} and $C_2O_4^{2-}$. Thus ψ and γ of urine have (independently of the actual concentration of CaOx in it) a nearly constant value [8] (typically $\psi = 0.3$ and $\gamma = 0.1$).

c) Thermodynamically important concentrations of ion complexing agents (such as Mg^{2+}, citrate ions, hippuric acid) are normally present in urine.

d) Ca^{2+} is usually present in great excess with respect to the concentration of $C_2O_4^{2-}$ in urine.

Taking into account these four specific features of CaOx as a solute and of urine as a solvent, the solubility S of CaOx at temperature T can be calculated according to procedures normally used in analytical chemistry [8] in the following way:

$$S = f(1/\gamma) L_{P_0} \alpha_a \alpha_b \tag{2}$$

where L_{P_0} is the solubility product of CaOx in pure water (at the same temperature T) and the α factors are determined from the concentrations C_L, C_J and solubility constants K_L, K_J of Ca^{2+} or $C_2O_4^{2-}$ binding ions in the investigated biological solution as

$$\alpha_a = 1 + \Sigma C_L \, \Sigma C_L K_L \tag{3a}$$

for Ca^{2-}-binding complexing cations and

$$\alpha_b = 1 + \Sigma C_J K_J \tag{3b}$$

for $C_2O_4^{2-}$-binding complexing anions.

In this way, as discussed in more detail in [8], simple formulas can be obtained describing the effect of complexing agents present in the solution at various concentrations on supersaturation, solubility and growth velocity of CaOx crystals growing or dissolving in a solution resembling human urine. It can be shown that if we introduce an increasing concentration C_H (e.g. hippuric acid) of Ca^{2+}-binding complexing agent having a solubility constant K_H into the solution, a linear dependence between the solubility S_H and C_H for $Ca \gg Ox$ will be predicted by

$$S_H = S(1 + K_H C_H/\alpha_c') \tag{4}$$

where α_c' is the α factor in the absence of the complexing agent H (hippuric acid).

Thus, the dependence of the supersaturation $\Delta\mu$ on C_H for the case $C_{Ca} \gg C_{Ox}$ is

$$\Delta\mu \simeq \Delta\mu_0 - \tfrac{1}{2} \ln (K_H C_H/\alpha_c') \tag{5}$$

where $\Delta\mu_0$ is the supersaturation without hippuric acid.

It is also of interest that in the case of the dissolution of CaOx concrements in the presence of a fixed initial concentration of CaOx (or – which in the case of $Ca^{2+} \gg C_2O_4^{2-}$ is the same – in the presence of constant concentration C_0^* of oxalic anions) we have to rewrite Eq. 4 as follows:

$$S_H = S(1 + K_H C_H/\alpha_c') - C_0^* \tag{6}$$

Thus a plot of S_H vs C_H should result in a straight line with a slope of $-S \cdot K_H/\alpha_c'$, cutting from the ordinate axis a segment $S_H(0) = S - C_0^*$. In this way, both S and K_H can be determined at a known value of α. According to data in Robertson et al. [15], α_c' for human urine is approximately 3.

Thus, depending on the concentration C_H, i.e. on the sign of $\Delta\mu$ (i.e. $\Delta\mu > 0$ during growth, $\Delta\mu < 0$ during dissolution), growth or dissolution of CaOx concrements can be achieved simply by changing the concentration C_H.

Let us also consider the inhibitory effect of an increasing concentration of hippuric acid on the overall precipitation kinetics, when a concentration N_0 of CaOx seed crystals is introduced into a metastable solution having an initial concentration C_0^* with respect of CaOx. If the growth rate in the solution is g, the overall volume of CaOx precipitated at the time t will be

$$V = N_0 (gt)^3 \tag{7}$$

Assuming a normal mode of crystal growth, we have to expect $g \sim \Delta\mu$. Thus the concentration of CaOx precipitated at time t will be given by

$$[M_0 - M(t)]^{1/3} \simeq \text{const}_1 [1 - \text{const}_2 (K_H C_H/\alpha_c')t] \tag{8}$$

This equation is used here for analysing the kinetics of inhibited precipitation (see also Fig. 5).

Experimental details

Two types of experiments were performed: (1) on the kinetics of dissolution of CaOx calculi in physiological solutions containing

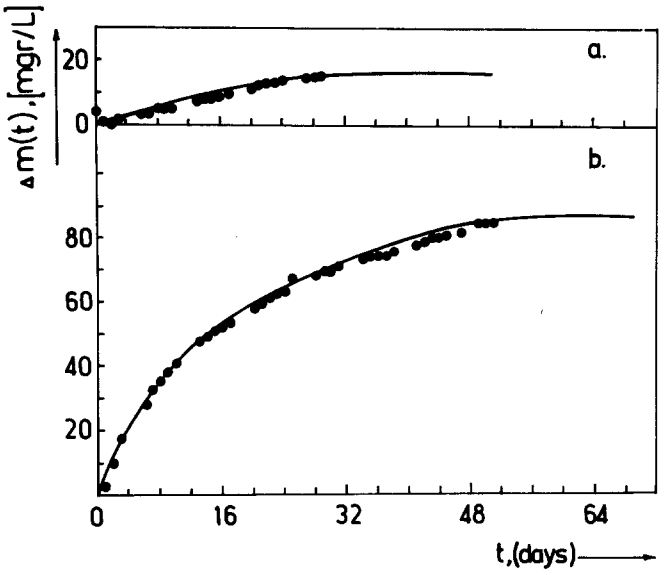

Fig. 1a, b. Weight change in the dissolution of calcium oxalate concrements in artificial urine with zero supersaturation. *a* Solution without hippuric acid; *b* solution with 15 mmol/l hippuric acid

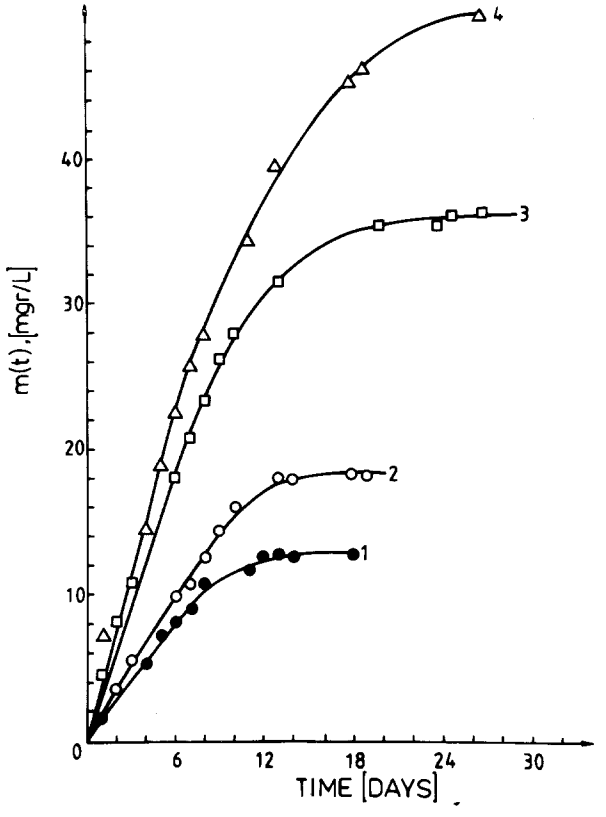

Fig. 2. Dissolution kinetics of calcium oxalate calculi in artificial urine under the influence of hippuric acid (Archimedean weight curves) in initially medium-supersaturated urine. *1*, With 5 mmol/l hippuric acid; *2*, with 10 mmol/l hippuric acid; *3*, with 15 mmol/l hippuric acid; *4*, with 25 mmol/l hippuric acid

various concentrations of hippuric acid, and (2) on the formation of CaOx precipitates in physiological solutions with different concentrations of hippuric acid [1].

The experiments on the kinetics of dissolution of CaOx calculi were performed at 25°C. The volume of solution used was 1500 ml and it was stirred by an electromagnetic stirrer [8]. The Archimedean weight $G(t)$ of the samples of CaOx calculi put in a platinum net basket was measured on a torsion balance with a sensitivity of ± 0.5 mg.

The CaOx calculi used had been formed in the urinary tract and eliminated by (or removed by operation from) patients with oxalate lithiasis. The calculi were selected to have a weight of 200–300 mg and to be of identical mineral composition – mainly $CaC_2O_4 2H_2O$ (weddellite). The composition of the calculi was checked by X-ray analysis, differential thermoanalysis.

We employed two different types of aqueous solutions:

1. Physiological solutions, in which the addition of NaCl in a concentration of 0.3 M ensured an ionic strength equivalent to that of urine (i.e. $\psi = 0.3$).
2. "Artificial urine", i.e. a solution with the mean ionic composition of human urine and $\psi = 0.3$. We employed a composition recommended by several authors [3, 5] but varied the concentration of oxalate ions in two different series of experiments (medium or normal) by introducing different concentrations of oxalate ions into it. In this way we attained a differentially supersaturated system. Because of the buffering action of the complex composition of artificial urine its pH value was in all cases 5.2.

Hippuric acid was aded to all four solutions in varying concentrations above the physiological norm for this substance in human urine.

Each experiment was repeated under identical conditions three or four times, a new calculus being employed each time. The shape of the dissolution curves, and especially the data on the change in sample weight, did not vary by more than 10%–15% in the repeat experiments.

The inhibitory effect of hippuric acid on CaOx precipitation was studied using a method similar to that of Fleisch, as described in [12]. A suspension of crystalline CaOx was added in the presence of hippuric acid to a metastable CaOx solution (with constant Ca^{2+} and $C_2O_4^{2-}$ ion concentration) which was sufficiently supersaturated to allow the added crystals to grow but no spontaneous precipitation of new crystals to occur. The overall growth kinetics were evaluated by centrifuging the suspension thus obtained and analysing the calcium content in the remaining solution by atomic absorption spectrometry.

Results

The solubility of CaOx in artificial urine with zero supersaturation (Fig. 1a) is considerably increased (8.7×10^{-5} mol/l) compared with its solubility in pure water (5.7×10^{-5} mol/l) due to the presence of complexing ions (Mg^{2+}, citrate ions, etc.) in this solution as predicted by the α_a, α_b coefficients in Eq. 2. When hippuric acid is introduced into the same physiological solution a dramatic change in solubility (up to 50×10^{-5} mol/l) is observed, as shown in Fig. 1b.

A similar effect of hippuric acid is also seen in artificial urine in which distinct supersaturation (due to the presence of a normal concentration [8] of Ca^{2+} and a medium concentration [8] of $C_2O_4^{2-}$ ions) has been maintained (Fig. 2). In accordance with Eq. 4 a linear dependence of the solubility on C_H is observed (Fig. 3) for each series of measurements, in which three different supersaturation values (zero, from Fig. 1; medium, from Fig. 2; and normal – from additional experiments) have been established.

The inhibiting effect of various concentrations of hippuric acid is evident from Fig. 4, which shows the dependence of Ca^{2+} concentration in the solution at given times t for different hippuric acid concentration. The

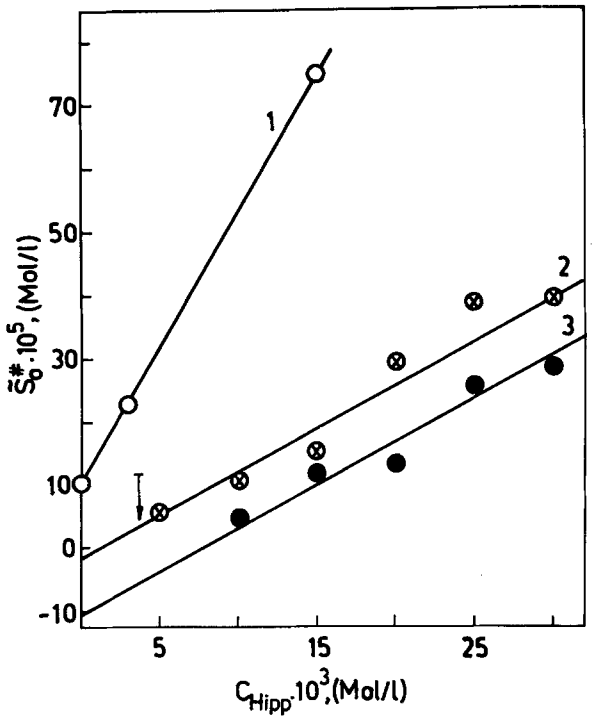

Fig. 3. Solubility of calcium oxalate concrements in artificial urine as a function of hippuric acid concentration. *1*, Solubility in artificial urine with zero supersaturation; *2*, solubility in artificial urine with medium supersaturation; *3*, solubility in artificial urine with normal supersaturation

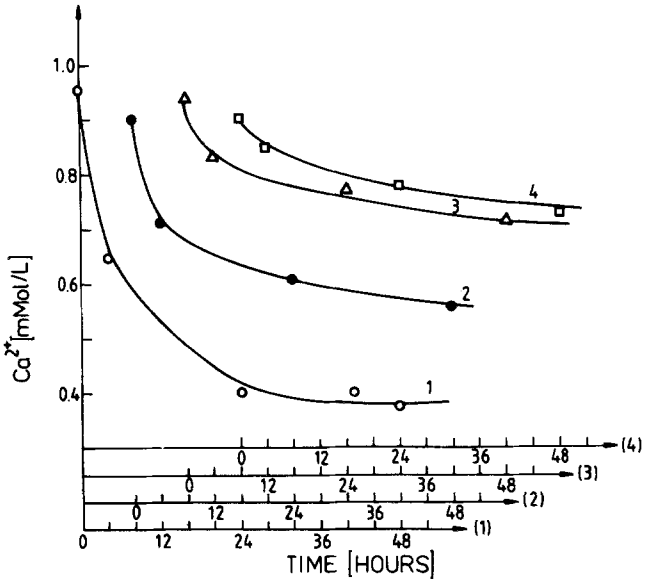

Fig. 4. Inhibiting effect of hippuric acid on the kinetics of precipitation of metastable calcium oxalate solutions. Concentration of Ca^{2+} after elapse of time t at the introduction of a suspension of CaOx: *1*, in the absence of hippuric acid; *2*, in the presence of 10 mmol/l hippuric acid; *3*, in the presence of 15 mmol/l hippuric acid; *4*, in the presence of 20 mmol/l hippuric acid. In drawing curves 1–4 they have been shifted as indicated by 2 h in order of increasing hippuric acid concentration

linear dependence of $\sqrt[3]{(M_0 - M_t)}$ on C_H in Fig. 5 (cf. Eq. 8) is an indication that in the presence of hippuric acid the supersaturation in the solution is diminished and that this causes a decrease in the CaOx crystal growth rate and the observed inhibition of CaOx precipitation.

Discussion

It has been found that hippuric acid is a very effective solvent of CaOx calculi in solutions with the composition and ionic strength of human urine. The detailed analysis performed in our previous study [8] indicates that hippuric acid is comparable in its solubility effect to the best-known classical complex binders of Ca^{2+} or $C_2O_4^{2-}$ ions in urine, i.e. Mg^{2+} and citrate anion. This is seen from Table 1, in which the stability constants K_H of hippuric acid calculated according to our results (Fig. 2 and Eq. 4) are compared with the K_H values of Mg^{2+}, Na-EDTA and other known complex-formers of CaOx.

We have found that the mechanism of dissolution of CaOx calculi follows the Nernst model of a diffusion-limited process as discussed in detail in [8].

The dissolution of CaOx with hippuric acid is a relatively slow process, taking approximately one month to dissolve a 100-mg stone. However, in assessing the efficiency of hippuric acid as an eventual clinical solvent of CaOx calculi it should be borne in mind that conditions will prevail (continuous flushing of the calculus in the renal tract with fresh urine) under which the time necessary for the dissolution effect will be much shorter, because it is determined by the initial value of the dissolution rate.

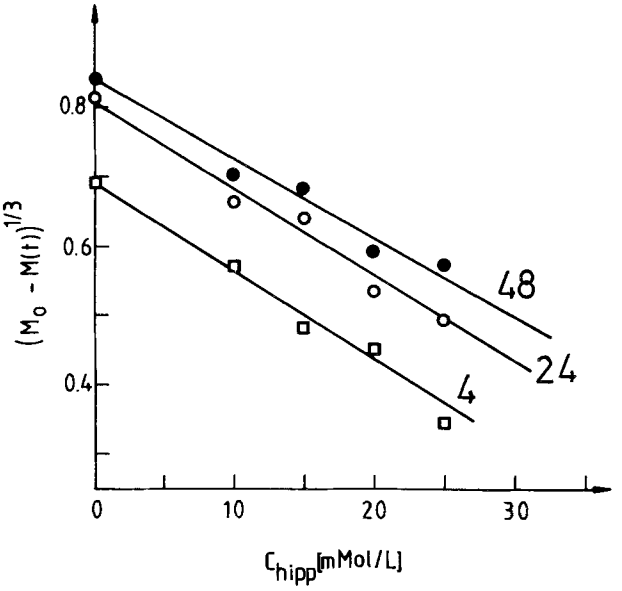

Fig. 5. The data on the precipitation kinetics from Fig. 4 in coordinates $(\Delta m)^{1/3}$ vs C_H according to Eq. 8. Each *straight line* has been drawn through experimental data corresponding to different times as indicated (in hours)

It is also of interest that while the food of herbivorous mammals contains considerable quantities of soluble oxalates (of lithium and sodium) the formation of CaOx calculi is practically unknown in these animals. In this

Table 1. Hippuric acid as a solvent of calcium oxalate: comparison with classical complex-formers

Ligand	Complex-former	Solution	T (°C)	pH	ψ	K (l/mol)	Authors
Na-EDTA	Ca^{2+}	Pure water	25	7.5	0	10×10^5	Gutzow [8]
Na-EDTA	Ca^{2+}	Physiological solution	25	7.0	0.3	5×10^5	Gutzow [8]
Mg^{2+}	$C_2O_4^{2-}$	Pure water	25	–	0	2.7×10^3	Robertson [15]
Mg^{2+}	$C_2O_4^{2-}$	0.3 M NaCl	37	–	0.3	5.6×10^3	Hammarsten [9]
Mg^{2+}	$C_2O_4^{2-}$	0.3 M NaCl	25	5.0	0.3	4×10^3	Gutzow [8]
Citric anion	Ca^{2+}	Pure water	25	–	0	5×10^2	Robertson [15]
Hippuric acid	Ca^{2+}	Pure water	20	–	0	2.7	Davies [4]
Hippuric acid	CaOx	Physiological solution	25	2.7–7.0	0.3	2×10^3	Gutzow [8]
Hippuric acid	CaOx	Zero supersaturated artificial urine	25	5.2	–	7.2×10^3	This study (Fig. 3)
Hippuric acid	CaOx	Artificial urine	25	5.2	–	6×10^4	This study (Fig. 3)

CaOx, Calcium oxalate

sense our results are perhaps an indication that hippuric acid could be a natural regulator of CaOx solubility in mammals. We should also keep in mind the possibility of provoking the formation of hippuric acid in human urine by the oral administration of substances containing benzene rings (e.g. sodium benzoate, as in Quick's test [2, 17].

In this sense it should also be expected that the urine of CaOx stone-forming patients should be characterized by a lower concentration of hippuric acid than in normals. In a recent detailed study [2] we have shown that on average 80% of chronic CaOx stone-formers have a pronounced hypoexcretion of hippuric acid, the mean value in 200 cases being 2.3 ± 0.6 mmol/day (corresponding to a concentration of 2.9 ± 0.5 mmol/l). On non-stone-forming patients an analysis of 100 cases gave a mean value of 25.4 ± 12.4 mmol/day (28.03 ± 14.3 mmol/l) in urine. Further investigations are planned, but nevertheless the above results speak for themselves.

As mentioned in the introduction to this paper, it is known that a number of amino acids also have an inhibitory effect on CaOx crystal growth. The results of these investigations show that this inhibitory effect is displayed at such small concentrations that it can be explained by adsorption. In the case of hippuric acid we have obtained growth inhibition which considerably surpasses that of other amino acids, thus assigning to hippuric acid a place among the most efficient and promising inhibitors of CaOx growth. In our studies however, this effect is due to the fact that hippuric acid considerably lowers the supersaturation.

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