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Experiences with the Measurement of Inhibitory Activity of Urine and Crystallisation Inhibitors by Different Techniques

J. M. Baumann and M. Wacker

Department of Urology and Stone Research Laboratory, Regional Hospital, CH-2500 Biel, Switzerland

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Summary. The effect of urine, pyrophosphate (PPi), citrate and diphosphonate on the formation, the growth rate or the aggregation of calcium oxalate or calcium phosphate crystals was measured by 4 different methods. The degree of supersaturation and the area of crystal surface present in the test systems were found to be limiting factors for the action of inhibitors. Citrate and PPi proved to be important urinary inhibitors of the growth of calcium oxalate crystals. Comparison between stone formers and healthy controls revealed a significant lack of PPi in urine of male patients. The inhibitory effect of PPi in urine of healthy people was enhanced by unknown factors. This enhancement could not be found in about 60% of the stone patients. Problems relating to measurement and clinical importance of inhibitors are discussed.

Key words: Inhibitors - Calcium stones - Pyrophosphate - Citrate - Diphosphonate.

Most investigators now consider stone formation to be a problem of crystal formation, growth and aggregation due to urinary supersaturation with stone minerals. Since urine is normally supersaturated with respect to calcium oxalate, octocalcium phosphate and hydroxyapatite (22) and sometimes brushite (21) the question arises why healthy people produce only crystalluria but no calcium stones. One possible answer is that crystallisation inhibitors in urine may protect from calcium urolithiasis. As early as 1958 it had been shown, that urine inhibits the crystallisation of calcium phosphate in vitro and that urine of some stone patients lacks this inhibitory activity (14).

Inorganic pyrophosphate (PPi) (7, 9, 17, 29) and citrate (17, 29) have been found to be potent physiological inhibitors of the formation and growth of calcium oxalate and calcium phosphate crystals. With ethane-1-hydroxy-1, 1-diphosphonate (EHDP) a synthetic compound related in structure to pyrophosphate a great deal of research has been done to elucidate the action of inhibitors (5, 13, 18, 19, 23, 30). Clinical use suggests that crystallisation inhibitors may prevent stone recurrence (2). Acid mucopolysaccharides, especially heparin and chondroitin sulphate selectively inhibit the crystal aggregation of both calcium oxalate (23) and calcium phosphate (13). Inhibitory activity to crystal aggregation found in urine seems to be due mainly to mucopolysaccharides whose structure has not yet been determined (25). In some stone patients their activity is probably decreased because of binding to urinary uric acid which may be present in increased quantities (25). Not all the inhibitory activity found in different test systems can be related to known compounds (11, 12, 15, 17, 29) and since the action of some inhibitors may be influenced by secondary factors (1, 25), the measurement of inhibitory activities seems to be most important for calcium stone research. Although many test systems have been developed (3-6, 9, 11-20, 23, 27, 29, 30) there is neither consensus about the test methods nor about the role that inhibitors play in stone formation. In this paper results with four different test systems are presented and problems relating to measurement and clinical importance of inhibitors are discussed.

Theoretical Background for the Measurement of Inhibitory Activities

Crystallisation processes are governed by the activity product of crystal forming ions and by promoters and inhibitors of crystallisation. Thermodynamic activity of ions is influenced by complex-

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ors which reduce free ionic concentration by forming soluble complexes and by the ionic strength of the solution. Determination of ionic activities in urine demands analysis of 9 important ions and computer calculations of at least 22 complexes (22). For clinical purposes it is more practicable to work with concentration products, determined before and after equilibration with stone forming salts from which the degree of urinary supersaturation can be estimated (21). Promoters of crystallisation are compounds present in minimal concentrations inducing epitactic crystal formation and growth (10). Contrary to complexors, inhibitors influence crystallisation processes by other means than decreasing ionic activities in the solution. Inhibitors of crystal formation and growth are supposed to block growing sites of crystal surfaces whereas aggregation inhibitors seem to change zetapotentials (10).

Measurement of urinary crystallisation inhibitors demands a clear distinction between the inhibitory activity to be measured and the unknown effect of complexors added with urine to the test system. Since inhibitors are more active than complexors the effect of complexors is eliminated in most test systems by urine dilution.

METHODS

1. Measurement of Minimal Concentration Product for the Formation of Calcium Oxalate (MCPox)

In buffered (pH 6.2) solutions containing calcium (1.2 mmol/l), oxalate (0.16 mmol/l) and NaCl (150 mmol/l) a 0.1 molar solution of sodium oxalate was infused at fixed rates by an infusion pump using a magnetic stirrer. The test was run with and without 10^{-5} mol/l EHDP. The time between the start of the infusion and the beginning of calcium oxalate precipitation was measured by a nephelometer connected to a strip chart recorder. From initial calcium and oxalate concentrations and from infused oxalate the MCPox was calculated.

2. Measurement of Minimal Concentration Product for the Formation of Calcium Phosphate (MCPphos) (1.6)

A series of buffered solutions with constant pH (7.4), ionic strength (0.165) and calcium (1.7 mmol/l) but increasing phosphate (0.46 to 5.57 mmol/l) were incubated on a mechanical shaker for 72 hours at 37°C. The solutions contained either 3% urine, known concentrations of PPi or an equal volume of KCL (150 mmol/l) as a control. Using a fall in calcium level as the indicator of precipitation the solution with the lowest initial calcium x phosphate product in which precipitation had occurred was determined (MCPphos). Inhibitory activity was calculated as the difference in $(mmol/l)^2$ between MCPphos with and without urine or PPi respectively. Urinary inhibitory activity was measured before (total inhibition) and after (residual inhibition) urinary PPi had been hydrolysed by incubation with inorganic pyrophosphatase. Urinary PPi was measured by the method of Fleisch and Bisaz (8).

3. Measurement of Growth Rate of Calcium Oxalate (GRox) (16)

In buffered (pH 6.7) solutions metastable with respect to calcium oxalate containing equimolar concentrations of calcium and oxalate (0.44 mmol/l) and NaCl (150 mmol/l) 0.07 mg/ml calcium oxalate crystals with an average diameter of 10 µ were incubated. At fixed times (5, 10, 15 min) after the addition of crystal seeds samples of the solution were Millipore-filtered (0.45 µ pore size) and analysed for calcium. The test was run with various concentrations of urine and known concentrations of PPi and citrate or an equal volume of NaCl (150 mmol/l) as a control. Crystal growth was measured by the decrease of calcium in the solution. The growth rate constant (K) was calculated from the formula: $d\,Ca/dt = (Ca_t - Ca_e)^2 \cdot (K)$; ($d\,Ca/dt$ = decrease of calcium per time, Ca_t = calcium at time t, Ca_e = calcium at equilibration with calcium oxalate). Inhibitory activity was expressed as a percentage from the formula: $100 \cdot (K_c - K_s) \cdot (K_c)^{-1}$; ($K_c$ = K of control, K_s = K of sample with urine or inhibitors).

4. Measurement of Aggregation and Growth of Calcium Oxalate (5, 23)

In buffered (pH 6.0) solutions metastable with respect to calcium oxalate containing calcium (1.0 mmol/l), oxalate (0.2 mmol/l), and NaCl (150 mmol/l) calcium oxalate crystals (0.01 mg/ml with average size of 10 µ) were incubated for 2 h at 37° on a rotating machine. The tests were run with 3% urine or equal volumes of NaCl (150 mmol/l) as a control. Volume/size distribution of crystals was measured by a Coulter Counter. From these data the percentage of volume of crystals with diameters above 12.7 µ was calculated for disaggregated crystals (before incubation) (Vd%) and aggregated crystals incubated with urine (Vu%) and without urine (Vc%). Urinary inhibitory activity was expressed as percentage from the formula: $100 \cdot (Vc\% - Vu\%) \cdot (Vc\% - Vd\%)^{-1}$.

RESULTS

1. Measurement of MCP for the formation of calcium oxalate with short incubation time and at high supersaturation showed a decrease of MCP

with reduction of infusion rate and increase of incubation time respectively (Fig. 1). 10^{-5} mol/l EHDP had a small influence on MCP only at the lowest degree of supersaturation produced by the test system. Therefore the method was not used for further investigations of inhibitors.

2. Measurement of MCP for the formation of calcium phosphate at low supersaturation and with 72 h incubation showed a marked increase of MCP (= inhibition) by only 3% urine (Fig. 2) and by PPi in micromolar concentrations (Fig. 3). Most of urinary inhibitory activity measured by this test system was due to PPi, since residual inhibition after hydrolysis of urinary PPi was very small. In a study conducted with patients with active recurrent stone disease who were compared with age and sex matched control subjects given an identical diet the urine of male but not of female stone formers showed a significant decrease of inhibitory activity (Fig. 2). This diminution was due to a lack of PPi in the urine of male patients (1) which was also demonstrated by an equal residual inhibition of patients and controls (Fig. 2).

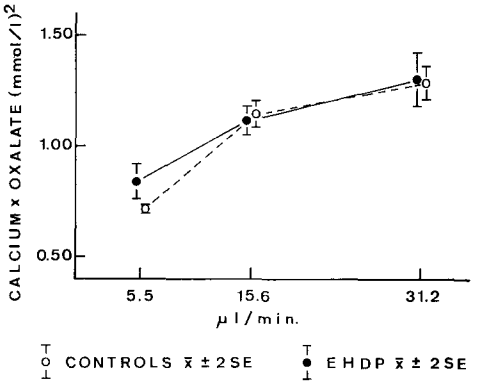

Fig. 1. MCPox ($\bar{x} \pm 2$ SE, n = 6) in test solutions with and without 10^{-5} mol/l EHDP plotted versus infusion rate of 0.1 molar sodium oxalate

From the difference between total and residual inhibition the inhibitory effect of urinary PPi was calculated and plotted against the PPi concentration added with 3% urine to the test system. Comparison between the calculated inhibitory effect of PPi in urine and the measured inhibition of PPi in test solutions disclosed that in 12 of 13 controls but only in 6 of 14 patients the effect of urinary PPi was higher than expected from results in test solutions (Fig. 3). The reason for this enhancement in urine of healthy people could not be elucidated.

3. In the same controlled study of stone patients and healthy subjects inhibitory activity of urine to the aggregation and growth of calcium oxalate was measured. A 3% dilution of 24 hour urines showed high inhibition in the test system without any significant difference between patients and controls (Table 1).

4. Measurement of GRox showed marked inhibition of the test system even with urine dilutions as low as 1%. Plotting reciprocal values of the inhibition of the different urine concentrations versus reciprocal values of concentrations according to Langmuir adsorption isotherm (17) revealed a linear function. The same was found valid for phys iological concentration of PPi and citrate (Fig. 4). Inhibitory activity of 3% urine was determined in 10 healthy men. The mean value of this inhibition was compared to calculated values of the inhibition due to PPi (1, 2) and citrate (22) added with 3% normal urine to the test system. Figure 5 shows that most of the urinary inhibition of GRox can be attributed to PPi and citrate, if an additive effect of these compounds is assumed (3).

DISCUSSION

Measurement of MCPox revealed, that a concentration of EHDP (10^{-5} mol/l), which has been reported to be most active in other test systems

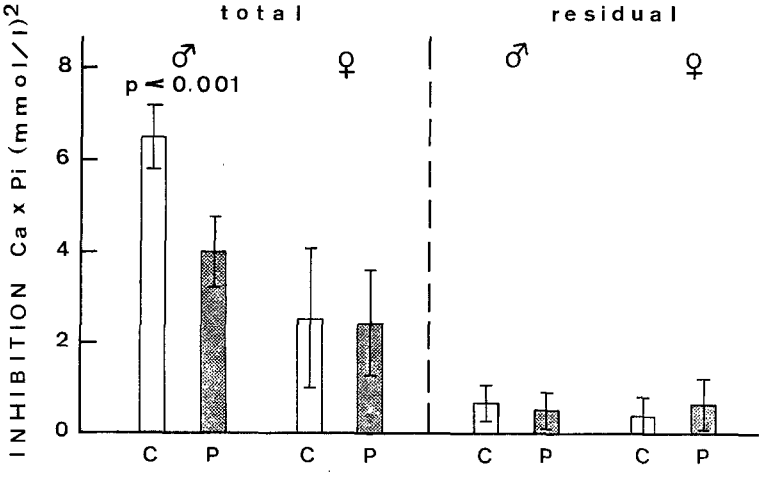

Fig. 2. Inhibitory activity ($\bar{x} \pm 2$ SE) of 3% urine from 18 stone patients (P) and 16 healthy controls (C) on the formation of calcium phosphate before (total) and after (residual) hydrolysation of urinary PPi

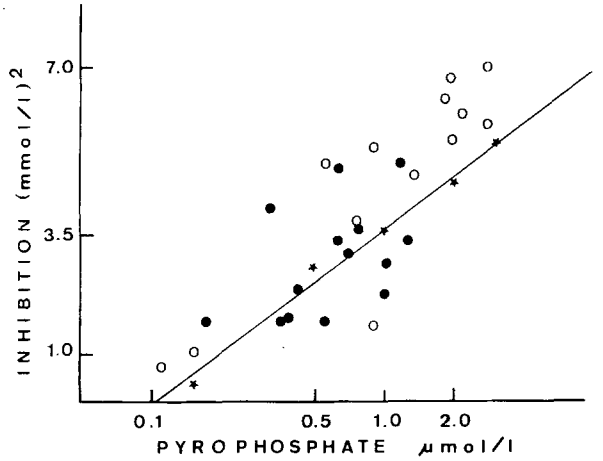

Fig. 3. Inhibitory effect of PPi in urine of stone patients (●) and controls (O), and of PPi in test solutions (*) on the formation of calcium phosphate plotted versus PPi concentration in the test system

Table 1. Inhibition of calcium oxalate crystals aggregation ($\bar{x} \pm 2$ SE %) by 3% 24 hour urine in stone formers and controls

Men		Women	
Patients n = 8	Controls n = 5	Patients n = 4	Controls n = 4
67 ± 5.6	72.8 ± 9.2	71.8 ± 1.7	73.8 ± 3.0

(5, 13, 18, 19, 23, 30) and to prevent stone recurrence in vivo (2), had no inhibitory effect at high supersaturation and with a short incubation time. Similar observations have been made by others (24) determining MCPphos. Since urinary supersaturation with respect to calcium salts is often high (21, 22) and since healthy people also show crystalluria, it can be assumed that crystallisation inhibitors are not always able to prevent crystal formation in urine. The potent effect of urinary inhibitors on crystal growth and aggregation ascertained by this study seems to be more relevant to conditions occurring in vivo. However, comparison between our results in the GRox-test and those reported by Meyer and Smith (17) who developed this method, reveals that the area of crystal surface to be protected from growth is another important limit for the effect of inhibitors. These authors used 0.06 mg/ml calcium oxalate crystals of diameters 1-5 µ and found a 50% inhibition in the test system by $1.6 \cdot 10^{-5}$ mol/l PPi and by 1.6% of urine. Reducing crystal surface by our seeding with crystals of an average diameter of 10 µ (0.07 mg/ml) increased the inhibitory effect of

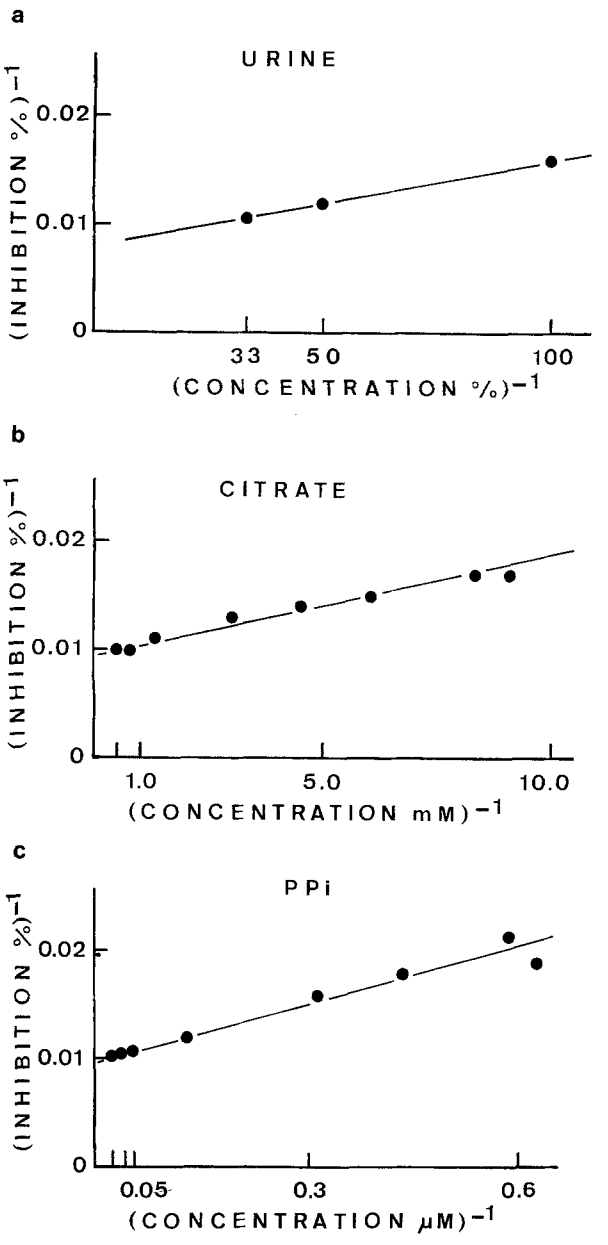

Fig. 4. Reciprocal values of inhibition of GRox plotted versus reciprocal values of concentration for various concentrations of 24 hour urines of a healthy man (Fig. 4a), citrate (Fig. 4b) and PPi (Fig. 4c)

urine more than twice (50% inhibition by 0.7% urine). This was due mainly to a ten fold elevated PPi effect (50% inhibition by $1.7 \cdot 10^{-6}$ mol/l PPi). Citrate and PPi proved to be the main urinary inhibitors of calcium crystal growth in this modification of the test system.

The role that crystallisation inhibitors play in stone formation is even less clear than their physiological effects. Citrate is not diminished in the urine of idiopathic calcium stone formers (22). But our study disclosed a significant lack of PPi

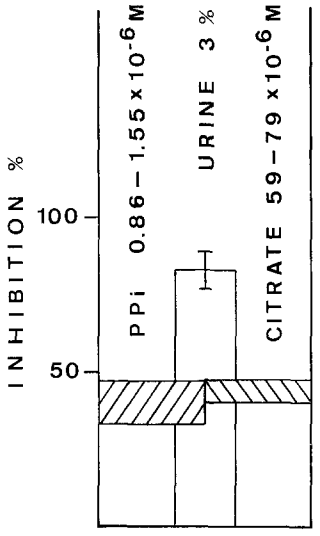

Fig. 5. Inhibitory activity on GRox of 3% 24 hour urine from 10 healthy men ($\bar{x} \pm 2$ SE)and of equal concentrations of PPi and citrate as added with 3% urine to the test system

in the urine of male stone formers (1). This lack, not found to such an extent by other investigators (28), was probably detected because not only sex and age but also an often neglected diet dependency of PPi excretion had been taken into account. Furthermore an enhancement of the inhibitory effect of PPi in urine of healthy people was shown. The absence of this enhancement in about 60% of patients might also have some importance in stone disease. Measurement of the inhibitory activity of 24 hour urine to the aggregation of calcium oxalate crystals revealed no significant difference between stone formers and controls. This is not necessarily contrary to Robertson et al. (26) who found differences by the analysis of single urine samples, since residual inhibition was decreased in some urine samples from stone formers taken during the day but not in the 24 hour urine (1).

The problems encountered in this study suggest that further progress in inhibitor research can only be achieved by standardising test methods as well as examination conditions for patients. More physiological test systems with respect to supersaturation, incubation time and surface area of seed crystals are needed. Methods for direct measurements of inhibitors in voided urine are now available (3) avoiding problematic extrapolations from results obtained with diluted urine.

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REFERENCES

1. Baumann, J.M., Bisaz, S., Felix, R., Fleisch, H., Ganz, U., Russell, R.G.G.: The role of inhibitors and other factors in the pathogenesis of recurrent calcium-containing renal stones. Clinical Science and Molecular Medicine 53, 141 (1977)
2. Baumann, J.M., Bisaz, S., Fleisch, H., Wacker, M.: Biochemical and clinical effects of ethane-1-hydroxa-1,1-diphosphonate in calcium nephrolithiasis. Clinical Science and Molecular Medicine 54, 509 (1978)
3. Bisaz, S., Felix, R., Neuman, W.F., Fleisch, H.: Quantitative determination of inhibitors of calcium phosphate precipitation in whole urine. Mineral Electrolyte Metabolism 1, 74 (1978)
4. Dent, C.E., Sutor, D.J.: Presence or absence of inhibitor of calcium-oxalate crystal growth in urine of normals and of stone-formers Lancet 2, 775 (1971)
5. Felix, R., Monod, A., Broge, L., Hansen, N.M., Fleisch, H.: Aggregation of calcium oxalate crystals: Effect of urine and various inhibitors. Urological Research 5, 21 (1977)
6. Fleisch, H., Neuman, W.F.: Mechanisms of calcification: role of collagen, polyphosphates, and phosphatase. American Journal of Physiology 200, 1296 (1961)
7. Fleisch, H., Bisaz, S.: Isolation from urine of pyrophosphate, a calcification inhibitor. American Journal of Physiology 203, 671 (1962)
8. Fleisch, H., Bisaz, S.: Die Pyrophosphatausscheidung im Harn beim gesunden Menschen. Helvetica Physiologica Acta 21, 88 (1963)
9. Fleisch, H., Bisaz S.: The inhibitory effect of pyrophosphate on calcium oxalate precipitation and its relation to urolithiasis. Experientia 20, 276 (1964)
10. Fleisch, H.: Inhibitors and promoters of stone formation. Kidney International 13, 361 (1978)
11. Gardner, G.L., Doremus, R.H.: Crystal growth inhibitors in human urine. Effect on calcium oxalate kinetics. Investigative Urology 15, 478 (1978)
12. Gill, W.B., Karesh, J.W., Garsin, L., Roma, M.J.: Inhibitory effects of urinary macrolole-cules on the crystallisation of calcium oxalate. Investigative Urology 15, 95 (1977)
13. Hansen, N.M., Felix, R., Bisaz, S., Fleisch, H.: Aggregation of hydroxyapatite crystals. Biochimica et Biophysica Acta 451, 549 (1976)
14. Howard, J.E., Thomas, W.C.: Some observations on rachitic rat cartilage of probable sigphonates on calcium phosphate crystallisation in actions of the American Clinical and Climatological Association 70, 94 (1958)
15. Howard, J.E.: Studies on urinary stone formation: a saga of clinical investigation. The Johns Hopkins Medical Journal 139, 239 (1976)

16. Meyer, J. L., Smith, L. H.: Growth of calcium oxalate crystals. I. A model for urinary stone growth. Investigative Urology 13, 31 (1975)
17. Meyer, J. L., Smith, L. H.: Growth of calcium oxalate crystals. II. Inhibition by natural urinary crystal growth inhibitors. Investigative Urology 13, 36 (1975)
18. Ohata, M., Pak, C. Y. C.: The effect of diphosphonates on calcium phosphate crystallization in urine in vitro. Kidney International 4, 401 (1973)
19. Pak, C. Y. C., Ohata, M., Holt, K.: Effect of diphosphonate on crystallisation of calcium oxalate in vitro. Kidney International 7, 154 (1975)
20. Pak, C. Y. C., Holt, K.: Nucleation and growth of brushite and calcium oxalate in urine of stone-formers. Metabolism 25, 665 (1976)
21. Pak, C. Y. C., Hayashi, Y., Finlayson, B., Chu, S.: Estimation of the state of saturation of brushite and calcium oxalate in urine: A comparison of three methods. Journal of Laboratory and Clinical Medicine 89, 891 (1977)
22. Robertson, W. G., Peacock, M., Nordin, B. E. C Activity products in stone-forming and non-stone-forming urine. Clinical Science 34, 579 (1968)
23. Robertson, W. G., Peacock, M., Nordin, B. E. C Inhibitors of the growth and aggregation of calcium oxalate crystals in vitro. Clinica Chimica Acta 43, 31 (1973)
24. Robertson, W. G.: Factors affecting the precipitation of calcium phosphate in vitro. Calficied Tissue Research 11, 311 (1973)
25. Robertson, W. G., Knowles, F., Peacock, M.: Urinary acid mucopolysaccharide inhibitors of calcium oxalate crystallisation. In: Urolithiasis Research. Fleisch, H., Robertson, W. G., Smith, L. H., Vahlensieck, W. (eds.), New York: Plenum Press 1976
26. Robertson, W. G., Peacock, M., Marshall, R. W., Marshall, D. H., Nordin, B. E. C.: Saturation-inhibition index as a measure of the risk of calcium oxalate stone formation in the urinary tract. New England Journal of Medicine 294, 249 (1976)
27. Rose, M. B.: Renal stone formation. The inhibitory effect of urine on calcium oxalate precipitation. Investigative Urology 12, 428 (1975)
28. Russell, R. G. G., Hodgkinson, H.: Urinary excretion of inorganic pyrophosphate by normal subjects and patients with renal calculus. Clinical Science 31, 51 (1966)
29. Smith, L. H., Meyer, J. L., McCall, J. T.: Chemical nature of crystal inhibitors isolated from human urine. In: Urinary calculi. Cifuentes, Delatte, L., Rapado, A., Hodgkinson, A. (eds.), p. 318. Basle: S. Karger 1973
30. Will, E. J., Bijvoet, O. L. M., te Brakevan der Linden, H.: Inhibition of calcium oxalate crystal growth - a simple method of measurement and preliminary results. In: Urolithiasis research. Fleisch, H., Robertson, W. G., Smith, L. H., Vahlensieck, W. (eds.). New York: Plenum Press 1976

Dr. J. Baumann
Urologische Abteilung und
Steinforschungslabor
Regionalspital
CH-2500 Biel, Switzerland