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Macrocyclic Polyamines as Calculi Solubilizers

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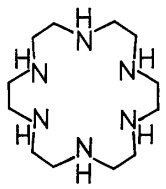
The novel complexing properties of macrocyclic polyamines with phosphate and oxalate anions, which we previously discovered, were tested against urinary calculi. An 18-membered hexaamine [18]ane N_6 and a 16-membered pentaamine [16]ane N_5 *in vitro* solubilized inorganic models of calculi ($Ca_3(PO_4)_2$ and $Ca(C_2O_4)$) and human urinary calculi in acidic solutions, implying that they may be useful as a new type of ingredient of irrigating fluids.

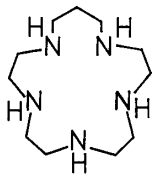
Keywords——urinary calculus; macrocyclic polyamine; citric acid; ethylenediamine tetraacetic acid; solubility

Urinary lithiasis is a disease in which calculi form in the urinary tract. Various theories have been proposed regarding its genesis[1-3] but no fundamental remedies have yet been established. Since Crowell first succeeded in solubilizing calculi from the renal pelvis with an irrigating fluid,[4] quite a few attempts have been made to make this technique more practical. The major components of calculi are calcium oxalate and calcium phosphate, and there are three types of solubilizers that are currently in clinical use against calculi: Solution G (a major component is citric acid),[5] Versene (EDTA),[6-9] and Renacidin (citric acid, glucuronic acid, *etc.*).[10-13] Their actions against calculi apparently occur through two mechanisms: one is chelation with the cations in calculi, *i.e.* Mg^{2+} and Ca^{2+}, and the other is acidification of urine to enhance the solubility of calcium oxalate and calcium phosphate.

Recently we have been developing macrocyclic polyamines such as [18]ane N_6 and [16]-ane N_5 (see Fig. 1) that can be either cation chelators[14] or anion chelators.[15-18] In alkaline



[18]ane N_6



[16]ane N_5

Fig. 1. Structures of Macrocyclic Polyamines
[18]ane N_6 = 1,4,7,10,13,16-hexaazacyclooctadecane.
[16]ane N_5 = 1,4,7,10,13-pentaazacyclohexadecane.

solutions, these macrocyclic polyamines are strong metal-chelators and can complex with Ca^{2+},[14] while in acidic solutions, they capture protons in the macrocyclic cavities and the resulting organic polycations tend to hydrogen-bond with polyoxyanions such as polycarboxylates,[15] inorganic and organic phosphates,[16] or carbonate.[17] These dual properties of macrocyclic polyamines might work against calculi by removing either Ca^{2+} or phosphate and oxalate anions. The present *in vitro* experiments indicate that these macrocyclic polyamines are indeed effective in solubilizing $Ca_3(PO_4)_2$, $Ca(C_2O_4)$ and human urinary calculi and that they may represent a new type of component of an irrigating fluid for treating calculi.

Experimental

Tests on Calculi Models $Ca_3(PO_4)_2$ and $Ca(C_2O_4)$. Materials——$Ca_3(PO_4)_2$ and $Ca(C_2O_4)$ (both Nakarai extra pure reagents) were used as calculi models. Solutions of ethylenediaminetetraacetic acid disodium salt (EDTA), citric acid (both Yoneyama guaranteed reagents), [18]ane $N_6 \cdot 6HCl$, and [16]ane $N_5 \cdot 5HBr$[19] were all 10 mM in concentration and pH 5—6 (adjusted with NaOH or HCl). The influence of free cations was studied with $MgCl_2$ (25 mM), $CaCl_2$ (25 mM) and the influence of free anions with NaH_2PO_4 (10 mM) in distilled water. Acetic acid–sodium acetate buffer (pH 4.1, 5.0 and 5.9), collidine–HCl buffer (pH 7.0) and Tris-HCl buffer (pH 8.0) were used at 0.1 M concentration for the optical density analysis and at 0.05 M for the isotachophoretic analysis. The buffer components (collidine, Tris or acetic acid) had little effect on the solubilizing and chelating activities of macrocyclic polyamines.

Procedure——Accurately weighed samples of $Ca_3(PO_4)_2$ (10 mg) or $Ca(C_2O_4)$ (2 mg) were placed in a group of test tubes, to which 0.2 ml of stock solution of chelating agent, *e.g.* EDTA, citric acid or macrocyclic polyamine, was added. The effect of free ions (0.2 ml of stock solutions) was investigated simultaneously. A suitable buffer was added to make the total volume 2.0 ml. After being shaken in a water bath at 37 °C for 30 min, the solutions were immediately filtered through filter papers (Toyo Roshi #2). The filtrates were subjected to analysis of phosphate or oxalate anions by the following two methods.

(i) The Fiske–Subbarow Method for Phosphate:[20] Since the polyamines were found to interfere (causing precipitation) with the interaction between phosphate ions and the molybdate reagent, we modified the standard method as follows: 3.0 ml of *n*-butanol–*tert*-butanol (1 : 1) was added to 1.0 ml of filtrate. The mixture was well shaken for 15 s, then aminonaphthol sulfonic acid and the molybdate reagents were added, and the whole was allowed to stand at 30 °C for exactly 30 min. The butanol layer (1.5 ml) was pipetted out and diluted to 5.0 ml with ethanol. The dissolved phosphate anion was determined spectrophotometrically at 800 nm. Standard curves were obtained using $NaH_2(PO_4)$ aqueous solutions of known concentrations.

(ii) Isotachophoretic Analysis (Shimadzu Isotachophoretic Analyzer IP-2A) for Oxalate and Phosphate Anions: A buffer solution of 5 mM HCl–*β*-alanine (pH 3.77) with 0.1% Triton X-100 was used as a leading solution. The terminal solution was 5 mM aqueous *n*-capronic acid. The analyzer was fitted with a pre-analyzing column (1 mm i.d. × 40 mm) and an analyzing column (0.5 mm i.d. × 100 mm), and a potential gradient detector was operated at 75 A and at 15 °C. Aliquots of 50 μl of sample solutions were injected for the analysis. Standard curves for phosphate or oxalate were obtained by using diluted $NaH_2PO_4 \cdot 2H_2O$ or $C_2O_4H_2 \cdot 2H_2O$ solutions.

Tests on Human Urinary Calculi *in Vitro* Materials——[18]ane $N_6 \cdot 6HCl$ was dissolved to give 10 mM aqueous solution and the pH was adjusted to 4.0 or 5.0 with NaOH. (Macrocyclic polyamines have buffering capacities at around pH 4.) EDTA stock solutions (10 mM) were prepared in both pH 4.0 and 5.0 acetate buffers. The acetate buffers (5 mM) were also used for blank tests. The three pieces of calculi (designated as A, B and C) used for the present experiment were taken from the urinary tracts of three urinary lithiasis patients.

Procedure——A 2.0 ml aliquot of the buffer with or without solubilizer was added to 5 mg of well-ground calculus. After being shaken at 37 °C for 6 h, the solution was immediately filtered through filter paper (Toyo Roshi #2). Phosphate and oxalate concentrations in the filtrates were determined by isotachophoretic analysis. In order to obtain separate potential gradients for phosphate and EDTA, we changed the pH value of the leading solution to 4.20. Standard curves were obtained by the same procedure using dilute $NaH_2PO_4 \cdot 2H_2O$ or $C_2O_4H_2 \cdot 2H_2O$ solutions. Other conditions were the same as those used for calculi models.

Results and Discussion

Solubilization of Calculi Models

The solubilities of the weakly acidic salts, $Ca_3(PO_4)_2$ and $Ca(C_2O_4)$, depend on the H^+ concentration. This trend in general persists in the presence of the solubilizers (see Table I). Citric acid remains relatively effective at higher pH, while the macrocyclic polyamines [18]ane N_6 and [16]ane N_5 are effective at lower pH. This opposite trend in maximum solubilities could be explained by the different mechanisms of solubilization: *i.e.* cation chelation with citric acid and anion chelation with the polyamines. To make citric acid a better donor ligand to cations, a higher pH is preferable. In contrast, chelation of polyamines with phosphate or oxalate anion would be more favorable with more protons attached. EDTA worked effectively at lower pH.

Urine contains various kind of free ions that may interfere with the solubilizing reagents. Therefore the effects of magnesium ion (added as $MgCl_2$), calcium ion ($CaCl_2$) and phosphate anion (NaH_2PO_4) were examined. The results, summarized in Table I, indicate that free calcium ion interferes with the solubilizing activities of all the chelators above pH 5.9, while it

TABLE I. Solubilization of Calcium Phosphate with Various Chelating Agents[a)]

	Solubility (mM) pH				
	4.1	5.0	5.9	7.0	8.0
1 Control[b)]	7.9±0.6	2.1±0.0	0.46±0.02	0.25±0.03	0.36±0.02
2 [18]ane N_6	13.0±1.3	3.2±0.0	0.65±0.06	0.30±0.03	0.29±0.01
3 +$CaCl_2$	20.2±2.0	4.2±0.4	0.46±0.02	0.19±0.03	0.19±0.11
4 +$MgCl_2$	25.2±3.7	5.5±0.4	0.89±0.17	0.32±0.03	0.27±0.09
5 [16]ane N_5 +NaH_2PO_4	13.3±0.5	2.9±0.1	0.47±0.20	0.26±0.13	0.41±0.00
6 [16]ane N_5	14.9±1.2	3.6±0.3	0.66±0.08	0.33±0.01	0.25±0.08
7 +$CaCl_2$	23.9±1.9	5.0±0.5	0.69±0.22	0.16±0.06	0.18±0.06
8 +$MgCl_2$	27.2±2.2	6.6±0.8	0.98±0.17	0.30±0.01	0.37±0.04
9 +NaH_2PO_4	14.7±0.0	3.7±0.6	0.85±0.11	0.18±0.02	0.41±0.05
10 EDTA	11.3±0.6	4.8±0.6	1.17±0.22	0.50±0.07	0.41±0.03
11 +$CaCl_2$	17.5±1.3	4.4±0.1	1.28±0.24	0.32±0.12	0.18±0.02
12 +$MgCl_2$	20.3±1.8	5.0±1.4	1.95±0.47	0.39±0.05	0.29±0.02
13 +NaH_2PO_4	11.7±0.8	3.3±0.4	1.17±0.02	—	—
14 $CaCl_2$	13.9±0.9	2.4±0.0	0.49±0.20	0.23±0.04	0.06±0.02
15 $MgCl_2$	13.9±1.4	3.6±0.4	0.87±0.02	0.36±0.17	0.22±0.02
16 NaH_2PO_4	7.8±0.0	1.8±0.2	1.16±0.02	0.16±0.04	0.44±0.21
17 Control[c)]	7.5±0.5	2.2±0.1	0.16±0.02	0.18±0.01	0.11±0.01
18 Citric acid	7.1±0.5	2.4±0.2	1.23±0.15	1.55±0.06	1.14±0.18
19 +$CaCl_2$	6.6±0.7	2.2±0.1	1.00±0.02	1.03±0.15	0.66±0.16
20 +$MgCl_2$	6.6±0.9	1.9±0.0	0.87±0.05	1.03±0.15	0.55±0.04
21 +NaH_2PO_4	6.3±0.5	2.5±0.1	1.52±0.08	2.33±0.44	2.30±0.10

a) For the concentration of additives, see the text. All the experiments were repeated at least three times and the values given are mean values.
b) 1—16 were determined by the isotachophoretic analysis.
c) 17—21 were determined spectrophotometrically, since isotachophoretic analysis was not satisfactory.

TABLE II. Solubilization of Calcium Oxalate with Chelators[a)]

	Solubility (mM) pH		
	4.1	5.0	5.9
Control	0.26±0.13	0.12±0.08	0.17±0.02
[18]ane N_6	0.29±0.11	0.21±0.10	0.19±0.10
[16]ane N_5	0.15±0.03	0.26±0.08	0.18±0.03
EDTA	0.26±0.01	0.43±0.00	0.56±0.05
Citric acid	0.24±0.05	0.17±0.03	0.21±0.01

a) See footnote *a*) in Table I.

promotes solubilization below pH 5.0.

Free calcium ion itself assists the dissolution of calcium phosphate in acidic solutions, as does free magnesium ion. The presence of magnesium ion increases the effect of macrocyclic polyamines substantially. Generally, addition of NaH_2PO_4 had a minor negative effect on the chelator action, especially in the low pH range, which could be accounted for by the common ion effect. Although multiprotonated macrocyclic polyamines are likely to bind to free phosphate anion, the solubilizing activities were not much affected at pH 4.0.

Oxalate is the major component of stones, and calcium oxalate is less water-soluble than

TABLE III. Solubilization of Human Urinary Calculi in Terms of Phosphate and Oxalate Anions with EDTA and [18]ane N_6[a]

		Solubility (mM)					
		A[b]		B[c]		C[d]	
		Phosphate	Oxalate	Phosphate	Oxalate	Phosphate	Oxalate
pH 4.0	Control	7.3±0.8	0	7.6±0.9	0	12.7±1.1	0
	EDTA	31.4±4.5	0.10±0.02	30.4±4.2	0	29.6±3.7	0
	[18]ane N_6	14.5±1.7	0.36±0.12	14.1±1.5	0	28.7±3.8	0.11±0.06
pH 5.0	Control	1.9±0.6	0	2.3±0.2	0	4.5±0.6	0
	EDTA	15.3±1.3	0.16±0.01	13.5±1.5	0.08±0.00	16.9±1.8	0
	[18]ane N_6	4.7±0.9	0.37±0.13	4.8±0.6	0.10±0.03	19.4±2.4	0

a) See footnote *a*) in Table I. *b*) Ureteral calculus. *c*) Renal calculus. *d*) Bladder calculus.

calcium phosphate. Therefore, solubilization of calcium oxalate poses the most important and interesting problem. Our results in Table II suggest that EDTA is the most effective at higher pH and that, at lower pH, [18]ane N_6 may be the best solubilizer.

Solubilization of Human Urinary Calculi

Human calculus contains calcium phosphate and calcium oxalate as major components, and also proteins, cystine, minerals, *etc.*,[3,21] which may interfere with the solubilization of the major components. We evaluated the activities of the chelators with free phosphate and oxalate anions in solution (Table III).

The amounts of the anions freed depended significantly on pH and almost paralleled the behavior of the calculi models described above. For the calculi, A, B and C, EDTA and [18]ane N_6 were effective solubilizers of phosphate anion. For liberation of oxalate anion, [18]ane N_6 showed a stronger activity than EDTA, although on the model calcium oxalate, EDTA worked better at higher pH. Complex effects of the various minor components in calculi may have reversed the trend observed in the model. Although producing less solubility enhancement than anticipated on the basis of the calcium phosphate experiments, the macrocyclic polyamines are still interesting as potential solubilizers of oxalate salts since none of the currently available irrigating fluids act successfully in this respect.[22]

Conclusions

From the present results on calculi models and human urinary calculi, the following conclusions can be drawn regarding the possible use of macrocyclic polyamines as calculi-solubilizers.

(1) Macrocyclic polyamines having the ability to complex with polyanions ("anion chelators") can dissolve urinary calculi. Probably because of their anion chelating actions, they work more effectively in acidic media than the conventional cation chelators such as citric acid and EDTA. (2) Free ions such as Mg^{2+}, Ca^{2+}, $(HPO_4)^{2-}$ present in urine may not greatly affect the chelating activities of the macrocyclic polyamines at low pH. (3) pH is the most important factor in the solubilization, and the macrocyclic polyamines should be more effective under acidic conditions. (4) For the treatment of urinary lithiasis using an indwelling catheter and irrigating fluid, the macrocyclic polyamines may be used in acidic solution and their action may not be much affected by other minor components. (5) To take

advantage of the different dissolution mechanisms, macrocyclic polyamines can be used together with EDTA or citric acid for maximum effect. We are now attempting (*e.g.*, by chemical modification of the anion chelators) to develop a practical irrigating fluid containing macrocyclic polyamines.

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