[Chem. Pharm. Bull. 33(2) 655—661 (1985)]

A New Macrocyclic Hexaamine Ligand for the Dissolution of Human Inorganic Calculi

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(Received June 11, 1984)

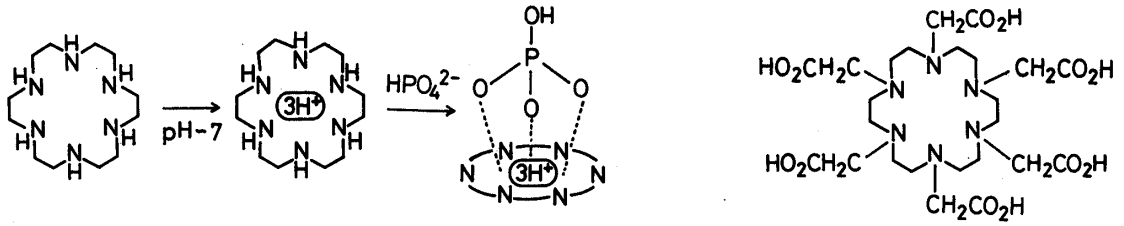
A newly synthesized 18-membered hexaamine carrying an acetate moiety on each nitrogen, L^2, has been demonstrated to be a more efficient dissolving agent that ethylenediaminetetraacetic acid for human urinary calculi whose major components are calcium phosphate and magnesium phosphate. L^2 is a good chelating agent for Ca^{2+} and Mg^{2+}, and this chelating action is presumably responsible for the dissolution of inorganic calculi.

Keywords——urinary calculus; macrocyclic polyamine; dissolution; ethylenediaminetetraacetic acid

Introduction

Of human urinary calculi, nearly 90% are composed of calcium phosphate, calcium oxalate, mixed calcium oxalate phosphate, or mixed calcium phosphate–magnesium ammonium phosphate.[1] Recently developed physical techniques such as the application of shock waves or laser irradiation can disintegrate calculi and may be alternatives to the surgical removal of renal calculi in many cases. However, the problem still remains of dealing with elusive residual particles. Hence chemical dissolution of inorganic stones by irrigation of the renal pelvis may still be a useful treatment. The main solubilizers in clinical use are ethylenediaminetetraacetic acid (EDTA) and citric acid.[2–7] Their clinical application, however, seems to be limited because of irritation or the need for prolonged hospitalization.[8] Thus, less irritating and more efficient solvents are required.

The conventional solvents can dissolve inorganic calculi by two mechanisms. Citric acid serves to lower the urinary pH, increasing the solubility of calcium and magnesium salts of weak acids. EDTA has a chelating action on calcium and magnesium cations. Previously, we reported that macrocyclic polyamines such as 18-azacrown-6, L^1, can dissolve inorganic



18-azacrown-6 L^1 | $L^1 \cdot 3H^+$ | $L^1 \cdot 3H^+$–phosphate 1 : 1 complex | L^2 1,4,7,10,13,16-hexacarboxymethyl 1,4,7,10,13,16-hexaazacyclooctadecane

calculi by an entirely different mechanism involving a polyanion chelating action.[9] The macrocyclic hexaamine L^1 can hold three protons within its cavity at weakly acidic to neutral pH and the resulting highly protonated cation H_3L^{3+} can bind with polyoxyanions such as phosphate and oxalate through electrostatic interaction as well as hydrogen bondings.[10,11] At acidic pH *ca.* 4, L^1 showed a higher ability to dissolve calculi than EDTA.[9] In this paper we report a new macrocyclic hexaamine carrying an acetate moiety on each nitrogen, L^2. This agent was developed in an attempt to combine the anion-seeking function of 18-azacrown-6 with the cation-seeking function of EDTA in order to obtain a more efficient agent for dissolving urinary calculi.

Experimental

Materials: Synthesis of $L^2 \cdot 6HCl$——Monochloroacetic acid (1.3 g) in 10 ml of water was added dropwise to a solution of sodium hydroxide (1 g) in 10 ml of ice-cooled water. Free base of L^1 (500 mg) in 10 ml of ethanol was then added dropwise to this solution. The reaction mixture was stirred in an oil-bath at 70 °C for 24 h. The solvent was evaporated off, and the residue was redissolved in water and acidified with hydrochloric acid. The mixture was extracted three times with methylene chloride to remove the excess monochloroacetic acid. The water layer was evaporated, and the residue was recrystallized from 6 N HCl to give 1 g of $L^2 \cdot 6HCl$ (63% yield) as colorless needles. Nuclear magnetic resonance (NMR) (D_2O); δ (external TMS) 3.63 (macrocyclic ring methylene, 24H) 4.02 (acetate methylene, 12H). *Anal.* Calcd for $C_{24}H_{42}N_{16}O_{12} \cdot 6HCl$: C, 34.92; H, 5.13; N, 10.18. Found. C, 34.70; H, 5.30; N, 10.23.

All the other chemicals used were of analytical reagent grade. The buffer solutions used in the present study were 0.1 M acetate buffer (pH 4.0, 5.0 and 5.7), 0.05 M collidine–HCl buffer (pH 7.0), and 0.05 M Tris–HCl buffer (pH 8.0). The buffers had negligible effects on the dissolving activities of macrocyclic polyamines. The human calculi used in this work were obtained from the urinary tract of patients with urinary lithiasis.

Dissolution Test for Calculi Model——An accurately weighed mineral calculi model $Ca_3(PO_4)_2$ (5 mg, 5.3 mM), $Ca(C_2O_4) \cdot H_2O$ (3 mg, 6.8 mM), or $Mg_3(PO_4)_2 \cdot 8H_2O$ (7 mg, 5.7 mM) was placed in triplicate in test tubes with a dissolving agent $L^1 \cdot 3H_2SO_4$, L^2 6HCl or EDTA (all at 10 mM) in 3 ml of a suitable buffer solution. Immediately after being shaken in a water bath at 37 °C for 1 h (90 times/min), the mixture was filtered through a filter paper (Toyo Roshi No. 2) and the appropriately diluted filtrates were subjected to ionic analysis as follows.

Atomic Absorption Spectrophotometry——Calcium and magnesium concentrations of sample solutions were determined with a Shimadzu AA-646 atomic absorption spectrophotometer. Acetylene was used as a fuel gas and compressed air as a support gas. Other conditions were as follows. For calcium determination: wavelength, 422.7 nm; lamp current, 8 mA; slit width 3.8 Å. For magnesium determination: wavelength, 285.3 nm; lamp current, 5 mA; slit width, 3.8 Å. Sample solutions were diluted suitably and injected for analysis. Standard curves for calcium and magnesium were obtained by using diluted $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$ solution, respectively.

Isotachophoretic Analysis——Phosphate concentrations of sample solutions were determined with a Shimadzu IP-ZA isotachophoretic analyzer, equipped with a pre-column (1 mm i.d. × 40 mm) and an analyzing column (0.5 mm i.d. × 100 mm), and a potential gradient detector. A buffer solution of 10 mM HCl–β-alanine (pH 3.7) was used as a leading solution, and the terminal solution was 10 mM aqueous *n*-capronic acid. Aliquots of 50 ml of sample solutions were injected and the analysis was done by applying 125 μA electric current for the first 10 min and then 100 μA at 20 °C. A standard curve for phosphate was obtained by using dilute $NaH_2PO_4 \cdot 2H_2O$ solution. All the solubility tests were performed in triplicate. The mean values (the deviation was within $\pm 10\%$) are listed in all the tables.

Dissolution Test for Human Urinary Calculi *in Vitro*——Well-ground human calculus (10 mg) and 5 mM chelating agent were placed in a group of test tubes, to which 50 mM collidine–HCl buffer (pH 7.0) was added to make up 3 ml of solution. After being shaken in a water bath at 37 °C for 1 h (90 times/min), each mixture was immediately filtered through a filter paper (Toyo Roshi No. 2) and the filtrate was subjected to analysis. Concentrations of dissolved calcium and magnesium ion were determined by atomic absorption spectrophotometry.

pH-Metric Titration——The aqueous solutions of $L^2 \cdot 6HCl$ were titrated potentiometrically with 0.1 N NaOH solution in the absence or in the presence of $MgCl_2$, $CaCl_2$ or NaH_2PO_4 under a nitrogen atmosphere at 25 °C and I = 0.2 M (see Fig. 1). The mixed protonation constants K_i were determined from pH readings. The values of $-\log[H^+]$ (for calculation of K_{ML}) were estimated from pH readings: $-\log[H^+] = pH - 0.13$.

Results and Discussion

A New Macrocyclic Hexaamine Ligand L^2

The mixed protonation constants log K_i defined by Eq. 1 were 10.10, 10.01, 8.96, and 8.20

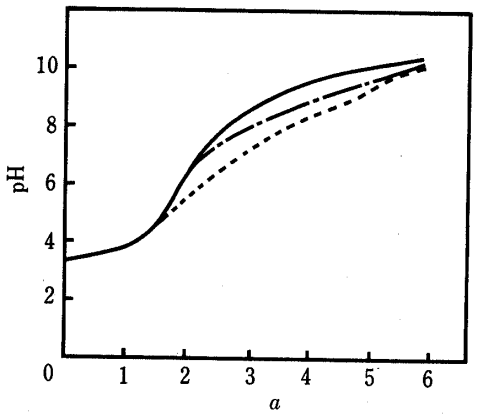
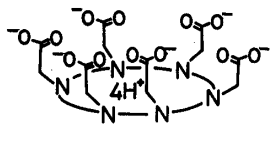


Fig. 1. pH-Titration Curves of $L^2 \cdot 6H^+$; 1 mM Ligand Only (——), in the Presence of Equivalent Mg^{2+} (– – – –) and Ca^{2+} (------) at 25 °C and I 0.2 M ($NaClO_4$)



I

$$K_i = \frac{[H_iL^{i+}]}{[H_{i-1}L^{(i-1)+}][H^+]} \quad (1)$$

for the four most basic amines K_1—K_4. The values for the other weaker bases including the six carboxylates were all less than 5. Since the $\log K_{1-6}$ values of the skeleton macrocycle 18-azacrown-6 L^1 are 10.19, 9.23, 8.73, 4.09, *ca.* 2, and *ca.* 1,[12] it can be deduced that the initial four protonations occur at the macrocyclic amines. Thus, the most abundant species of L^2 at pH *ca.* 7 can be depicted as I.

The potentiometric titration curves in the presence of equimolar calcium or magnesium ion (Fig. 1) showed that calcium ion binds more strongly than magnesium. The 1 : 1 complexation constant K_{ML} was calculated by using Eq. 1.[12]

$$K_{ML} = \frac{\{\alpha(\alpha_H)_L - \beta_H C_L\}\{6(\alpha_H)_L - \beta_H\}}{(6C_L - \alpha)^2(\alpha_H)_L} \quad (2)$$

The plots of the titration curve for Mg^{2+} ($8 < a < 12$) fit Eq. 2 and give a K_{MgL} value of $1.7 \times 10^8\ M^{-1}$. On the other hand, the plots of titration data for Ca^{2+} ($8 < a < 11$) are not consistent with Ca^{2+}–L (unprotonated form) complex formation, but rather fit Eq. 3[12] for the

$$K_{MHL} \cdot [H^+]K_1 = \frac{\{5(\alpha_H)_L - \beta_H\}\{\alpha(\alpha_H)_L - \beta_H C_L\}}{(5C_L - \alpha)^2(\alpha_H)_L} \quad (3)$$

$$\text{where} \quad K_{MHL} = \frac{[MHL]}{[M][HL]} \quad (4)$$

Ca^{2+}–HL^+ (monoprotonated form) complex to give the K_{MHL} value of 8.6×10^7. However, one cannot totally exclude 2 : 1 Ca^{2+}–L^2 complexation, especially when a large excess (11 times) Ca^{2+} is used under basic conditions. The Dreiding model suggests that two Ca^{2+} ions can be placed on opposite sides of the N_6 plane, each being accommodated by three N's and three carboxylates.

The calculus dissolution ability of L^2 was compared with those of EDTA and the macrocyclic hexaamine L^1. EDTA is currently the best litholytic agent in clinical use.[6] Since the three ligands can all bind with alkaline earth metal salts in 1 : 1 ratio at physiological pH, we prepared ligand solutions at the same molar concentrations and measured [Ca^{2+}] or [Mg^{2+}] dissolved from inorganic calculi models or human calculi.

The present work constitutes the first *in vitro* investigation of the intrinsic solvability of inorganic materials to provide basic data for studies on calculi, which may contain a proteinaceous matrix.

Dissolution of Calculi Models, $Ca_3(PO_4)_2$, $Ca(C_2O_4)$ and $Mg_3(PO_4)_2$

1. Cation Concentration and Anion Concentration in Dissolved $Ca_3(PO_4)_2$——In the dissolution of calcium phosphate with various chelating agents, the concentration of freed calcium cation was measured by atomic absorption spectrophotometry and the concentration of freed phosphate anion by isotachophoretic analysis. The results (Table I) were consistent with the expected 3:2 Ca^{2+}–PO_4^{3-} ratio in the solution. A similar result was obtained in acetate buffer (pH 4). Hence, we subsequently estimated the extent of dissolution of inorganic salts and calculi on the basis of cation concentration.

2. Effects of Reaction Time on the Dissolution of $Ca_3(PO_4)_2$——Upon exposure to the chelating agents L^2 and EDTA, the insoluble $Ca_3(PO_4)_2$ was seen to begin disappearing, but then no further dissolution was visually apparent. The quantitative measurements (Table II) indicate that the dissolution of $Ca_3(PO_4)_2$ with EDTA almost stops at the stage of 1:1 Ca^{2+}–

TABLE I. Concentrations of Ca^{2+} and PO_4^{3-} Released from Insoluble $Ca_3(PO_4)_2$ Powder (10 mg)[a] by Various Chelating Agents (10 mM) in 0.05 M, pH 7.0 Collidine Buffer (3 ml) at 37 °C for 1 h

Ligand	Free $[Ca^{2+}]$[b] in ppm	Free $[PO_4^{3-}]$[c] in ppm	$[Ca^{2+}]/[PO_4^{3-}]$ ratio
None	21	16	
	(0)	(0)	
L^1	157	92	3.6:2
	(136)[d]	(76)[d]	
L^2	832	556	3:2
	(811)[d]	(540)[d]	
EDTA	354	512	3:2
	(333)[d]	(496)[d]	

a) 10 mg of $Ca_3(PO_4)_2$, if completely dissolved, corresponds to 10.7 mM.
b) Measured by atomic absorption spectrophotometry.
c) Measured by isotachophoretic analysis.
d) Concentration of ion dissolved by complexation with ligand.

TABLE II. Time Course of Dissolution (in ppm)[a] of $Ca_3(PO_4)_2$ Powder (20 mg) by L^2 and EDTA (Both 3 mM) at pH 7 (Collidine Buffer) and 37 °C

Ligand	Reaction time					
	20 min	40 min	1 h	2 h	4 h	6 h
None	14	15	16	16	23	43
	(0)	(0)	(0)	(0)	(0)	(0)
L^2	202	212	219	232	254	285
	(188)[b]	(197)[b]	(203)[b]	(216)[b]	(231)[b]	(242)[b]
EDTA	134	128	131	139	145	166
	(120)[b]	(113)[b]	(115)[b]	(123)[b]	(122)[b]	(123)[b]

a) The theoretical value is 134 ppm, provided that a ligand forms a 1:1 complex with Ca^{2+}.
b) Ca^{2+} concentration dissolved by complexation with ligand.

TABLE III. Concentration (in ppm) of Ca^{2+} Released from $Ca_3(PO_4)_2$ (5 mg) by Chelating Agents (10 mM) at Various pHs[a]

Chelate	pH				
	4.0[b]	5.0[b]	5.9[b]	7.0[c]	8.0[d]
None	252	176	18	23	13
	(0)	(0)	(0)	(0)	(0)
L^1	437	212	99	113	72
	(185)	(36)	(81)	(90)	(59)
L^2	374	463	400	510	432
	(122)	(287)	(382)	(487)	(419)
EDTA	315	453	370	307	282
	(63)	(277)	(352)	(284)	(269)

a) At 37 °C; reaction time 1 h. If a ligand (10 mM) shows complete 1 : 1 complexation with Ca^{2+} in solution, the theoretical [Ca^{2+}] value should be 401 ppm.
b) 0.2 M acetic acid–sodium acetate buffer (3 ml).
c) 0.05 M collidine–HCl buffer (3 ml).
d) 0.05 M Tris–HCl buffer (3 ml).

TABLE IV. Dissolution of $Ca_3(PO_4)_2$ (5 mg), $Ca(C_2O_4)$ (3 mg) and $Mg_3(PO_4)_2$ (7 mg) by Chelating Agents (10 mM) in pH 7.0 Collidine Buffer (3 ml)

Chelate	Calculus models		
	$Ca_3(PO_4)_2$ Free [Ca^{2+}] in ppm	$Ca(C_2O_4)$ Free [Ca^{2+}] in ppm	$Mg_3(PO_4)_2$ Free [Mg^{2+}] in ppm
Control	23	17	126
	(0)	(0)	(0)
L^1	113	28	302
	(90)[a]	(11)[a]	(176)[b]
L^2	510	222	316
	(487)[a]	(205)[a]	(190)[b]
EDTA	307	309	303
	(284)[a]	(292)[a]	(177)[b]

a) If a ligand (10 mM) captures Ca^{2+} in a 1 : 1 complex in solution, the theoretical [Ca^{2+}] value would be 401 ppm.
b) The theoretical [Mg^{2+}] value is 243 ppm.

L complexation. With L^2, the dissolution continues beyond 1 : 1 complexation stoichiometry.

3. Effects of pH Change on the Dissolution of $Ca_3(PO_4)_2$——Dissolution of $Ca_3(PO_4)_2$ with the chelating agents at various pH values is shown in Table III. The macrocyclic polyamine ligand L^1 promotes dissolution of calcium phosphate at acidic pH (due to the phosphate-chelating action). On the other hand, the caxboxylated ligand L^2 and EDTA are more effective (due to cation-chelating actions) at higher pH, and most significantly L^2 has the optimum pH at 7.0.

4. Dissolution of $Ca_3(PO_4)_2$, $Ca(C_2O_4)$ and $Mg_3(PO_4)_2$——The effects of cation and anion composites were tested by comparing the solubilizations of $Ca_3(Po_4)_2$, $Ca(C_2O_4)$, and $Mg_3(PO_4)_2$. The results are summarized in Table IV. L^2 is more effective in dissolving the calcium salt than the magnesium salt, which is also the case with EDTA. Most interestingly, the dissolution of Ca^{2+} from $Ca_3(PO_4)_2$ is achieved more effectively with L^2 than with EDTA.

TABLE V. $[Ca^{2+}]$ and $[Mg^{2+}]$ Dissolved from Human Urinary Calculi[a] by Various Chelating Agents at 37 °C for One Hour in 3 ml of 0.05 M Collidine Buffer (pH 7)

Ligand 5 mM	Calculus-1 Composition[b] $Ca_3(PO_4)_2$ 78% $CaCO_3$ 12%	Calculus-2 $Ca(C_2O_4)$ 69% $Ca_3(PO_4)_2$ 31%	Calculus-3 $MgNH_4PO_4$ 93% $CaCO_3$ 7%	Calculus-4 $Ca_3(PO_4)_2$ 85% $CaCO_3$ 15%	Calculus-5 $Ca(C_2O_4)$ >98%
	$[Ca^{2+}]$ in ppm				
None	22	21	18	19	26
L^1	71	69	31	43	31
	(59)	(48)	(13)	(24)	(5)
L^2	254	290	224	342	82
	(232)	(269)	(206)	(323)	(56)
EDTA	143	168	89	140	173
	(121)	(147)	(71)	(121)	(147)
	$[Mg^{2+}]$ in ppm				
None	31	3	90	37	2
L^1	43	4	103	51	2
	(12)	(8)	(13)	(14)	(0)
L^2	54	8	149	73	2
	(23)	(5)	(59)	(36)	(0)
EDTA	45	5	131	54	2
	(14)	(2)	(41)	(17)	(0)

a) Calculus weight 10 mg, except calculus No. 1 (6 mg).
b) Analyzed by infrared spectral measurement (Japan Special Reference Laboratory, Inc.).

The fact that L^2 takes nearly two equivalents of Ca^{2+} seems to indicate that L^2 is capable of forming a 2:1 Ca^{2+} complex. However, when the anion is oxalate, dissolution of Ca^{2+} is drastically reduced with L^2. On the other hand, EDTA can dissolve $Ca(C_2O_4)$ to the same extent as $Ca_3(PO_4)_2$, *i.e.*, the anion effect is insignificant.

Dissolution Tests of Human Urinary Calculi *in Vitro*

Five human urinary calculi containing various proportions of $Ca_3(PO_4)_2$, $Ca(C_2O_4)$, $CaCO_3$ and $MgNH_4(PO_4)$ were subjected to dissolution tests at pH 7.0 in the same manner as described for the models (Table V). The dissolution patterns of the model phosphate and oxalate stones were found to resemble those of real phosphate and oxalate calculi. For the dissolution of the phosphate stones No. 1—4, L^2 was more effective than L^1 and EDTA. For oxalate stone No. 5, EDTA was superior. These chelating agents were all found to be ineffective for organic stones containing uric acid, 2,8-dihydroxyladenine, or cystine.

Conclusion

(1) The newly synthesized macrocyclic hexaamine hexaacetate ligand L^2 is a strong chelating agent for Ca^{2+} and Mg^{2+}. The 1 : 1 complexation constants are comparable to those for EDTA.[13] However, L^2 can capture more Ca^{2+}, probably by accommodating 2 mol of Ca^{2+}. Thus, L^2 is a better dissolving agent for $Ca_3(PO_4)_2$ than EDTA. The dissolution by these chelating agents occurs immediately upon mixing.

(2) In dissolving inorganic calculi, L^2 is most effective at pH 7.

(3) Its mechanism is probably a cation-chelating action like that of EDTA, in contrast to the anion-chelating mechanism of skeleton ligand L^1.[9]

(4) As a litholytic agent for phosphate stones, L^2 is generally more efficient than EDTA.

However, for oxalate stones, EDTA is better.

We are now investigating the toxicity of L^2 with the aim of further development of this compound for use as a novel litholytic agent.

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