MIXED CHELATES OF Ca(II)-PYRIDINE-2,6-DICARBOXYLATE WITH SOME AMINO ACIDS RELATED TO BACTERIAL SPORES

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ABSTRACT The high resistance of bacterial spores to heat has been repeatedly postulated to be due to stabilization of spore biopolymers by metal chelate compounds. Binding of calcium dipicolinic acid (Ca(II)-DPA) with spore proteins and amino acids has been discussed in the literature, but equilibrium data are generally lacking. By means of potentiometric pH titrations at 25°C and an ionic strength of 1.0 (KNO_3), the formation of Ca(II)-DPA (1:1 and 1:2) chelates and the interactions of Ca(II)-DPA chelate with a mole of each of three typical amino acids viz., cysteine, alanine, and glycine has been investigated. Analysis of the potentiometric data indicates that calcium and DPA forms 1:1 and 1:2 chelates with log K_{ML_1} = 4.39 ± 0.01 and log K_{ML_2} = 2.25 ± 0.01. In the presence of an equimolar amount of each of the amino acids under consideration, the Ca(II)-DPA chelate forms mixed ligand (ternary) chelate yielding the following stepwise stability constants: log K_1 = 4.17 ± 0.01, log K_2 = 0.78 ± 0.01 for cysteine, log K_1 = 4.06 ± 0.01, log K_2 = 0.65 ± 0.01 for alanine, and log K_1 = 4.30 ± 0.02, log K_2 = 0.11 ± 0.01 for glycine. Methods for calculating the stability constants of the mixed ligand system have been developed. On the basis of the potentiometric equilibrium data, possible structures for the various calcium chelate species are discussed. The data suggest that the differences in heat resistance of various strains of bacterial spores may conceivably be related to the differences in composition and stability of coordination complexes in the spore.

INTRODUCTION

There are two major lines of reasoning used to explain the mechanism of the high resistance of bacterial spores to heat: (*a*) a dehydrated or hydrophobic spore interior has been suggested since the turn of the century (6, 10, 20) and (*b*) stabilization of spore biopolymers by cross-linking between macromolecules (2) or by chelation involving pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA), calcium and spore amino acids, and peptides (13, 9, 17, 7).

These two ideas are not necessarily contradictory; in fact, it has been suggested that chelation may be responsible for the low hygroscopicity of spores (8). The

hypothesis postulates that coordination bonds involving DPA, amino acids, and cations such as Ca(II), Mg(II), etc., may form a protective cement of low polarity, low hygroscopicity, and generally low chemical reactivity. This spore cement may protect vital spore biopolymers against thermal denaturation by a "masking" or "caging" effect.

The molecular masking hypothesis is based on the unique abundance of DPA and calcium in bacterial spores. The spores contain 5–15% of DPA (on dry weight basis) whereas the parent vegetative cells contain no DPA at all. DPA is extremely rare in nature and its biological function is not known. The calcium content of spores is about 10 times that of parent vegetative cells.

All bacterial spores contain DPA and calcium (or other cations) and yet spores of different strains vary greatly in heat resistance. The amount of Ca(II)-DPA could not be correlated with heat resistance (5, 29, 12). It seems logical to assume that the hypothetical "coordination cement" in the spore might consist of more than just calcium and DPA. The involvement of amino acids or peptides might explain individual variations in heat resistance between strains since different quantities of amino acids would be expected to be found in different strains.

Past studies of spore coordination complexes (17, 7) have been restricted to DPA-cation interactions. Thermodynamic studies of mixed ligands involving amino acids and peptides in addition to cation-DPA became complicated because of the occurrence of overlapping equilibrium and of the necessary mathematical treatments of the equilibrium data. The present study, therefore, attempts to evaluate the interaction of some amino acids with Ca(II)-DPA chelates and particularly to develop methods for the mathematical evaluation of mixed ligand chelates of this general nature.

Three amino acids of special importance to bacterial spores have been selected for study, viz; cysteine, alanine, and glycine. Cysteine is known to be predominant in the spore coats and has been postulated to play an outstanding role in resistance of spores to heat and radiation (28, 3). Alanine is a major component of spore glycopeptide which is a unique spore component and may be related to heat resistance (12). Furthermore, alanine has been frequently linked to spore germination (23); this suggests the possibility that alanine may induce germination by competing for spore cations and in this manner breaking the "spore cement." Glycine is a major component of the spore coat (11) which may be viewed as the first barrier in the defensive structure of the spore against deleterious environmental effects.

MATERIALS AND METHODS

Stock solutions of calcium ion were prepared by dissolving appropriate quantities of reagent grade calcium chloride in distilled water and standardized by means of the complexometric titration method described by Schwarzenbach (21). Aqueous solutions of ligand acids, i.e. dipicolinic acid, cysteine, alanine, and glycine were standardized potentiometrically. All stock solutions were 0.02 M in concentration.

The experimental procedure consisted of measuring the equilibrium hydrogen ion concentrations of the ligand acids in the absence of, and in the presence of, equimolar concentrations of calcium ion. A Corning Model 12 Research pH meter (Corning Glass Works, Corning, New York) fitted with glass and calomel extension electrodes was used to determine the hydrogen ion concentration. Measurements were made at 25°C and at an ionic strength of 1.00 maintained constant by the addition of KNO_3. The electrode system was calibrated with HCl and NaOH to give $-\log [H^+]$ values directly. Carbon dioxide free NaOH was used for the titration. The titration cell consisted of a jacketed beaker through which thermostated water was circulated to maintain any desired constant temperature. Prepurified nitrogen was bubbled into the titration cell throughout the entire experimental process.

RESULTS AND CALCULATIONS

The interaction of calcium ion in the following three systems was studied: (1) Ca(II)-DPA-chelate system, (2) Ca(II)-amino acid chelate system, and (3) mixed ligand system of Ca(II)-DPA-amino acid.

1. Calcium(II)-DPA Chelate System

Fig. 1 shows the potentiometric titration curves of the free ligand DPA and its calcium(II) chelate having 1:1 and 1:2 molar ratios. The free ligand titration curve (curve L) exhibits two distinct inflections after the addition of one and two equivalents of base. These inflections represent the dissociation of protons from the two carboxyl groups of the ligand. Acid dissociation constants for the free ligand were calculated by a simple algebraic method and the results are presented in Table I.

In the 1:1 chelate titration curve (Fig. 1, curve ML) the inflection at $m = 2$ could be accounted for by assuming the interaction of calcium ion with one mole of the ligand through the dissociation of two hydrogen ions from the two carboxyl groups of DPA. The depression of the two equivalent buffer regions in the 1:1 curve as compared to the free ligand curve (Fig. 1, curve L) is significant; it is a clear qualitative indication of the coordination of the metal ion with the ligand. The reaction of calcium ion and DPA which takes place between $m = 0$ to $m = 2$ could be represented by the following equation:

$$Ca^{2+} + H_2L \overset{K_{eq}}{\rightleftharpoons} CaL + 2H^+.$$

Therefore, the equilibrium constant K_{eq} for the above equation is:

$$K_{eq} = \frac{[CaL]\,[H^+]^2}{[Ca^{2+}]\,[H_2L]}$$

where H_2L represents DPA with its two dissociable hydrogen ions and CaL is the Ca(II)-DPA 1:1 chelate. The formation constant of Ca(II)-DPA chelate is represented by the following equation:

$$Ca^{2+} + L^{2-} \underset{}{\overset{K_{ML}}{\rightleftharpoons}} CaL \qquad (1)$$

where L^{2-} is the dissociated form of DPA. By employing the conventional material balance and electro-neutrality expressions, equation 2 could be deduced. Equation 2 contains all known quantities and thus enables the calculation of the formation

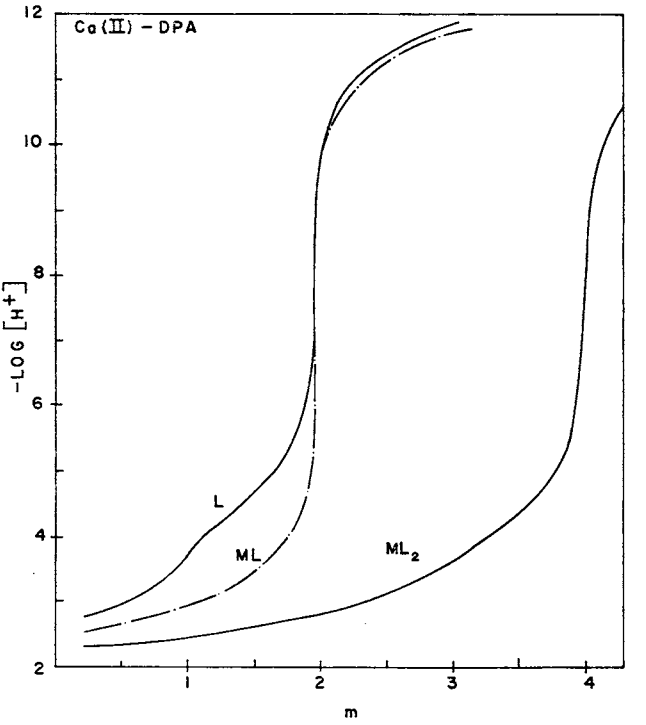


FIGURE 1 Potentiometric titration of dipicolinic acid. L = 4.0732 × 10^{-3} M dipicolinic acid; ML = Ca(II)-dipicolinate chelate (1:1), 4.0732 × 10^{-3} M; ML_2 = Ca(II)-dipicolinate chelate (1:2) 8.1464 × 10^{-3} M DPA and 4.0732 × 10^{-3} M Ca(II); μ = 1.0 (KNO_3); t = 25°C; m = moles of base added per mole of ligand.

constant, K_{ML}, of 1:1 calcium(II)-DPA chelate

$$K_{ML} = \frac{T_M - [L^{2-}]X}{[L^{2-}]^2 X} \qquad (2)$$

where

$$[L^{2-}] = \frac{2T_L - T_{OH} - [H^+]}{2\,[H^+]^2/k_1 k_2 + [H^+]/k^2}$$

$$X = \frac{[H^+]^2}{k_1 k_2} + \frac{[H^+]}{k_2} + 1.$$

The designations of each of the terms in the above expressions are as follows: (*a*) k_1 and k_2 are the acid dissociation constants of DPA. (*b*) T_M and T_L are the total molar concentration of the metal ion and ligand acid respectively. In the 1:1 ratio, these quantities are identical with each other. (*c*) T_{OH} is the total molar concentration of base added to the solution. The log of the formation constant of 1:1 calcium-(II)-DPA chelate calculated by equation 2 is presented in Table I.

The titration curve of 1:2 molar ratio of Ca(II) to DPA (Fig. 1, curve ML_2) shows an inflection at $m = 4$, which corresponds to the displacement of four hydrogen ions from two moles of the ligand. Furthermore, the single low buffer region

TABLE I

ACID DISSOCIATION CONSTANTS AND STABILITY CONSTANTS OF CALCIUM CHELATES

Ligand	Positive ion	Reaction	Log of formation constant
Dipicolinic acid, H_2L	H^+	$H^+ + L^{2-} \rightleftharpoons HL^-$	4.59 ± 0.02
		$H^+ + HL^- \rightleftharpoons H_2L$	2.28 ± 0.01
	Ca^{2+}	$Ca^{2+} + L^{2-} \rightleftharpoons CaL$	4.39 ± 0.01
		$CaL + L^{2-} \rightleftharpoons CaL_2^{2-}$	2.25 ± 0.01
Cysteine, H_2A	H^+	$H^+ + A^{2-} \rightleftharpoons HA^-$	8.28 ± 0.01
		$H^+ + HA^- \rightleftharpoons H_2A$	2.26 ± 0.02
	Ca^{2+}	$Ca^{2+} + HA^- \rightleftharpoons CaHA^+$	1.19 ± 0.11
Alanine, H_2A	H^+	$H^+ + A^{2-} \rightleftharpoons HA^-$	9.69 ± 0.02
		$H^+ + HA^- \rightleftharpoons H_2A$	2.55 ± 0.05
	Ca^{2+}	$Ca^{2+} + HA^- \rightleftharpoons CaHA^+$	1.56 ± 0.16
Glycine, H_2A	H^+	$H^+ + A^{2-} \rightleftharpoons HA^-$	9.65 ± 0.01
		$H^+ + HA^- \rightleftharpoons H_2A$	2.62 ± 0.02
	Ca^{2+}	$Ca^{2+} + HA^- \rightleftharpoons CaHA^+$	1.47 ± 0.14

$t = 25°C$; $\mu = 1.0$ (KNO_3).

exhibited in the 1:2 titration curve indicates that the interaction of calcium(II)-DPA chelate with the second mole of DPA took place in two continuous overlapping steps. The stepwise formation of 1:1 and 1:2 calcium(II)-DPA chelate is represented by the following equations:

$$Ca^{2+} + L^{2-} \overset{K_{ML_1}}{\rightleftharpoons} CaL \tag{3}$$

$$CaL + L^{2-} \overset{K_{ML_2}}{\rightleftharpoons} CaL^{2-}{}_2. \tag{4}$$

The stepwise formation constants K_{ML_1} and K_{ML_2} were calculated by a modification of Bjerrum's successive approximation method (1). A plot of the formation function is presented in Fig. 2.

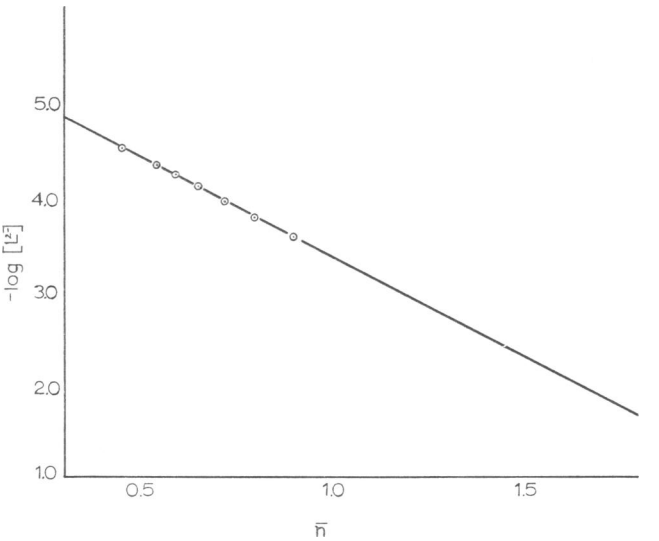


FIGURE 2 Stepwise formation constants of 1:1 and 1:2 Ca(II)-DPA chelate. $\bar{n}$ = average number of moles of ligand bound per mole of Ca(II) ion. L^{2-} represents the binegative anion of DPA.

From the material balance equations, K_{ML_1} and K_{ML_2} are expressed by the following related equations:

$$K_{ML_1} = \frac{1}{[L^{2-}]}\left[\frac{\bar{n}}{(1-\bar{n}) + K_{ML_2}[L^{2-}](2-\bar{n})}\right] \tag{5}$$

$$K_{ML_2} = \frac{1}{[L^{2-}]}\frac{\bar{n}/K_{ML_1}[L^{2-}] + (\bar{n}-1)}{(2-n)} \tag{6}$$

where

$$[L^{2-}] = \frac{2T_L - T_{OH} - [H^+]}{2[H^+]^2/k_1k_2 + [H^+]/k_2}$$

and

$$\bar{n} = \frac{T_L - [L^{2-}]X}{T_M}.$$

All the terms, i.e. T_M, T_L, T_{OH}, X, k_1, and k_2 have the same meaning as designated previously. $\bar{n}$ is the average number of moles of ligand bound per mole of metal at each pH reading on the titration curve. The first approximated value for K_{ML_1} would correspond to the reciprocal value of L^{2-} at $\bar{n} = 0.5$. Similarly, the first approximated value for K_{ML_2} would correspond to the value of $1/L^{2-}$ at $\bar{n} =$

1.5. By substituting these approximate values of K_{ML_1} and K_{ML_2}, equations 5 and 6 were solved by means of successive approximations based on two sets of experimental data.

The K_{ML_1} and K_{ML_2} for the interaction of calcium(II) and DPA thus determined and reported in Table I are the averaged values from two sets of approximations. It can be seen that the interaction of calcium(II)-DPA chelate with the second mole of DPA has a smaller affinity than that of the first mole.

2. *Calcium(II)-Amino Acid Chelate System*

The titration curves of free amino acids, cysteine, alanine, and glycine are presented in Figs. 3, 4, and 5, respectively (curve *A* in each figure). The inflection at $m = 1$ represents the dissociation of the carboxyl hydrogen ion from the amino acid. The dissociation constants of each of the amino acids studied are presented in Table I. Titration curves of the 1:1 Ca(II)-cysteine, Ca(II)-alanine, and Ca(II)-glycine are also presented in Figs. 3, 4, and 5 (curves *MA*).

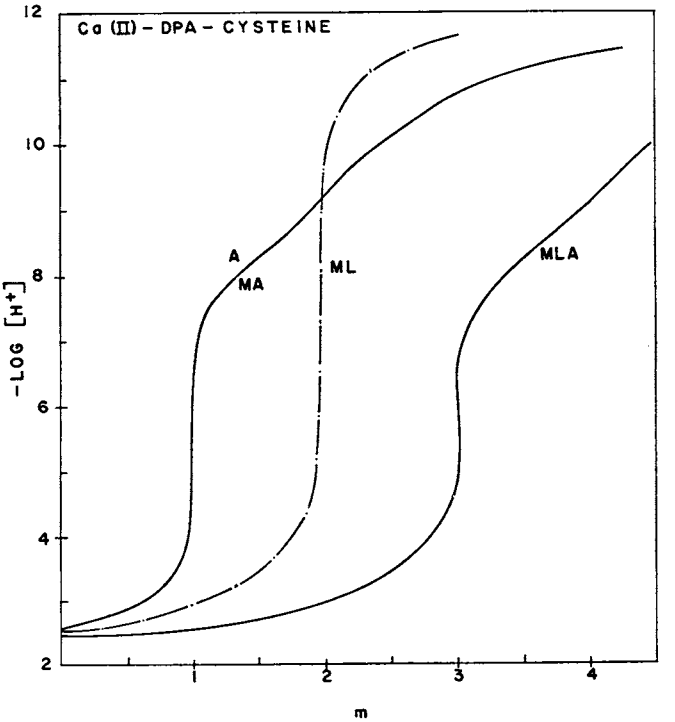


FIGURE 3 Potentiometric titration of cysteine and related systems. $A = 4.2014 \times 10^{-3}$ M cysteine; MA = Ca(II)-cysteine chelate (1:1), 4.2014×10^{-3} M; ML = Ca(II)-dipicolinate chelate (1:1), 4.0732×10^{-3} M; MLA = DPA-Ca(II)-cysteine chelate (1:1:1), 4.2014×10^{-3} M; m = moles of base added per mole of ligand; $\mu = 1.0$ (KNO_3); $t = 25°C$.

The discussion presented here is applicable to all three systems. The Ca(II)-amino acid chelate curve (*MA*) from $m = 0$ to $m = 1$ was depressed to a very small extent over that of the free ligand (*A*), although this is not distinctly shown in the titration curves traced in Figs. 3, 4, and 5. The interpretation that the carboxyl oxygen is involved in coordination with calcium is based on this slight depression of the Ca(II)-amino acid curve. It should be noted that the free ligand is the

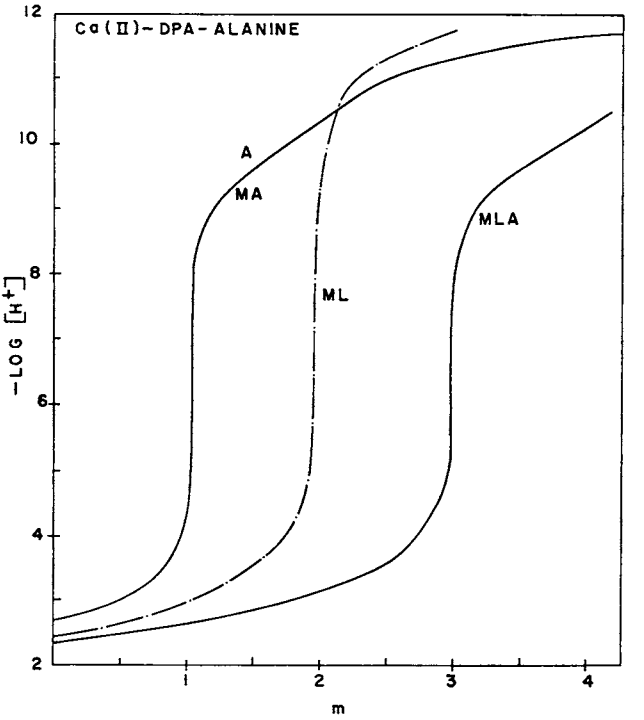


FIGURE 4 Potentiometric titration of alanine and related systems. $A = 4.0007 \times 10^{-3}$ M alanine; *MA* = Ca(II)-alanine chelate (1:1), 4.0007×10^{-3} M; *ML* = Ca(II)-dipicolinate chelate (1:1), 4.0732×10^{-3} M; *MLA* = DPA-Ca(II)-alanine chelate (1:1:1), 4.0007×10^{-3}; m = moles of base added per mole of ligand; $\mu = 1.0$ (KNO_3); $t = 25°C$.

protonated form of the respective amino acid and as such the first buffer region corresponds to the dissociation of the proton from the carboxylic acid group.

The reaction of calcium(II) and amino acid taking place at $m = 0$ to $m = 1$ could be represented by the following equation:

$$Ca^{2+} + HA^{-} \underset{}{\overset{K_{MA}}{\rightleftharpoons}} CaHA^{+}$$

therefore

$$K_{MA} = \frac{[CaHA^{+}]}{[Ca^{2+}][HA^{-}]} \qquad (7)$$

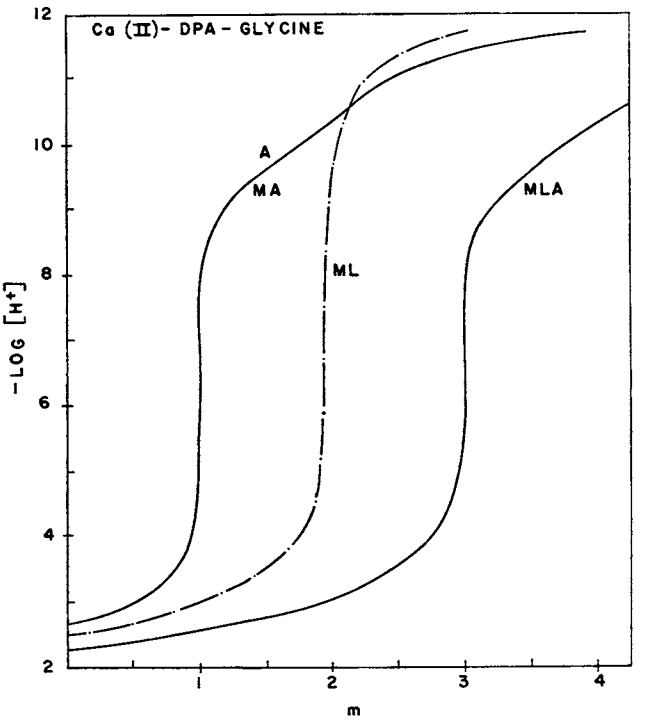


FIGURE 5 Potentiometric titration of glycine and related systems. A = 4.7520 × 10^{-3} M glycine; MA = Ca(II)-glycine chelate, 4.7520 × 10^{-3} M; ML = Ca(II)-dipicolinate chelate (1:1), 4.0732 × 10^{-3} M; MLA = DPA-Ca(II)-glycine chelate (1:1:1), 4.7520 × 10^{-3} M; m = moles of base added per mole ligand; μ = 1.0 (KNO_3); t = 25°C.

From the usual material balance equations and electroneutrality equation, equation 8 was obtained and the formation constant of calcium(II)-amino acid chelate, K_{MA}, was calculated:

$$K_{MA} = \frac{T_M - [HA^-]X}{[HA^-]^2X} \tag{8}$$

where

$$HA^- = \frac{T_L - T_{OH} - [H^+]}{\frac{[H^+]}{k_1}}; \qquad X = \frac{[H^+]}{k_1} + 1.$$

The meaning of the terms T_M, T_L, T_{OH}, and k_1 is the same as previously described. Formation constants of the 1:1 calcium(II)-amino acid studied are presented in Table I. It is clear from Table I that Ca(II) forms a much stronger chelate with DPA than with cysteine, alanine, and glycine.

3. *Mixed Ligand System of Ca(II)-DPA-Amino Acid*

Titration curves of the mixed ligand system of Ca(II)-DPA-cysteine, Ca(II)-DPA-alanine, and Ca(II)-DPA-glycine are shown in Figs. 3, 4, and 5 (curves *MLA*); the discussion presented is applicable to all three systems investigated.

Horizontal addition of the 1:1 Ca(II)-DPA curve (curve *ML*) and the curve of free amino acid (curve *A*) indicated that there was a weak but definite tendency to form a mixed ligand chelate. Potentiometric equilibrium data of all three systems were then analyzed by a suitable mathematical treatment. The inflection at $m = 3$ represents the dissociation of one hydrogen ion from the carboxyl group of amino acid and two hydrogen ions from the DPA molecule. Furthermore, the single uniformly low pH buffer region exhibited by the mixed ligand curve is indicative of the fact that the formation of Ca(II)-DPA chelate and its subsequent interaction with the amino acid took place in two overlapping steps. The solution of the stepwise formation constants was done by the unique intercept method reported by Rajan and Martell (15).

The following basic reactions were assumed:

$$Ca^{2+} + L^{2-} \overset{K_1}{\rightleftharpoons} CaL$$

$$CaL + HA^{-} \overset{K_2}{\rightleftharpoons} CaLHA^{-}$$

therefore:

$$K_1 = \frac{[CaL]}{[Ca^{2+}][L^{2-}]} \tag{9}$$

$$K_2 = \frac{[CaLHA^{-}]}{[CaL][HA^{-}]}. \tag{10}$$

The material balance equations and electro-neutrality equation could be set up as follows:

$$T_{Ca} = [Ca^{2+}] + [CaL] + [CaLHA^{-}] \tag{11}$$

$$T_L = [CaLHA^{-}] + [CaL] + [H_2L] + [HL^{-}] + [L^{2-}] \tag{12}$$

$$T_A = [CaLHA^{-}] + [H_2A] + [HA^{-}] + [A^{2-}] \tag{13}$$

$$C_{OH} = T_{OH} + [H^{+}] - [OH^{-}] = 2\,[CaL] + 3\,[CaLHA^{-}] + [HL^{-}] + 2\,[L^{2-}] + [HA^{-}] \tag{14}$$

where H_2L represents DPA and H_2A represents the amino acid.

From equations 9, 10, 11, 12, 13, and 14 all the unknown terms could be elimi-

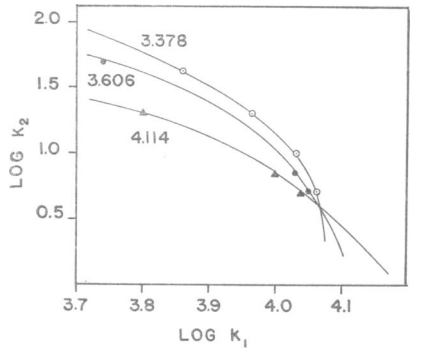


FIGURE 6 Solution of stepwise formation constants for the mixed chelate of DPA, Ca(II), and cysteine. The number on the curve represents the negative logarithm of hydrogen ion concentration.

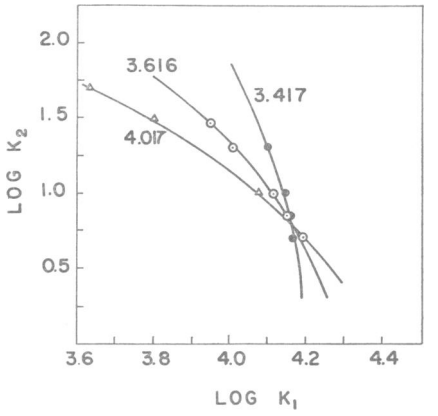


FIGURE 7 Solution of stepwise formation constants for the mixed chelate of DPA, Ca(II), and alanine. The number on the curve represents negative logarithm of hydrogen ion concentration.

nated except three terms, i.e., K_1, K_2, and L^{2-}. Their relation to one another is expressed in the following two equations.

$$[L^{2-}]^2[\tfrac{2}{3}X^2 \cdot K_2 - \tfrac{1}{3}X' \cdot X \cdot K_2] + [L^{2-}][\tfrac{1}{3}C_{OH} \cdot X \cdot K_2 - \tfrac{5}{3}T_{Ca} \cdot X \cdot K_2 - \tfrac{2}{3}X \cdot T_L K_2 + \tfrac{1}{3}X' \cdot T_L \cdot K_2 - \tfrac{2}{3}X \cdot Y + \tfrac{1}{3}X' \cdot Y] + [\tfrac{5}{3}T_{Ca} \cdot T_L \cdot K_2 - \tfrac{1}{3}C_{OH} \cdot T_L \cdot K_2 - \tfrac{1}{3}C_{OH} \cdot Y + \tfrac{5}{3}T_{Ca} \cdot Y + \tfrac{1}{3}T_{Ca} - T_{Ca} \cdot Y] = 0 \quad (15)$$

$$\frac{1}{K_1} = \frac{[L^{2-}]}{T_L - [L^{2-}]X}\left[\frac{T_{Ca} \cdot Y}{Y + T_L \cdot K_2 - K_2[L^{2-}]X} + [L^{2-}]X - T_L\right] \quad (16)$$

where:

$$X = \frac{[H^+]^2}{k_1 k_2} + \frac{[H^+]}{k_2} + 1 \qquad X' = \frac{[H^+]}{k_2} + 2$$

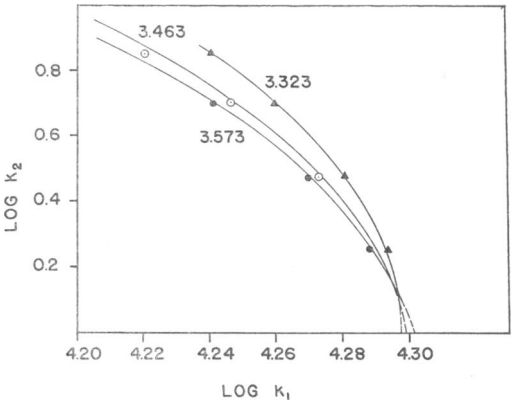


FIGURE 8 Solution of stepwise formation constants for the mixed chelate of DPA, Ca(II), and glycine. The number on the curve represents negative logarithm of hydrogen ion concentration.

TABLE II

FORMATION OF MIXED LIGAND CHELATES OF CALCIUM ION WITH DIPICOLINIC ACID (H_2L) AND ONE MOLE OF AN ADDITIONAL LIGAND (H_nA)

Added ligand	Equilibrium quotient	Stepwise formation constants	
		Log K_1	*Log* K_2
Cysteine	$\frac{[CaL]}{[Ca^{2+}][L^{2-}]}$	4.17 ± 0.01	
	$\frac{[CaLHA^{-}]}{[CaL][HA^{-}]}$		0.78 ± 0.01
Alanine	$\frac{[CaL]}{[Ca^{2+}][L^{2-}]}$	4.06 ± 0.01	
	$\frac{[CaLHA^{-}]}{[CaL][HA^{-}]}$		0.65 ± 0.01
Glycine	$\frac{[CaL]}{[Ca^{2+}][L^{2-}]}$	4.30 ± 0.02	
	$\frac{[CaLHA^{-}]}{[CaL][HA^{-}]}$		0.11 ± 0.01

$t = 25°C$; $\mu = 1.0$ (KNO_3).

k_1 and k_2 in the expressions of X and X' are the acid dissociation constants of DPA

$$Y = \frac{[H^+]}{k_1'} + 1$$

k_1' in the expression Y is the acid dissociation constant of amino acid.

Equation 15 is a quadratic equation. By assuming a value for K_2, the roots of $[L^{2-}]^2$ could be calculated for any given point along the titration curve. Therefore, by varying the assumed values for K_2, a series of the corresponding values could be calculated for $[L^{2-}]$. Each of the $[L^{2-}]$ values was then substituted into equation 16 to solve for K_1. Plotting the values of log K_2 against the corresponding values of log K_1 resulted in a curved line. A line was obtained for each of the pH readings on the potentiometric titration curve. The intercept of the various lines from the plot of log K_2 vs. log K_1 is the unique solution to equations 15 and 16.

Figs. 6, 7, and 8 illustrate the series of intersecting lines for Ca(II)-DPA-cysteine, Ca(II)-DPA-alanine, and Ca(II)-DPA-glycine, respectively. The reaction sequence for the formation of mixed ligand chelates as well as the values obtained for K_1 and K_2 are presented in Table II. It can be seen that the reaction of Ca(II) and DPA shows a stronger affinity as evidenced by the high log K_1 for all systems, whereas the formation of Ca(II)-DPA-amino acid chelate is characterized by the much lower log K_2 values.

DISCUSSION

The stability constants calculated from our potentiometric equilibrium data for Ca(II)-DPA and Ca(II)-amino acids are in good agreement with values reported in the literature (22). The slight differences may be due to differences in ionic strength and method of evaluation. The precision of our stability constants for the mixed ligand chelates of Ca(II)-DPA-amino acids is of the same order as those for the single systems. No literature reports are currently available on mixed ligand systems described in this paper.

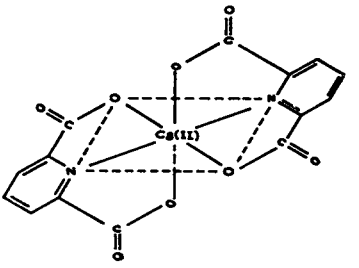
Taking into consideration the stability constants determined in this study, the structures of the various chelates could be postulated (Figs. 9, 10, 11).

In the Ca(II)-DPA chelate (Fig. 9) the attachment of ligand to metal ion is through the carboxyl groups and ring nitrogen, thus forming two 5-membered rings. The two rings account for the high stability of this chelate (log K = 4.4).

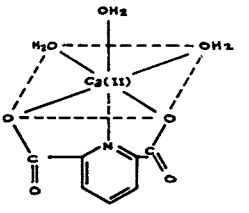
The stability constants for interaction of amino acids with calcium (II) either in the single or in the mixed system suggest that the attachment of ligand to the metal ion in all three cases is through the α-amino and α-carboxyl group thus forming a single 5-membered chelate ring (Figs. 10, 11).

The question arises, how do these observations relate to the typical (and unique) characteristics of bacterial spores. At present the relationship is quite speculative. There are three lines of evidence which support the in vivo existence of a hypothetical complex of DPA-Ca(II)-amino acid in the spore: (*a*) DPA, Ca(II), amino acids, and peptides appear to be always released together: all methods for release of DPA, Ca(II), amino acids, and peptides destroy at the same time the typical spore characteristics, i.e., optical refractility, high buoyant density, resistance to stains, etc. The methods capable of breaking the spore state include natural germi-

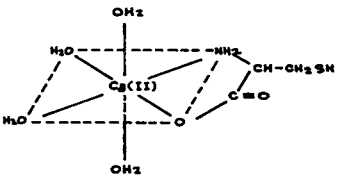
nation in nutrient media, exposure to chelating agents, extraction by hot or cold water, solvent extraction, killing by wet heat, etc. (14, 17, 19, 30). In spore exudates the released compounds are found in a complexed form. But it has not been demonstrated conclusively whether these complexes are present in the spore in vivo or whether they are released individually and then combine after they leave the spore.



Calcium (II) Dipicolinate (1:2)



Calcium (II) Dipicolinate (1:1)



Calcium (II) Cysteine Chelate

FIGURE 9 Postulated structures of chelates.

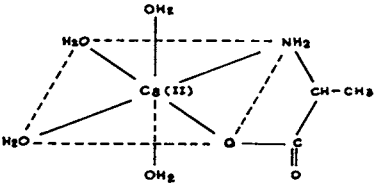
(*b*) The rate of release of DPA and Ca(II) during heating is approximately proportional to the rate of killing of spores (25, 26, 24). This observation seems to indicate that gradual solution of the DPA-Ca(II)-amino acid complexes by hot water unmasks the vital spore biopolymers which subsequently become subject to thermal denaturation.

(*c*) Germination of spores, i.e. loss of dormancy and heat resistance as well as release of DPA, Ca(II), amino acids, and peptides can be induced by many chelating agents including EDTA, DPA, sodium bicarbonate, etc. (4, 16, 18). This seems

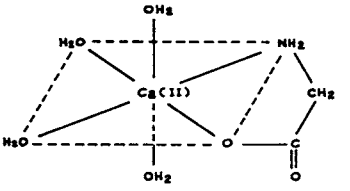
to suggest that the heat resistant dormant state is related to a metal-ligand coordination complex, and breaking of dormancy by chelating agents seems to involve competitive displacement reactions.

Based on the idea of *spore cement*, it could be postulated that stabilization of spores may occur either by embedding of vital spore polymers into the cement or by direct coordination bonding between Ca(II)-DPA and spore molecules.

The stability of the cement would depend on its composition, e.g., the relative proportion of different amino acids in the chelate matrix. The data presented in this paper demonstrate that there are considerable differences in the stability of various chelate compounds related to bacterial spores. The interaction of Ca(II)



Calcium (II) - alanine



Calcium (II) - glycine

FIGURE 10 Postulated structures of chelates.

with DPA was stronger than with any of the amino acids tested either singly or in the mixed system. The stability constants of the three amino acids tested in this study are relatively close to each other, however, complexes of Ca(II) with other amino acids (such as α, ϵ-diaminopimelic acid, aspartic acid, and glutamic acid) have considerably lower stability tested in single or in the mixed system (27).

The amino acids in the spore are probably bound in peptides although the existence of an appreciable free amino acid pool has been demonstrated and the constituent amino acids have been identified (30). At present, the number of different spore peptides and their composition are largely unknown. Therefore, the interaction of Ca(II)-DPA with free amino acids was studied as a first step toward understanding of the role of coordination complexes in heat resistance.

Although the observed differences in stabilities of the mixed ligand chelates are rather small, it should be emphasized that they do not rule out a possible contribu-

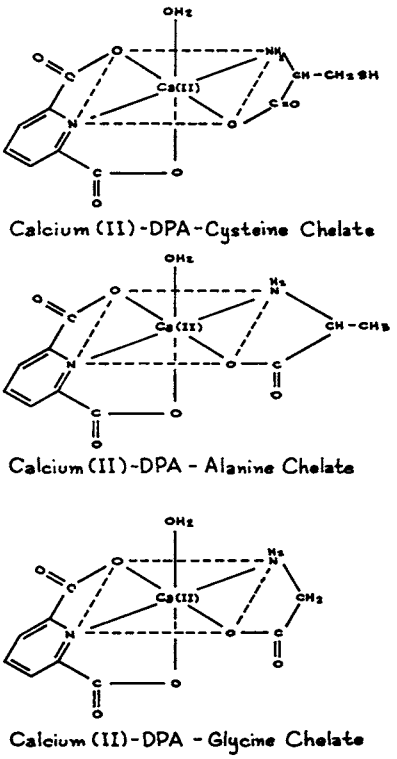


FIGURE 11 Postulated structures of chelates.

tion to heat resistance. However, more work is needed to substantiate this hypothesis. In particular, studies should be directed to the measurement of the size and composition of the free amino acid pool as related to heat resistance. Further supporting evidence in favor of the "mixed ligand chelate hypothesis" could be considered by examining the interaction of Ca(II)-DPA with some typical peptides related to spores.

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