COORDINATIVE BINDING OF DIVALENT CATIONS WITH LIGANDS RELATED TO BACTERIAL SPORES

EQUILIBRIUM STUDIES

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ABSTRACT It has been repeatedly postulated that the high heat resistance of bacterial spores is due to stabilization of biopolymers in the spore interior by a solid deposit of protective cement consisting of coordination complexes of ligands with divalent metal ions. This report presents data on metal-binding characteristics of some of the ligands related to spores as determined by means of potentiometric equilibrium measurements under conditions of temperature and ionic strength (t = 25.0°C; μ = 1.0 KNO_3) identical with those reported earlier by the authors in order to facilitate correlation by using comparable data. The spore ligands investigated in this study included 2,6-pyridinedicarboxylic acid (DPA), α,ϵ-diaminopimelic acid, D-glutamic acid, and D-alanine in a ratio of 1:1 with metal ions which are known to play a role in heat resistance of spores. Stability constants of the chelates of these spore ligands with metal ions such as Ca(II), Mg(II), Cu(II), Ni(II), Zn(II), Co(II), and Mn(II) have been determined. In general the metal chelates of DPA exhibited the greatest stability. On the basis of a consideration of the stability data together with the known configurations of the ligand and the coordination requirements of the metal ions, possible structures indicating the coordinate binding of the spore ligands with the metal ions are presented. All the metal chelates except those of Ca(II) were found to undergo hydrolysis and separation of solid phase in the pH range 7–8.5. The relatively greater hydrolytic stability of Ca(II) chelates and the high affinity of DPA for metal ions appear to be of biological significance insofar as these two spore components are more widely associated with the heat resistance of bacterial spores.

INTRODUCTION

The hypotheses attempting to explain the high resistance of bacterial spores to heat center around two principal ideas: (*a*) low water content or hydrophobic nature of the spore interior (7, 15, 23), and (*b*) stabilization of spore components either by cross-linking between cell biopolymers (2) or by "calcification" of the spore interior

involving chelation of divalent ions by spore ligands (21, 10, 8, 22, 30). Recently it has been suggested that the two ideas may not be contradictory because the hydrophobic nature of spores may be the result of their high content of coordinated complexes (9). The metal chelates of typical spore ligands and various divalent cations may perhaps form a "cementing" or stabilizing structure somewhat analogous to bone or dental substance in higher animals.

Bacterial spores contain large amounts of divalent metals such as Ca, Co, Cu, Mg, Mn, Ni, and Zn (6, 26, 19, 17, 13). The amounts of the metal ions accumulated by spores depend mostly on the concentration of metals in the growth medium. The amount of individual metals in bacterial spores may vary widely. Furthermore, the metals are exchangeable to a certain extent (26). Among all divalent metals, calcium has been observed to be most closely related to heat resistance. Replacement of calcium with other divalent ions resulted in a decrease of heat resistance (26, 16, 17, 32).

In addition to the accumulation of metal ions, spores also contain a relatively large amount of 2,6-pyridinedicarboxylic acid (dipicolinic acid, DPA) (21, 20) as well as a specific spore peptidoglycan (21, 18). DPA is present in amounts of 5–15% (on dry weight basis) (5, 3) and is distinguished by its ability to strongly chelate many divalent metals (27, 31, 1).

The peptidoglycan consists of a hexosamine backbone with a peptide chain of glutamic acid, alanine, and α,ϵ-diaminopimelic acid (18). Free amino and carboxyl groups have been identified which may conceivably be involved in coordinative binding with metal ions. It has been repeatedly postulated that the peptidoglycan (18) and DPA (5, 12, 14, 34, 28–30) may be related to heat resistance of bacterial spores.

The present study has been directed toward a quantitative evaluation of the metal-binding affinities of the spore ligand so as to develop a quantitative and comparable basis for discussing the possible role of metal chelates in the heat resistance of bacterial spores. It is thought that differences in heat resistances between spores may perhaps have some correlation with the predominant occurrences of different metals and ligands in the individual spores. The manner in which these metals and ligands are bound in the spore may be of major importance.

Past investigations along this line have been restricted to interactions between DPA and a few divalent ions (8, 22). Mixed chelates of Ca(II)-DPA with some amino acids of importance to bacterial spores have also been reported (30). The present paper extends these studies to the interactions of seven divalent metal ions characterized for their role in spore heat resistance (26) with DPA and three other ligands, D-alanine, α,ϵ-diaminopimelic acid, and D-glutamic acid, all primary components of the spore peptidoglycan which has been postulated to play a major role in spore heat resistance (18). The stability constants of a number of the chelate systems under investigation have been determined and reported before (30); the present investigations are in continuation of our earlier work and are carried out under identical

conditions of temperature and ionic strength, viz., $t = 25.0°C$ and $\mu = 1.0$ (KNO_3) so as to enable correlation of comparable data.

MATERIALS AND METHODS

Aqueous solutions of DPA, α,ϵ-diaminopimelic acid (DAP), D-glutamic acid, and D-alanine were freshly prepared from reagent grade materials and were standardized potentiometrically. Stock solutions of metal (II) ions were prepared by dissolving appropriate quantities of reagent grade metal chlorides or metal nitrates in distilled water and standardized by complexometric titrations (24). All solutions were 0.02 M in concentration.

The experimental method consisted of potentiometric equilibrium measurements of the hydrogen ion concentrations of the ligand acids in the absence of and in the presence of an equimolar amount of metal ion. A radiometer pH meter (type pH 26) fitted with glass and calomel extension electrodes was used for measuring hydrogen ion concentrations. Potentiometric measurements were made at 25°C and an ionic strength of 1.00 maintained constant by the addition of KNO_3. The electrode system was calibrated with HCl and NaOH in such a way that the observed pH meter reading was compared with actual hydrogen ion concentration determined on the basis of the data tabulated by Harned and Owen (11). Carbon dioxide–free NaOH was used for the titration. Experimental solutions were kept stirring in the thermostated cell, and the equilibrium pH's were measured after incremental additions of base. Each titration was continued until a pH of 11–11.5 was reached.

RESULTS

Titration curves are illustrated in Figs. 1–4 for the DPA, DAP, D-glutamic acid, and D-alanine chelates of Ca(II), Co(II), Cu(II), Mg(II), Mn(II), Ni(II), and Zn(II) for 1:1 ratio of ligand to metal ion. The titration curves were obtained by plotting the equilibrium pH's against corresponding values of equivalents, base added per mole of ligand or metal salt. In all the figures broken lines indicate separation of solid phase. On the basis of these titration curves, it is possible to assess qualitatively the nature and extent of their interaction. Furthermore, on the basis of these curves, assumptions regarding the possible reactions are made and these form the basis for mathematical treatment and quantitative calculations. In the following sections each system will be described separately.

Metal (II) Ion-DPA Systems

Fig. 1 shows the potentiometric titration curves of DPA and bivalent metal DPA systems containing one mole of ligand per mole of metal ion. The ligand curve shows two inflections at one and two moles of base per mole of ligand, which indicates the dissociation of proton from each of the two carboxyl groups of the ligand. The acid dissociation constants were calculated on the basis of the titration data by a direct algebraic method. The equilibrium reactions involved and dissociation constants are represented in Table I.

The titration curve of the 1:1 metal-DPA system shows a pronounced inflection at two moles of base per mole of metal ion. This inflection indicates that metal ions

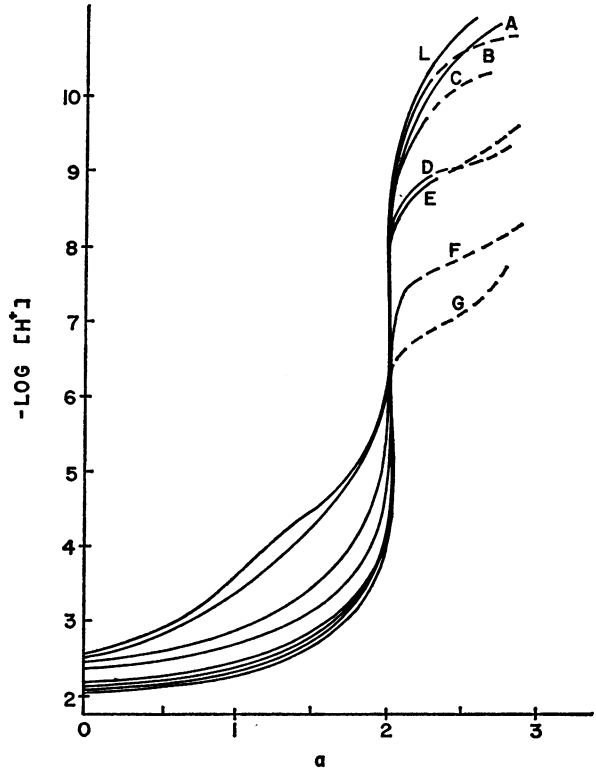


FIGURE 1 Potentiometric titrations of L, 3.867×10^{-3} M DPA; and 3.867×10^{-3} M DPA in the presence of equimolar amounts of Ca(II), A; Mg(II), B; Mn(II), C; Co(II), D; Zn(II), E; Ni(II), F; and Cu(II), G; a = moles of base added per mole of ligand; μ = 1.0 (KNO_3); t = 25°C.

TABLE I

DISSOCIATION CONSTANTS OF LIGAND ACIDS AT 25°C AND μ = 1.0 (KNO_3)

Ligand	Reaction	$-\log K$
Dipicolinic acid, H_2L	$H_2L \rightleftharpoons HL^- + H^+$	2.07 ± 0.02
	$HL^- \rightleftharpoons L^{2-} + H^+$	4.53 ± 0.02
Diaminopimelic acid, H_4L^{2+}	$H_4L^{2+} \rightleftharpoons H_3L^+ + H^+$	1.98 ± 0.02
	$H_3L^+ \rightleftharpoons H_2L + H^+$	2.54 ± 0.01
	$H_2L \rightleftharpoons HL^- + H^+$	8.95 ± 0.01
	$HL^- \rightleftharpoons L^{2-} + H^+$	10.00 ± 0.01
D-glutamic acid, H_3L^+	$H_3L^+ \rightleftharpoons H_2L + H^+$	2.04 ± 0.02
	$H_2L \rightleftharpoons HL^- + H^+$	4.15 ± 0.01
	$HL^- \rightleftharpoons L^{2-} + H^+$	9.49 ± 0.01
D-alanine, H_2L^+	$H_2L^+ \rightleftharpoons HL + H^+$	2.33 ± 0.02
	$HL \rightleftharpoons L^- + H^+$	9.72 ± 0.02

combine with DPA through the dissociation of two protons from the two carboxylic acid groups of the ligand, corresponding to the equilibrium:

$$M^{2+} + H_2L \rightleftharpoons ML + 2H^+,$$

where H_2L represents DPA. The formation constants K_{ML} of the 1:1 metal chelates represented by the following equilibrium were calculated as shown:

$$M^{2+} + L^{2-} \overset{K_{ML}}{\rightleftharpoons} ML,$$

where L^{2-} is the dissociated form of DPA. By using the appropriate material balance and electroneutrality expressions, the following equation can be obtained:

$$K_{ML} = \frac{T_M - [L^{2-}]x}{[L^{2-}]^2 x}, \tag{1}$$

where

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

and

$$x = ([H^+]^2/k_1k_2) + ([H^+]/k_2) + 1. \tag{3}$$

The designation of each of the terms are: T_M and T_L represent the total molar concentration of the metal ion and ligand respectively. T_{OH} denotes the total molar concentration of the base added to the experimental solution. k_1 and k_2 are the acid dissociation constants of the ligand.

The log of the formation constants of 1:1 metal (II)-DPA chelates are summarized in Table II.

TABLE II

STABILITY CONSTANTS OF METAL CHELATES OF DIPICOLINIC ACID (DPA) DIAMINOPIMELIC ACID (DAP), D-GLUTAMIC ACID, AND D-ALANINE AT 25°C AND μ = 1.0 (KNO_3).

Metal	DPA log K_{ML}	DAP log K_{ML}	D-glutamic acid log K_{ML}	D-alanine log K_{ML}
Ca(II)	4.05 ± 0.02	2.06 ± 0.20	1.38 ± 0.18	0.99 ± 0.12
Co(II)	7.10 ± 0.03	5.58 ± 0.18	4.25 ± 0.03	4.36 ± 0.03
Cu(II)	7.97 ± 0.06	12.96 ± 0.04*	8.05 ± 0.04	8.45 ± 0.04
Mg(II)	1.66 ± 0.04	1.95 ± 0.26	1.46 ± 0.20	1.37 ± 0.15
Mn(II)	4.72 ± 0.02	3.09 ± 0.06	2.43 ± 0.05	2.61 ± 0.05
Ni(II)	7.45 ± 0.04	7.38 ± 0.22	5.27 ± 0.03	5.41 ± 0.03
Zn(II)	7.29 ± 0.03	6.89 ± 0.22	4.45 ± 0.04	4.59 ± 0.05

* Log k^H_{MHL} = 5.09; log K_{MHL} = 8.06; log K_{ML} = 12.96.

Metal (II) Ion–DAP Systems

Fig. 2 shows the potentiometric titration curves of DAP and bivalent metal–DAP systems containing one mole of ligand per mole of metal ion. The ligand curve shows an inflection at two moles of base per mole of ligand, indicating that the dissociations of the two carboxyl hydrogens occur in an overlapping manner to give a single low pH buffer region. From these data, the determinations of the acid dissociation constants were carried out by means of the method outlined by Schwarzenbach et al. (25). The following equation was used to evaluate k_1 and k_2:

$$\frac{1}{k_1} = k_2 \frac{(T_L/[H^+])(2 - a) - 1}{[H^+](aT_L + [H^+])} - \frac{T_L(a - 1) + [H^+]}{[H^+](aT_L + [H^+])}, \quad (4)$$

where a represents the number of equivalents of base added per mole of the ligand. Likewise, the dissociation of the two α-amino hydrogens are represented by a single high pH buffer region. The dissociation constants were calculated from the following expression:

$$\frac{1}{k_3} = k_4 \frac{(T_L/[H^+])(4 - a) + [OH^-]/[H^+]}{[H^+](T_L(a - 2) - [OH^-])} - \frac{T_L(a - 3) - [OH^-]}{[H^+](T_L(a - 2) - [OH^-])}, \quad (5)$$

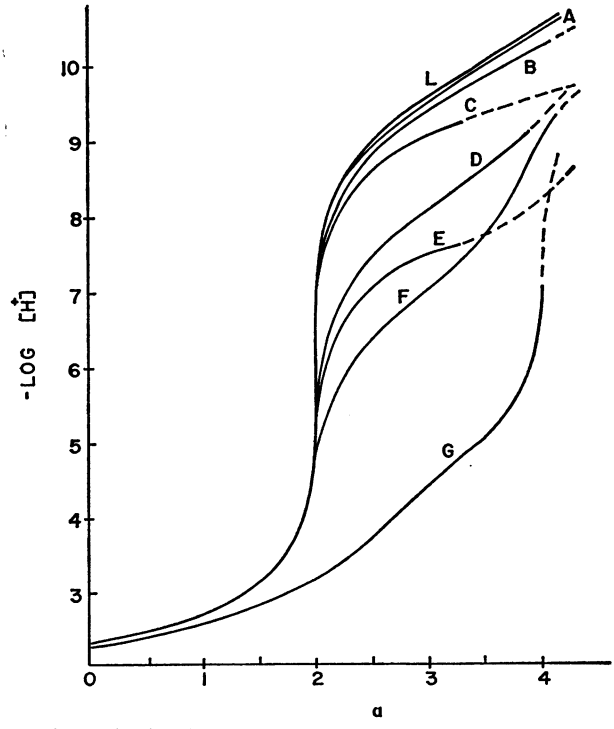


FIGURE 2 Potentiometric titration of L, 4.136×10^{-3} M DAP; and 4.136×10^{-3} M DAP in the presence of equimolar amounts of Ca(II), A; Mg(II), B; Mn(II), C; Co(II), D; Zn(II), E; Ni(II), F; and Cu(II), G; a = moles of base added per mole of ligand, μ = 1.0 (KNO_3); t = 25°C.

where $[OH^-] = k_w/[H^+]$ and k_w is the ionization constant of water at 25.0°C and $\mu = 1.0$ (KNO_3).

The dissociation constants k_1, k_2, k_3, and k_4 were determined by means of graphical solutions of equations 4 and 5. The results are presented in Table I.

The titration curves of DAP with the different bivalent metal ions given in Fig. 2 show that Ca(II), Co(II), Mg(II), Mn(II), Ni(II), and Zn(II) chelate curves overlap with that of the free ligand curves between $a = 0$ and $a = 2$. The equilibrium reaction involved in this region may be represented by the following reaction:

$$H_4L^{2+} + 2OH^- \rightleftharpoons H_2L + 2H_2O.$$

Consequently, little interaction occurs between these metals and the ligand.

The depression of the metal-DAP curves at high buffer region between $a = 2$ and $a = 4$ indicates the formation of 1:1 metal chelates in accordance with the following reaction:

$$M^{2+} + H_2L \rightleftharpoons ML + 2H^+,$$

where H_2L represents the DAP with its two dissociable hydrogen ions of the amino groups. The chelate formation constants were calculated by using equations 1–3. It should be noted that the dissociation constants employed in the expressions are k_3 and k_4.

In the case of Cu(II)-DAP, it is apparent that the first buffer region is also appreciably lowered. This indicates that the interaction of DAP with Cu(II) is much stronger than with other metal (II) ions. Apparently, Cu(II) can displace the two carboxyl hydrogens from the ligand at this low pH buffer region. The appearance of a weak inflection at $a = 3$ and a sharp inflection at $a = 4$ could be explained by formation of a monoprotonated complex, MHL^+, which subsequently dissociates into ML. The reactions of Cu(II) ion occurring in the titration process may be represented by the following reactions:

$$M^{2+} + HL^- \overset{K_{MHL}}{\rightleftharpoons} MHL^+,$$

$$MHL^+ \overset{k^H_{MHL}}{\rightleftharpoons} ML + H^+.$$

The over-all reaction for the binding of L^{2-} by the metal ion, i.e.

$$M^{2+} + L^{2-} \rightleftharpoons ML,$$

could be calculated by means of the following relationship:

$$K_{ML} = (K_{MHL})(k^H_{MHL})\left(\frac{1}{k_4}\right). \qquad (6)$$

Expressions of K_{MHL} and k^H_{MHL} could be obtained by employing the usual material

balance and electroneutrality equations, that are

$$K_{MHL} = \frac{T_L - [HL^-]x}{[HL^-]^2 x}, \tag{7}$$

where

$$[HL^-] = \frac{3T_L - T_{OH} - [H^+] + [OH^-]}{(3[H^+]^3/k_1k_2k_3) + (2[H^+]^2/k_2k_3) + ([H^+]/k_3)}, \tag{8}$$

and

$$x = \frac{[H^+]^3}{k_1k_2k_3} + \frac{[H^+]^2}{k_2k_3} + \frac{[H^+]}{k_3} + 1, \tag{9}$$

$$k^H_{MHL} = \frac{(T_{OH} + [H^+])[H^+]}{T_L - T_{OH} - [H^+]}. \tag{10}$$

Metal (II) Ion–D-Glutamic Acid Systems

In Fig. 3 are shown the titration curves of the ligand, D-glutamic acid, and of the bivalent metal (II)–D-glutamic acid systems containing one mole of ligand per mole of metal ion. The inflections at one and two moles of base per mole of ligand indicate the dissociation of two carboxylic acid groups. Acid dissociation constants are also calculated by direct algebraic methods and are presented in Table I.

The potentiometric titration curve of 1:1 metal–D-glutamic acid systems (Fig. 3) shows an inflection at $a = 2$ and a lowering of the buffer region between $a = 2$ and $a = 3$ for Ca(II), Co(II), Mg(II), Ni(II), and Zn(II) as in the case of DAP. The lowering of pH between $a = 2$ and $a = 3$ indicates that the chelation of these divalent metal ions involved the displacement of the amino hydrogen. The equilibrium reaction may be expressed as

$$M^{2+} + HL^- \rightleftharpoons ML + H^+.$$

Therefore, the region from $a = 2$ to $a = 3$ of the titration curves was used for the determination of metal chelate formation constants for the following reaction:

$$M^{2+} + L^{2-} \overset{K_{ML}}{\rightleftharpoons} ML,$$

where L^{2-} represents the dissociated form of D-glutamic acid. The relationships for the calculation of stability constants are similar to the ones used in the metal (II)-DPA system. But this system involved the dissociation constant of the amino hydrogen of D-glutamic acid:

$$K_{ML} = \frac{T_L - [L^{2-}]x}{[L^{2-}]^2 x}, \tag{11}$$

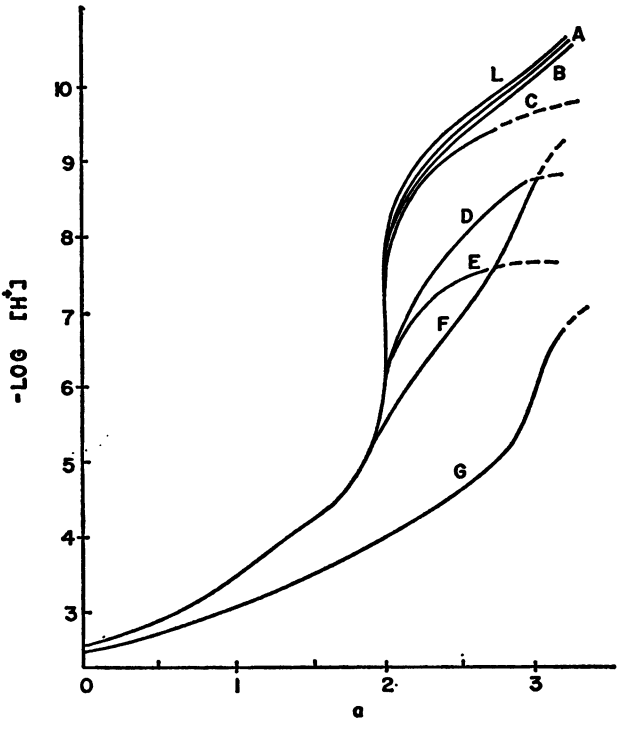


FIGURE 3 Potentiometric titration of L, 4.020×10^{-3} M D-glutamic acid; and 4.020×10^{-3} M D-glutamic acid in the presence of equimolar amounts of Ca(II), A; Mg(II), B; Mn(II), C; Co(II), D; Zn(II), E; Ni(II), F; and Cu(II), G; a = moles of base added per mole of ligand; μ = 1.0 (KNO_3); t = 25°C.

where

$$[L^{2-}] = \frac{T_L - T_{OH} - [H^+] + [OH^-]}{[H^+]/k_3}, \tag{12}$$

$$x = \frac{[H^+]}{k_3} + 1. \tag{13}$$

In the case of Cu(II)–D-glutamate, the first buffer region is also appreciably lowered as in the case of Cu(II)-DAP. This shows that Cu(II) ion can displace the two carboxyl hydrogens from the ligand at this low pH region. Hence, the equilibrium reaction may be expressed as:

$$M^{2+} + H_3L^+ \rightleftharpoons ML + 3H^+.$$

The stability constant of Cu(II)–D-glutamate was calculated by using equations 7–9 and is listed in Table II.

Metal (II) Ion–D-Alanine Systems

The inflection at $a = 1$ and a lowering of the buffer region after $a = 1$ in the 1:1 metal (II)–D-alanine system (Fig. 4) could be accounted for by assuming the interaction of metal (II) ion with D-alanine through the dissociation of the carboxyl and the α-amino hydrogen from the ligand. This corresponds to the following reaction:

$$M^{2+} + HL \rightleftharpoons ML^{+} + H^{+}.$$

Alternatively, the metal chelate formation equilibrium may be expressed as:

$$M^{2+} + L^{-} \overset{K_{ML}}{\rightleftharpoons} ML^{+}.$$

The chelate formation constants of all metal ions under consideration except Cu(II) were calculated using the following relationships:

$$K_{ML} = \frac{T_L - [L^-]x}{[L^-]^2 x}, \tag{14}$$

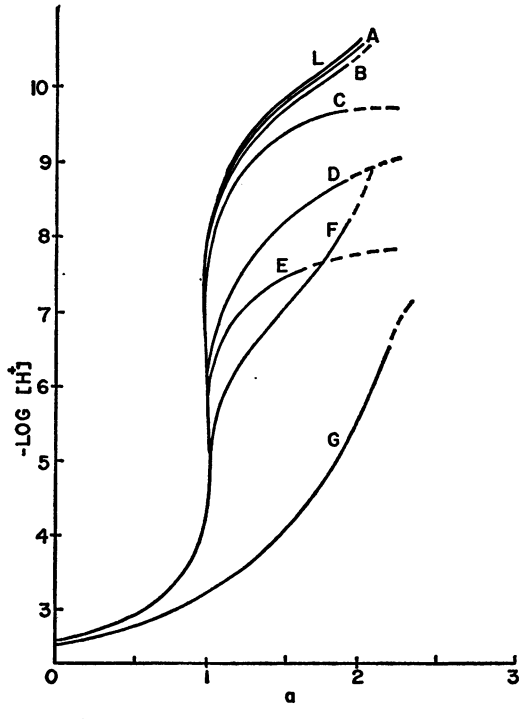


FIGURE 4 Potentiometric titration of L, 4.105×10^{-3} M D-alanine, and 4.105×10^{-3} M D-alanine in the presence of equimolar amounts of Ca(II), A; Mg(II), B; Mn(II), C; Co(II), D; Zn(II), E; Ni(II), F; and Cu(II), G; a = moles of base added per mole of ligand; μ = 1.0 (KNO_3); t = 25°C.

where

$$[L^-] = \frac{T_L - T_{OH} - [H^+] + [OH^-]}{[H^+]/k_2}, \quad (15)$$

$$x = \frac{[H^+]}{k_2} + 1. \quad (16)$$

In the case of Cu(II) ion the following equilibrium reaction was assumed:

$$M^{2+} + H_2L^+ \rightleftharpoons ML^+ + 2H^+.$$

Therefore the chelate stability constant was calculated by equations similar to equations 1–3.

DISCUSSION

A consideration of the titration curves of bivalent metal ion–ligand systems indicates that all systems tested formed 1:1 chelates. Transition metals and magnesium indicated hydrolysis and separation of solid phase at pH's above 8.5 except for Cu(II) which hydrolyzed at about pH 7.0. Notable exceptions were the Ca(II) chelates of these ligands, which showed remarkable hydrolytic stability. This observation may be of biological significance particularly from the point of view of the possible involvement of Ca(II) ion, DPA, and other spore ligands in the molecular mechanism of the heat resistance of bacterial spores. Because of the hydrolytic stability of Ca(II) chelates, it is possible to rationalize the accumulation of high concentrations of Ca(II) ion in spores in the form of its chelates and their possible role in heat resistance, a fact repeatedly postulated in the literature.

Formation of polynuclear complexes was thought to be a possible factor contributing to increased stabilization of the hypothetical "spore cement". However, on the basis of the constancy of the chelate formation constants of metal ion with DPA, D-glutamic acid, and D-alanine, the metal species present in these systems are considered to be mononuclear. On the other hand, the trend in the variation of the chelate formation constants of metal (II)-DAP chelates with increasing additions of base, may perhaps indicate formation of polynuclear species. However, the postulation of polymerization for DAP and the calculation of formation constants require additional detailed experimental studies and complex mathematical treatments.

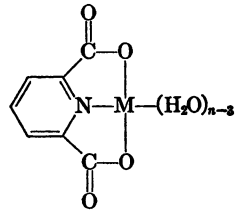
Depression of buffer regions of individual chelates in Figs. 1–4 relative to those of free ligands showed an apparent trend in metal-binding affinities of these ligands. For the four chelating agents described in this paper, the positions of the metal curves and the stability constants indicate the following order of metal chelate stabilities:

$$Cu > Ni > Zn > Co > Mn > Mg \approx Ca.$$

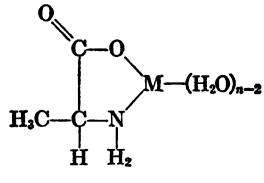
This represents the usual order of metal chelate stabilities reported in the literature.

The stability increases with decreasing basicity of the metal ion (4), the weakly basic metals Mg(II) and Ca(II) forming the weakest chelates.

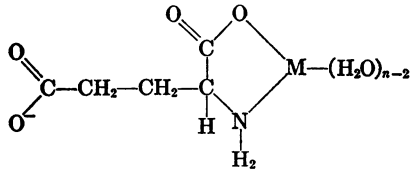
On the basis of the equilibrium data determined in this study, and taking into account the coordination requirements of the metal ions and the configurations of the ligands, the following structures of various chelates could be postulated:



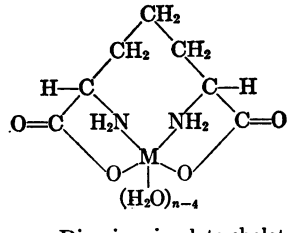
Dipicolinate chelate



D-Alanate chelate



D-Glutamate chelate



α, ε-Diaminopimelate chelate

The formation of two five-membered rings of DPA with metal ions and the resonance stabilization due to the conjugated ring system probably accounts for the relatively high stability of DPA-metal chelates.

On the basis of the equilibrium data obtained in this study, it may be stated that metal chelate formation in vivo is possible provided that the metal ions and ligands are available and that they are in close physical proximity. When the fact of the chelate stability is considered together with the often reported correlation between

the heat resistance of bacterial spores and the occurrence of Ca(II), DPA, and other amino acids, one may consider the possibility of attributing heat resistance to the stabilization of biopolymers through chelation. However, at present, the location of metal ions in the spore and the nature of an in vivo metal chelate polymer formation are still subjects of speculation. The existence of chelates in vivo is strongly suggested by EPR (electron paramagnetic resonance) spectra of spores produced on a manganese-enriched medium (33). Additional evidence supporting the existence of chelates in the spore has been summarized and discussed previously (30, 35).

The possible in vivo involvement of metal ions in heat resistance of spores is of prime importance with respect to the hypothetical spore cement. Slepecky and Foster (26) have reported that the content of individual metals in spores of *Bacillus megaterium* varies within a very wide range and depends on their relative abundance in the growth medium. Their studies with metal ions other than Ca(II) were restricted by (*a*) metal ion toxicity and (*b*) poor incorporation into spores. The high accumulation of Ca(II) in spores may be due to its high hydrolytic stability shown in this study, to its relatively great abundance in ordinary bacteriological media, as well as to its nontoxic nature. Mn(II) appeared to substitute for Ca(II) to some degree in conferring heat resistance, although Mn(II) as well as Zn(II) and especially Ni(II) were toxic to the cells and could not be added in high concentrations (26).

It may be pointed out that the two metal ions, Ca(II) and Mg(II), which show the lowest chelate stabilities with typical spore ligands, are also those ions which have been most closely related to heat resistance. This seems to indicate that the mechanism of heat resistance may perhaps depend on a delicately balanced, weak, and (probably) reversible binding of these metal ions within mixed chelate systems (30) which may gradually dissociate under the impact of the usual thermal energy. Thus, the heat energy requirement for complex dissociation may in effect provide a protective energy sink contributing to spore heat resistance. On the other hand, the more strongly bound metal ions may not be easily dissociated, a fact which would explain the biological toxicity of these ions.

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REFERENCES

1. ANDEREGG, G. 1960. *Helv. Chim. Acta.* **43**:414.
2. BLACK, S. H., and P. GERHARDT. 1962. *J. Bacteriol.* **83**:960.
3. BYRNE, A. F., T. H. BURTON, and R. B. KOCH. 1960. *J. Bacteriol.* **80**:139.

4. CHABEREK, S., and A. E. MARTELL. 1959. Organic Sequestering Agents. John Wiley & Sons, Inc., New York.
5. CHURCH, B. S., and H. HALVORSON. 1959. *Nature (London)*. **183**:124.
6. CURRAN, H. R., B. C. BRUNSTETTER, and F. T. MEYERS. 1943. *J. Bacteriol.* **45**:484.
7. DRYMONT, A. 1886. *Arch. Exp. Pathol. Pharmakol.* **21**:309.
8. FLEMING, H. P. 1963. Chelation properties of bacterial spore components and spore responses to ionic environment. Ph.D. Thesis. University of Illinois, Urbana, Illinois.
9. GRECZ, N., and R. SMITH. 1966. *Bacteriol. Proc.* 32.
10. HALVORSON, H. O. 1953. The Physiology of the Bacterial Spore. Technical University of Norway, Trondheim, Norway.
11. HARNED, H. S., and B. B. OWEN. 1950. The Physical Chemistry of Electrolyte Solutions. Reinhold Publishing Corp., New York. 2nd edition.
12. HASHIMOTO, T., S. H. BLACK, and P. GERHARDT. 1960. *Can. J. Microbiol.* **6**:203.
13. KOLODZIEJ, B. J., and R. A. SLEPECKY. 1964. *J. Bacteriol.* **88**:821.
14. LECHOWICH, R. V., and Z. J. ORDAL. 1960. *Bacteriol. Proc.* 44.
15. LEWITH, S. 1890. *Arch. Exp. Pathol. Pharmakol.* **26**:341.
16. MURRELL, W. G. 1961. *Sympos. Soc. Gen. Microbiol.* **11**:100.
17. MURRELL, W. G. 1967. *In* Advances in Microbial Physiology. A. H. Rose and J. F. Wilkinson, editors. Academic Press Inc., New York. **1**.
18. MURRELL, W. G., D. F. OHYE, and R. A. GORDON. 1969. *In* Spores IV. L. L. Campbell, editor. American Society for Microbiology, Bethesda, Maryland. 1–19.
19. MURRELL, W. G., and A. D. WARTH. 1965. *In* Spores III. L. L. Campbell and H. O. Halvorson, editors. American Society for Microbiology, Ann Arbor, Mich. 1–24.
20. PERRY, J. J., and J. W. FOSTER. 1955. *J. Bacteriol.* **69**:337.
21. POWELL, J. F. 1953. *Biochem. J.* **54**:210.
22. RIEMAN, H. 1963. Germination of bacterial spores with chelators. Ph.D. Thesis. Th. Bang, Copenhagen, Denmark.
23. ROSS, K. F. A., and E. BILLINGS. 1957. *J. Gen. Microbiol.* **16**:418.
24. SCHWARZENBACH, G. 1955. Die komplexometrische Titration. Ferdinand Enke, Verlag, Stuttgart, Germany.
25. SCHWARZENBACH, G., A. WILLI, and R. O. BACH. 1947. *Helv. Chim. Acta.* **30**:1303.
26. SLEPECKY, R. A., and J. W. FOSTER. 1959. *J. Bacteriol.* **78**:117.
27. SUZUKI, K., and K. YAMASAKI. 1957. *Naturwissenschaften.* **44**:396.
28. TANG, T., and N. GRECZ. 1965. *Bacteriol. Proc.* 7.
29. TANG, T., and N. GRECZ. 1966. *Bacteriol. Proc.* 32.
30. TANG, T., K. S. RAJAN, and N. GRECZ. 1968. *Biophys. J.* **8**:1458.
31. TICHANE, R. M., and W. E. BENNETT. 1957. *J. Amer. Chem. Soc.* **79**:1293.
32. WEINBERG, E. D., and S. M. TONNIS. 1966. *Appl. Microbiol.* **14**:850.
33. WINDLE, J. J., and L. E. SACKS. 1963. *Biochim. Biophys. Acta.* **66**:173.
34. WOOLEY, B. C., and R. E. COLLIER. 1965. *Can. J. Microbiol.* **11**:279.
35. YOUNG, E. I. 1959. *Can. J. Microbiol.* **5**:197.