

## Binding of Nickel and Zinc Ions to Bacitracin A<sup>†</sup>

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**ABSTRACT:** Bacitracin A is a cyclic dodecapeptide antibiotic produced by *Bacillus licheniformis*. Bacteriocidal activity requires the presence of divalent cations such as  $Zn^{2+}$ . The metal-bacitracin A complex binds to bactoprenyl pyrophosphate, a lipid intermediate required for cell wall biosynthesis which is found within the bacterial membrane. In this paper, the pH dependence of the metal binding to bacitracin A is investigated in an effort to define the sites of metal coordination. Most of the studies described in this report were performed with  $Ni^{2+}$  and  $Zn^{2+}$ . Metal binding was monitored by observing changes in the ultraviolet absorption spectrum of bacitracin A and by monitoring the proton release which is concomitant with metal binding to the peptide. It was determined that both  $Ni^{2+}$  and  $Zn^{2+}$  form 1:1 complexes with bacitracin A in solution. These complexes are soluble in acidic solutions, but above approximately pH 5.5 they become in-

soluble. On the basis of the data reported as well as results previously reported from other laboratories, a model for divalent metal ion binding to bacitracin is suggested. It is proposed that the metal coordinates directly to the glutamate carboxyl, the histidine imidazole, and the thiazoline ring. The aspartate carboxyl and N-terminal amino group are not directly involved in metal binding. It is further proposed that due to the proximity of the metal, the pK of the N-terminal amino is shifted from 7.7 to 5.7 upon metal binding. Deprotonation of this group is suggested to cause precipitation of the bacitracin A-metal complex. This model is consistent with all the metal binding data and, furthermore, is consistent with the <sup>1</sup>H NMR data presented in the accompanying paper [Mosberg, H. I., Scogin, D. A., Storm, D. R., & Gennis, R. B. (1980) *Biochemistry* (following paper in this issue)].

**B**acitracin consists of a mixture of closely related polypeptide antibiotics produced by *Bacillus licheniformis*. The major component, bacitracin A, is a cyclic dodecapeptide (see Figure 1). Bacitracin A is a potent antibiotic directed against gram-positive bacteria. The antibiotic activity of bacitracin is apparently due to inhibition of bacterial cell wall biosynthesis. The bacitracin-sensitive step is the dephosphorylation of the lipid carrier intermediate  $C_{55}$ -bactoprenyl pyrophosphate to form bactoprenyl phosphate (Siewert & Strominger, 1967). Strominger and co-workers have demonstrated that bacitracin forms a strong complex with long-chain polyisoprenyl pyrophosphates (Stone & Strominger, 1971; Storm & Strominger, 1973). This is a stoichiometric 1:1 complex and requires a divalent metal cation such as  $Zn^{2+}$ . The antibiotic activity of bacitracin has also been shown to require the presence of a divalent metal cation (Adler & Snoke, 1962). This general mechanism is supported by the data of Storm & Strominger (1974), who determined that the number of bacitracin molecules bound to *M. lysodeikticus* cells is similar to the number of bactoprenyl pyrophosphate molecules present.

The complex formed between bacitracin A and polyisoprenyl pyrophosphates can be viewed as a model for studying protein-lipid interactions. Before studying this complex, it is necessary to understand the role played by the divalent metal cation. It is well established that bacitracin binds divalent cations such as zinc, nickel, cobalt, and copper (Garbutt et al., 1961; Craig et al., 1969; Cornell & Guiney, 1970; Wasylshen & Graham, 1975). Previous reports on the metal binding properties of bacitracin are generally conflicting.

Virtually every available group on bacitracin has been suggested as being directly involved in metal coordination. These include (1) the thiazoline ring, (2) the histidine imidazole, (3) the terminal isoleucine amino group, (4) the histidine peptide nitrogen, and (5) both carboxyl groups. There is universal agreement that the ornithine amino group is not directly involved in metal coordination.

In this paper, potentiometric and optical spectroscopic techniques are employed to examine the pH dependence of metal binding to bacitracin A. It is demonstrated for the first time that in solution the stoichiometry of the bacitracin-metal complex is 1:1, and a model is proposed for the coordination sites on the peptide. In the accompanying paper (Mosberg et al., 1980) <sup>1</sup>H NMR data are presented in support of this model.

### Materials and Methods

Bacitracin USP was a gift from IMC Chemical Group, Inc., Terre Haute, IN. Bacitracin A was separated from the other components of the mixture by countercurrent distribution (Barry et al., 1948). The solvent system used, 1 part 0.1 M sodium phosphate buffer (pH 5.1)-1 part 1-butanol (v/v) (Janet Li Chen, personal communication), is a modification of one used successfully by Craig & co-workers (Craig & Konigsberg, 1957; Craig et al., 1969). An automatic machine (H. O. Post Scientific Instruments Co., New York) containing 400 tubes, each of 10 mL/phase capacity, was used. Phosphate was subsequently removed from bacitracin A by means of an anion-exchange resin, Dowex 1-X8 (200-400 mesh, chloride form). Use of the phosphorus assay described by Bartlett (1959) shows that the Dowex treatment results in less than 0.01 mol of phosphate per mol of bacitracin A. Atomic absorption measurements at 213.8 nm showed that both the Dowex-treated and untreated bacitracin A preparations are zinc free. The phosphate-free bacitracin A, a pale yellow powder after lyophilization, was stored in a freezer at about -13 °C.

The concentration of bacitracin A in aqueous solution was determined by absorption measurement using the extinction

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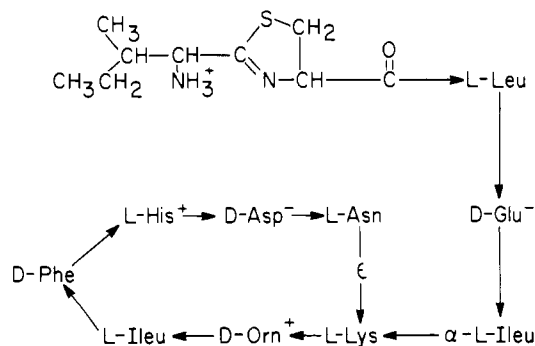


FIGURE 1: Structure of bacitracin A. The charges shown are for the molecule at neutral pH.

coefficient at 252 nm,  $\epsilon_{1\text{mg/mL}} = 2.0$  (Craig et al., 1969). The solution was free of divalent metal ions. Water used to make all solutions was deionized and subsequently distilled in glass. The bacitracin A concentration in the proton release (pH stat) and optical titrations was usually 0.2–0.3 mM. Precipitation of the bacitracin A–metal complex was prevented by using concentrations as low as 0.01 mM at  $\text{pH} \geq 6$ . The proton release method was more useful than the optical method with such low concentrations.

Reagent grade crystalline chlorides of nickel, zinc, and cobalt were used to prepare solutions for use in the binding experiments.

#### Metal Binding Monitored by Proton Release Measurement.

A solution of metal(II) chloride of known concentration was adjusted to exactly the same pH as that of a solution of bacitracin A in 0.15 M NaCl at 25 °C. Aliquots of the metal solution were then added by means of 5- and 25- $\mu\text{L}$  Hamilton syringes to the bacitracin solution (3-mL volume). The resulting pH changes, typically less than 0.1 unit, were back titrated with 0.1 N NaOH. The average proton release was calculated for each addition of metal by dividing the amount of  $\text{OH}^-$  added to restore the pH to the original value by the amount of bacitracin A in the sample. A combination pH meter/titrator was used in conjunction with an automatic motor-driven buret (Radiometer/Copenhagen). Separate glass and calomel reference electrodes were used to measure pH. For proton release experiments done at  $\text{pH} \geq 7$ , titrations were performed under argon or nitrogen.

**Metal Binding Monitored by UV Absorption.** Metal binding was also monitored by observing changes in the ultraviolet absorption spectrum of bacitracin A. In the absence of divalent cations, the peptide absorbs strongly at 252 nm due to the thiazoline ring, with an extinction coefficient of 2900  $\text{M}^{-1} \text{cm}^{-1}$  (see Figure 2). As small aliquots of a metal(II) chloride solution were consecutively added to a bacitracin A solution, the extinction coefficient increased until the bacitracin was saturated with the metal. With saturating concentrations of  $\text{Zn}^{2+}$  or  $\text{Ni}^{2+}$  the extinction coefficient in the 250-nm region was increased by  $\sim 50\%$ . The pH was held constant by use of a buffer (0.1 M sodium acetate or 0.05 M Pipes) or by use of a pH stat with no buffer. The absorbance was measured with a double-beam recording spectrophotometer (Varian 219 or Beckman Acta III). Absorbance of the bacitracin A solution was corrected for dilution, divided by the initial absorbance, and then plotted against the total metal concentration. See Figure 4 for typical binding isotherms.

Several metal binding experiments were performed in solutions containing 25% methanol in an attempt to reduce precipitation of the complex. This effort was not successful, but the methanol had no significant effect on either the proton release or the metal binding constant to bacitracin A.

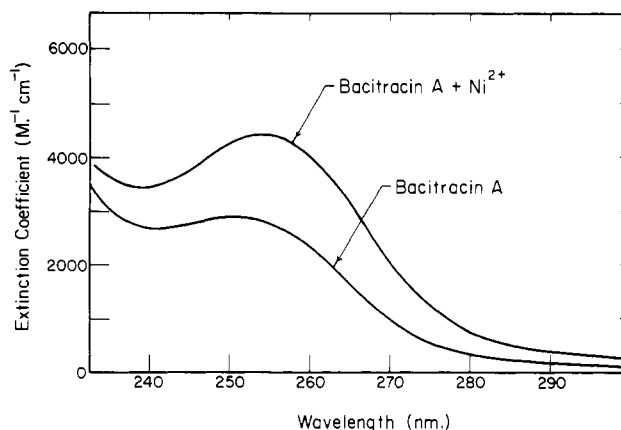


FIGURE 2: Effect of a saturating concentration of  $\text{Ni}^{2+}$  on the ultraviolet absorption spectrum of bacitracin A at pH 6.0 and 25 °C.  $\text{Zn}^{2+}$  is observed to have a similar effect.

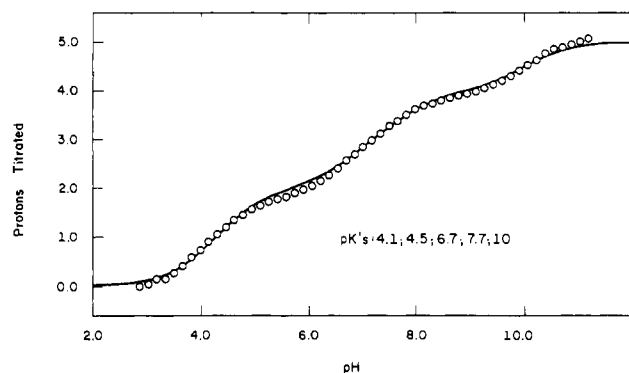


FIGURE 3:  $\text{H}^+$  titration curve of bacitracin A in 0.15 M NaCl at 25 °C. The smooth curve is calculated by assuming five independently titrating groups having the following  $\text{pK}$  values: 4.1, 4.5, 6.7, 7.7, and 10.

## Results

A number of residues on bacitracin are potential metal coordination sites. Many of these groups can be protonated and have  $\text{pK}$  values in the range pH 3–8. The strong pH dependence of the metal binding to bacitracin provides a powerful analytical technique for identifying the metal coordination sites. The  $\text{H}^+$  titration curve of bacitracin A is shown in Figure 3. The data are fit satisfactorily by assuming five independently titrating groups with the following  $\text{pK}$  values: 4.1 (aspartate), 4.5 (glutamate), 6.7 (histidine imidazole), 7.7 (isoleucine  $\text{NH}_3^+$ ), 10.0 (ornithine  $\text{NH}_3^+$ ).  $^1\text{H}$  NMR has been used to confirm these assignments (Mosberg et al., 1980). The values are in agreement with those previously reported (Newton & Abraham, 1953; Garbutt et al., 1961).

Two methods have been used to monitor metal binding to bacitracin A: (1) UV absorption and (2) proton release. Upon binding to divalent cations the extinction coefficient of bacitracin A in the 250-nm region increases by  $\sim 50\%$  (see Figure 2). This has been ascribed to the direct interaction of the metal with the thiazoline ring near the N terminus of bacitracin A and has been used previously to study metal binding (Craig et al., 1969; Garbutt et al., 1961). Binding isotherms for  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Co}^{2+}$  to bacitracin A were obtained at 25 °C at several pH values. As reported by other laboratories, all the metal–bacitracin complexes are insoluble at neutral pH. At the bacitracin A concentrations required for these experiments, the precipitation and ensuing turbidity make quantitation of the metal binding difficult by using this technique. However, it was found that in more acidic solutions, below approximately

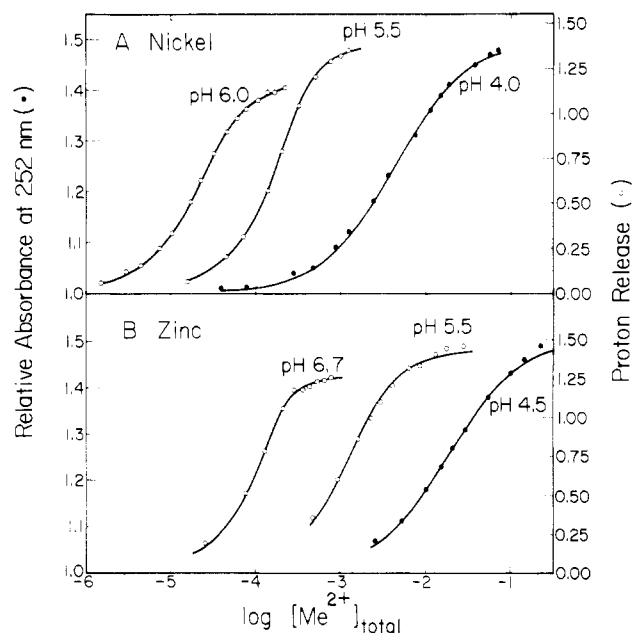


FIGURE 4: Effect of pH on the (A)  $Ni^{2+}$ - and (B)  $Zn^{2+}$ -bacitracin A binding isotherms, as monitored by spectrophotometric (●) and proton release (○) methods. Proton release is the average number of protons displaced by metal ions per bacitracin molecule. Total  $Me^{2+}$  is the sum of free and bacitracin-bound  $Me^{2+}$ . The experiments were done in 0.15 M NaCl at 25 °C. All curves are calculated by assuming a simple 1:1 binding equilibrium and optimizing the apparent binding constant.

pH 5.5, the metal-bacitracin complexes are fully soluble. Because of the competition between the metal ions and protons for common sites on bacitracin, the binding between the metal ions and bacitracin A is quite weak under acidic conditions. Several examples of  $Ni^{2+}$  and  $Zn^{2+}$  binding isotherms with bacitracin A are shown in Figure 4.

Upon metal binding to bacitracin A there is a concomitant release of protons. By monitoring proton release with a pH stat, the interaction between the metal and bacitracin A can be measured. Aliquots of  $Ni^{2+}$  or  $Zn^{2+}$  solutions were added to a solution of bacitracin A, and NaOH was added to neutralize any protons which were released. Resulting metal binding isotherms all show saturation and are in full agreement with the isotherms obtained at the same pH values by using the UV absorption changes as a monitor. Because of the sensitivity of the pH electrode, very low concentrations of bacitracin can be used at more alkaline pH values (pH greater than 6). The problem of precipitation of the metal-bacitracin complexes at neutral pH is thus eliminated or at least minimized by using this potentiometric technique. Several examples of  $Ni^{2+}$  and  $Zn^{2+}$  binding isotherms to bacitracin A are shown in Figure 4. For those cases shown, ~1–1.5 protons are released per bacitracin A molecule upon metal saturation. It should be noted that the axis in Figure 4 is not the free metal cation concentration but rather the total metal concentration including both free metal and that which is bound to the bacitracin. The curves drawn through the data points in Figure 4 are calculated based on a simple binding equilibrium with only one metal binding site per bacitracin A molecule.

It has been previously assumed that the stoichiometry of the metal-bacitracin complex is 1:1 (Craig et al., 1969). This has been based on the elemental analysis of the zinc-bacitracin A precipitate (Gross, 1954; Chornock, 1957). Figure 5 presents data which clearly demonstrate that the stoichiometry of both the  $Ni^{2+}$ - and  $Zn^{2+}$ -bacitracin A complexes in solution

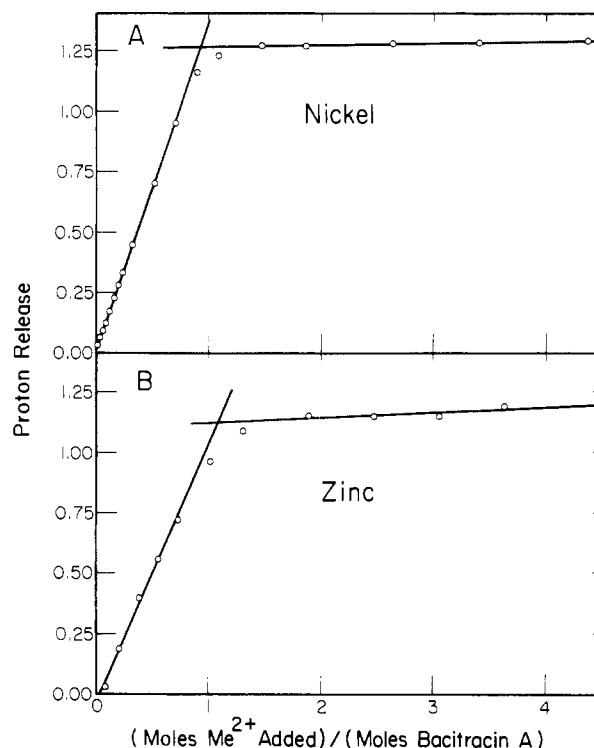


FIGURE 5: 1:1 complex formation between bacitracin A and (A)  $Ni^{2+}$  at pH 6.6 and (B)  $Zn^{2+}$  at pH 7.0. The experiments were done in 0.15 M NaCl at 25 °C.

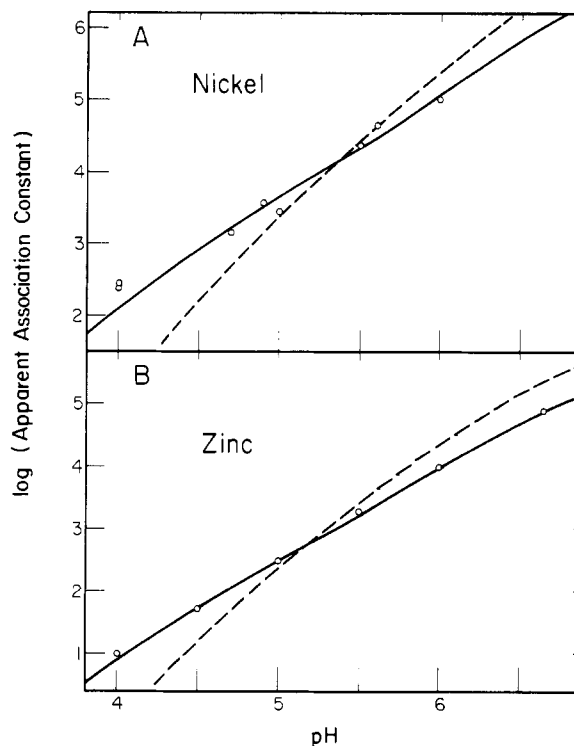


FIGURE 6: pH dependence of the apparent association constants for  $Ni^{2+}$ -bacitracin A and  $Zn^{2+}$ -bacitracin A. Data points were obtained by a curve-fitting procedure, and each represents a best fit to a set of binding data obtained at the indicated pH by either the spectrophotometric or the proton release method. The continuous curves are calculated on the basis of the model described under Discussion. The dashed curves represent an alternative model in which the metal is directly coordinated to the N-terminal amino position as well as to the His, Glu, and thiazoline residues.

is 1:1. Near pH 7, the binding between the  $Ni^{2+}$  or  $Zn^{2+}$  and bacitracin A is sufficiently strong so that all the added metal

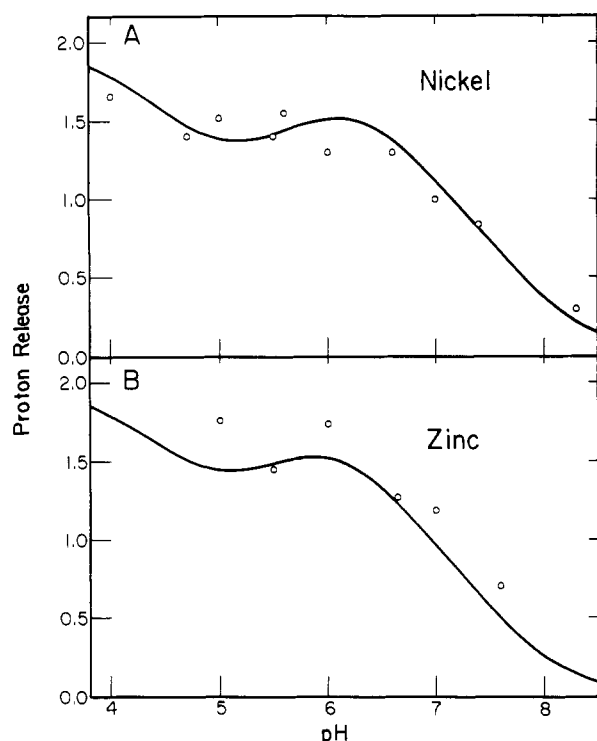


FIGURE 7: Dependence of proton release from bacitracin A on pH. The ordinate refers to protons released per molecule of bacitracin A on saturation with excess (A)  $\text{Ni}^{2+}$  and (B)  $\text{Zn}^{2+}$ .

is bound to the antibiotic up to the point of saturation. With both  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$  it is clear that the binding stoichiometry is 1:1 with bacitracin A.

Figures 6 and 7 summarize the data on the pH dependence of  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$  binding. On the assumption that the binding stoichiometry remains 1:1 for the experimental conditions employed, the binding data such as those shown in Figure 4 can be replotted in terms of the free metal ion concentration rather than the total metal ion concentration (not shown). From this plot can be obtained the apparent association constant of the metal for the bacitracin. Figure 6 shows the pH dependence of the apparent association constants for both  $\text{Zn}^{2+}$ -bacitracin A and  $\text{Ni}^{2+}$ -bacitracin A. In both cases, the apparent association constant is reduced by nearly 3 orders of magnitude when the pH is lowered from 6.5 to 4.

An alternate but equivalent way of quantitating the pH dependence of the metal binding to bacitracin is shown in Figure 7. The number of protons released upon saturation of bacitracin A with the metal cations is plotted against the pH of the solution. In fact, the curve described by the data in Figure 7 should simply be the derivative of the curve described by the data in Figure 6 (Laskowski & Finkenstadt, 1972). The data points shown in Figure 7 were, however, determined experimentally. The data obtained with both  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$  are quite similar. The number of protons released upon metal saturation appears to approach zero above pH 8. The data do not extend to more alkaline pH values due to the formation of insoluble metal hydroxides. Between approximately pH 4 and 6 the number of protons released upon metal saturation appears to reach a plateau at  $\sim 1.5$  protons/molecule of bacitracin. The data do not extend to lower pH values due to the inability to saturate bacitracin A with the metal at these acidic conditions.

The binding of  $\text{Co}^{2+}$  to bacitracin A was also examined (not shown). The data were similar to those obtained with  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$ , suggesting that all three metals bind to the peptide in a similar manner.

## Discussion

The goal of this work was to arrive at a plausible and testable model for the metal binding to bacitracin A by examining the pH dependence of that binding. It is shown for the first time that in solution the stoichiometry of divalent metal cation binding to bacitracin A is in fact 1:1. The use of potentiometric techniques enabled this experiment to be performed with sufficiently low bacitracin A concentrations so that precipitation of the complex did not occur. The agreement between the potentiometric and spectroscopic titrations (e.g., Figure 4) suggests that additional metal binding does not occur beyond that indicated by the proton release technique. It is possible, of course, that metal binding is occurring which results in no proton release and no spectral perturbation.

The strong pH dependence of the binding of both  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$  to bacitracin A makes it likely that the divalent cations and protons are competing for common sites on bacitracin A. Any model must be designed to fit the experimental data described in Figures 6 and 7. In addition, it is known that the metal-bacitracin complex is insoluble above approximately pH 5.5 but is fully soluble at more acidic pH values. Hence, there must be some group on the metal-bacitracin A complex which titrates in the range pH 5 to 6. The ionizable residues of bacitracin A are the glutamate, the aspartate, the histidine imidazole, the thiazoline ring, the  $\delta$ -amino group of ornithine, and the amino group of the N-terminal isoleucine. There is general agreement that the  $\delta$ -amino group of ornithine is not involved in metal coordination. The data presented in this paper are in agreement with this idea. For example, at pH 7.5 it is clear that neither  $\text{Ni}^{2+}$  nor  $\text{Zn}^{2+}$  is displacing a proton from the ornithine  $\delta$ -amino group (Figure 7). The pK of the thiazoline ring is reported to be below pH 2 (Newton & Abraham, 1953). Hence, the pH dependence of the metal binding to bacitracin A does not approach the question of the direct involvement of the thiazoline ring. However, the large effect of metal binding on the UV absorption spectrum coupled with the NMR data presented in the accompanying paper (Mosberg et al., 1980) makes it likely that the thiazoline ring provides one coordination site. There is also a considerable body of evidence that the histidine is directly coordinated to the metal in the complex (Garbutt et al., 1961; Cornell & Guiney, 1970; Wasylshen & Graham, 1975).

Examination of the data presented in Figures 6 and 7 indicates that a simple, straightforward model will not suffice. For example, the significant proton release observed above pH 7.5 implicates the amino of the N-terminal isoleucine. The increase in the number of protons released below pH 5.5 is considered to be due to the involvement of one of the carboxyl groups. Yet, if the metal is binding to both the histidine and the N-terminal amino residue, then at least two protons should be released per bacitracin A molecule near pH 5.5. This is certainly not the case, at least for nickel. Furthermore, the metal-bacitracin A complex must contain a titratable group responsible for the observed precipitation. With the metal coordinated directly to both the histidine and the N-terminal amino group, it is difficult to explain the origin of the pH-dependent precipitation of the complex.

The following model has been devised which is consistent with all the data presently available (see Figure 8). It is proposed that the metal binds directly to (1) the glutamate carboxyl, (2) the thiazoline ring, and (3) the histidine imidazole. The aspartate carboxyl and the amino group of the N-terminal isoleucine are not directly involved in metal coordination. It is further proposed that due to an electrostatic

$$\text{apparent } K_{\text{assn}} = \frac{\left(1 + \frac{[\text{H}^+]}{\alpha K_3}\right) / K_M}{1 + \frac{[\text{H}^+]}{K_1} + \frac{[\text{H}^+]}{K_2} + \frac{[\text{H}^+]}{K_3} + \frac{[\text{H}^+]^2}{K_1 K_2} + \frac{[\text{H}^+]^2}{K_2 K_3} + \frac{[\text{H}^+]^2}{K_1 K_3} + \frac{[\text{H}^+]^3}{K_1 K_2 K_3}} \quad (1)$$

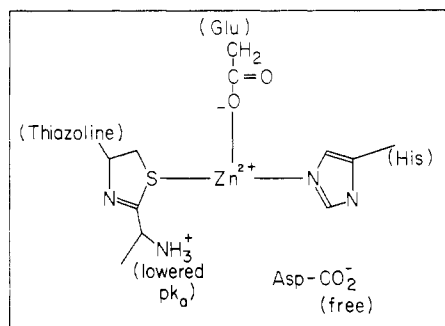


FIGURE 8: Schematic illustration of the model proposed for  $\text{Zn}^{2+}$ -bacitracin A. The same model would apply to the complexes formed with  $\text{Ni}^{2+}$  and  $\text{Co}^{2+}$ .

effect when the  $\text{Ni}^{2+}$  or  $\text{Zn}^{2+}$  binds to bacitracin, the  $pK$  of the N-terminal amino is lowered from 7.7 to  $\sim 5.7$ . It is deprotonation of this group which results in precipitation of the complex. The net charge on the complex at neutral pH would be +1. The pH dependence of the metal binding to bacitracin A can be easily calculated by using this model. The experimentally determined  $pK$  values of the glutamate carboxyl, the histidine imidazole, and the N-terminal amino group are utilized in this calculation. The adjustable parameters are the intrinsic binding constant  $K_M$  of the metal to bacitracin A and the parameter  $\alpha$  which quantitates the negative cooperativity between metal binding to bacitracin A and proton binding to the N-terminal amino group. Equation 1 was used for these calculations.

$K_1$ ,  $K_2$ ,  $K_3$  are the acid dissociation constants for the His imidazole, the Glu carboxyl, and the N-terminal amino group, respectively, and  $[\text{H}^+]$  is the hydronium ion concentration. The solid curves shown in Figures 6 and 7 were calculated by using the model described. The microscopic binding constants used to obtain the curves were  $K_{\text{Ni}^{2+}} = 4.0 \times 10^{-8}$  M and  $K_{\text{Zn}^{2+}} = 1.0 \times 10^{-6}$  M. For both  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$ ,  $\alpha = 100$  resulted in the optimum fit of the data. It is evident that this model provides a reasonable explanation for all the experimental data.  $^1\text{H}$  NMR data presented in the accompanying paper (Mosberg et al., 1980) provide direct evidence for metal binding to the glutamate, the sulfur on the thiazoline ring, and the histidine imidazole. The NMR data also indicate that the aspartate carboxyl is not involved in metal coordination. The NMR experiments are, however, equivocal concerning the role of the N-terminal amino. Experiments are in progress to test this aspect of the model.

It should be mentioned that the metal coordination by the glutamate, the thiazoline ring, and the histidine imidazole is easily accommodated by a space-filling molecular model. The requirement of having the divalent cation coordinated simultaneously to these three positions in large part defines the conformation of the peptide-metal complex. This information should be of particular value in understanding at a molecular level the nature of the ternary complex involving the lipid pyrophosphate. The model which has been described is offered as being the most plausible model for describing the data. It is not necessarily unique. However, a number of alternate models were tested and found to be markedly inferior in providing a fit to the experimental data. One example is shown

in Figure 6. The pH dependence of the apparent association constant was calculated for a model in which the metal was directly coordinated to the histidine, the glutamate, and the N-terminal amino position. Postulating direct coordination to the N-terminal amino residue predicts a pH dependence of the apparent association constant which is significantly steeper than that experimentally determined for both  $\text{Ni}^{2+}$  and  $\text{Zn}^{2+}$ . The primary experimental evidence in favor of direct coordination of the isoleucine amino group to the metal has been the report that the presence of excess  $\text{Zn}^{2+}$  protects this amino residue from chemical modification by 1-fluoro-2,4-dinitrobenzene (Craig et al., 1969). However, molecular model building shows that metal coordination to the histidine, thiazoline, and glutamate residues would leave the isoleucine amino position significantly less accessible to chemical reagents. Craig et al. (1969) also reported that  $\text{Zn}^{2+}$  binding to bacitracin A at pH 6.3 resulted in the release of four protons. This is not compatible with the data documented in this paper. The reason for the discrepancy is not known. It has also been pointed out that the binding of bactoprenyl pyrophosphate to bacitracin F is very weak and is not affected by divalent cations (Storm & Strominger, 1973). Bacitracin F differs from bacitracin A in that the amino residue at the N terminus is replaced by a ketone group and the thiazoline is replaced by a thiazole ring. It is not clear why bacitracin F differs in its properties from bacitracin A. Future experiments should clarify these ambiguities.

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