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Interactions between Polymyxin B and Divalent Nickel in Near-Neutral Aqueous Media

MORTON L. BARR and KENNETH KUSTIN ˣ

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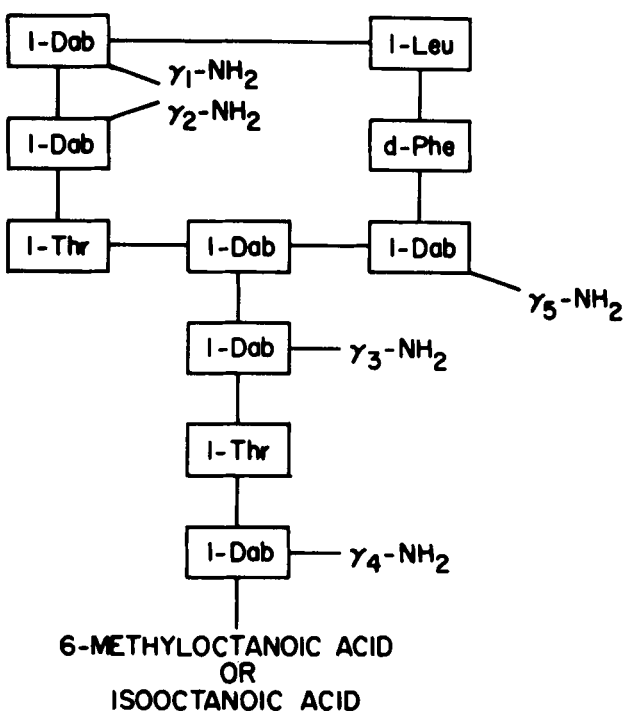
Abstract □ Stopped-flow and temperature-jump methods were used to determine the complexation constants for the reactions of polymyxin B with Ni^{2+} at 25° and 0.1 *M* ionic strength [$N(CH_3)_4NO_3$]. At pH 6.4–7.1 and with the ligand in excess, the formation of a 1:1 metal–ligand complex according to the reaction scheme $Ni^{2+} + H_3L^{3+} \rightleftarrows NiH_3L^{5+}$ and $Ni^{2+} + H_4L^{4+} \rightleftarrows NiH_3L^{5+} + H^+$ (k_1 and k_1' are respective forward rate constants; k_{-1} and k_{-1}' are respective reverse rate constants) is consistent with the data. The determined rate constants are $k_1 = (3.8 \pm 0.2) \times 10^3$ M^{-1} sec^{-1} and $k_{-1} = (1.2 \pm 0.1) \times 10^{-1}$ sec^{-1}. Upper and lower limits for k_1' of 2.4 and 0.6 M^{-1} sec^{-1}, respectively, were set. The stability constant ($K_1 = k_1/k_{-1}$) of the 1:1 complex determined by potentiometric titration is $(3.3 \pm 0.1) \times 10^4$ *M*; the four pKa values of polymyxin B agree with the literature values. At pH 7.1–7.9 and with the metal ion in excess, the described scheme and a binuclear complex reaction step, $Ni^{2+} + NiHL^{3+} \rightleftarrows Ni_2HL^{5+}$ (k_2 is the forward rate constant; k_{-2} is the reverse rate constant), fit the data. Upper and lower limits for k_2 of 1×10^4 and 3×10^3 M^{-1} sec^{-1}, respectively, were set for the formation of the binuclear complex. Comparison with model system complex formation rate constants indicates that the forward rate constants evaluated in this study are enhanced. This effect may be interpreted in terms of an internal conjugate base mechanism.

Keyphrases □ Polymyxin B—complexation with nickel(II) in aqueous media, effect of pH and ligand concentration □ Nickel(II)—complexation with polymyxin B in aqueous media, effect of pH and ligand concentration □ Complexation—polymyxin B and nickel(II) in aqueous media, effect of pH and ligand concentration □ Antibacterials—polymyxin B, complexation with nickel(II) in aqueous media, effect of pH and ligand concentration □ Metals—nickel(II), complexation with polymyxin B in aqueous media, effect of pH and ligand concentration

Polymyxin B is a surface-active polypeptide antibiotic exhibiting potent bactericidal activity against most Gram-negative bacilli (1). The addition of polymyxin B to cell suspensions of these bacilli results in a leakage from the cells of pentose, phosphate, and substances with absorption maxima at 260 nm (1, 2). The mechanism of action is thought to involve combination of the antibiotic with charged groups on the cell membrane surface, resulting in disorganization of the membrane structure (1–3). The bactericidal activity of polymyxin B is influenced strongly by various metal ions in several complicated modes of interaction (1, 2).

The proposed structure of polymyxin B is shown in Structure I and Fig. 1 (4, 5). Polymyxin B is a cyclic hexapeptide with a tripeptide side chain *N*-acylated by 6-methyloctanoic acid (polymyxin B_1) or isooctanoic acid (polymyxin B_2); it is a mixture of B_1 and B_2 in the ratio 65:35, respectively (6). Each component has one D-amino



I: Amino acid sequence of polymyxin B. Key: l-Dab, *l*-α,γ-diaminobutyric acid; l-Thr, *l*-threonine; d-Phe, *d*-phenylalanine; and l-Leu, *l*-leucine.

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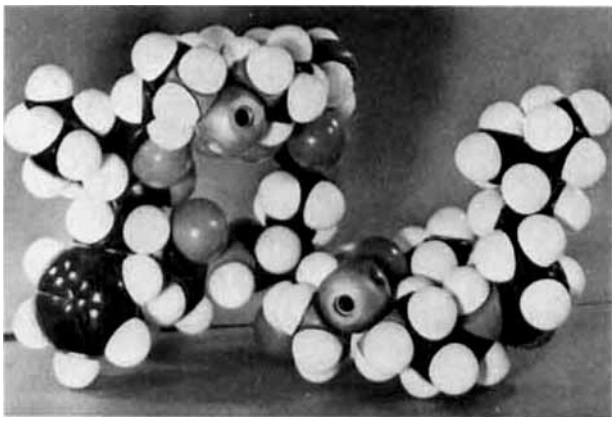

Figure 1—*Corey–Pauling model of one configuration for the bimetallic polymyxin B complex. The metal ions are represented by the gray octahedral units.*

acid (phenylalanine) and six residues of α,γ-diaminobutyric acid, which are responsible for its complexation behavior toward the divalent transition metal ions. From this behavior, it will be shown that polymyxin B represents a physiologically active analog of the simple polyamines, of which the complexation properties with transition metal ions are well known (7, 8).

EXPERIMENTAL

Reagent grade nickel(II) nitrate hexahydrate[1], phenol red (phenolsulfonphthalein), and *p*-nitrophenol[2] were used without further purification. Tetramethylammonium nitrate[2] was used as the supporting electrolyte in all studies, because preliminary investigations indicated complex formation to occur between polymyxin and the alkali metal ions. The nitrate salt was twice recrystallized from 75% aqueous ethanol and dried *in vacuo* under phosphorus pentoxide.

Polymyxin B was obtained commercially as the sulfate[3]; the peptide was dissolved in a minimum amount of distilled water, precipitated as the free base by passing gaseous ammonia through the solution at −5°, centrifuged, filtered on a fritted Büchner funnel, and washed three times with cold water. The light, fluffy white precipitate was then suspended in water and twice lyophilized. The stoichiometric amount of hydrochloric acid was added, and the solution was again twice lyophilized.

The resulting pentahydrochloride migrated identically with an authentic sample of polymyxin B[4] when placed on a thin-layer plate in the solvent system 1-butanol–pyridine–acetic acid–water (30:20:5:24). Both materials migrated as single components. The optical rotatory dispersion curves of the nickel complexes of the two materials also compared favorably in the 350–600-nm region. The chloride content of polymyxin B (pentahydrochloride) was confirmed by the Volhard method. Elemental analysis corresponded to $C_{56}H_{98}N_{16}$, as predicted.

In addition to the characteristic UV absorption near 260 nm, polymyxin B revealed an anomalous absorption tailing into the visible region, with a maximum near 280 nm. Thus, solutions of polymyxin with a concentration greater than 10^{-3} *M* exhibited a yellow hue. This apparent impurity was not detected within the experimental error of elemental analysis and was not removed by treatment of polymyxin B on chromatographic columns of Sephadex G-15 or G-25, Biorex-70 cation-exchange resin, and diethylaminoethylcellulose anion-exchange resin. Attempts to remove this impurity by countercurrent distribution in 1-butanol–pyridine–acetic acid–water (8:2:1:9) (5), in which polymyxins B_1 and B_2 were separated, were unsuccessful. Emission spectra of the purified pentahydrochloride confirmed the absence of metal ions. Therefore, if an impurity was indeed present, its concentration was sufficiently small not to preclude the experimental studies undertaken.

Stock solutions of nickel nitrate and indicators were prepared by weight. The nickel content of the stock solutions was confirmed by complexometric titration with ethylenediaminetetraacetic (edetic) acid

[1] Fisher.
[2] Eastman.
[3] Calbiochem.
[4] Supplied by Dr. R. O. Studer, Hoffmann–La Roche, Basel, Switzerland.

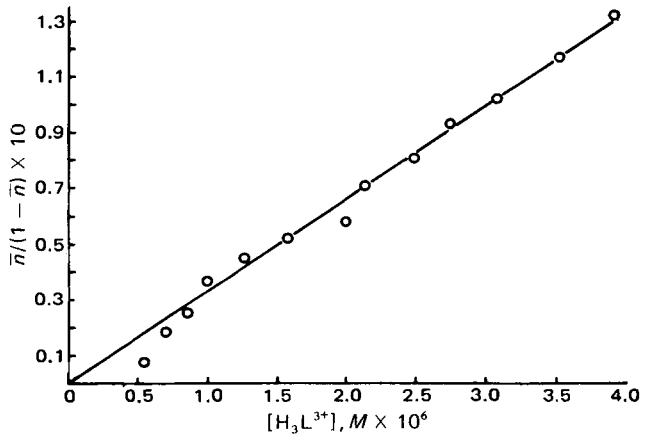

Figure 2—*Plot of* $\bar{n}/(1-\bar{n})$ versus *free ligand species concentration. The straight line was obtained by least-squares data analysis. (Data points close to the origin appear to lie on a curve. However, these points are collected at the start of the titration and have a higher intrinsic relative error. The apparent curvature actually reflects greater scatter and not a systematic trend.)*

with murexide as the indicator (9). Solutions to be studied were prepared by mixing the desired volumes of stock solutions with weighed amounts of polymyxin B and tetramethylammonium nitrate. All pH values were measured to ±0.02 pH unit. Hydrogen-ion concentrations were obtained by dividing the measured hydrogen-ion activity by $\gamma_{\pm} = 0.739$ from the Davies equation (10). The temperature in all experiments was 25 ± 1°. Commercially available distilled water was used in all experiments.

The stopped-flow and temperature-jump apparatuses were described previously (11–14). In the stopped-flow studies, one syringe contained polymyxin solution and the other contained Ni(II). Both syringes contained the same concentrations of the supporting electrolyte, indicator, and hydrogen ion (adjusted by dropwise addition of dilute acid and base). The pH values reported were determined shortly after mixing. Blank experiments of *p*-nitrophenol with nickel and polymyxin gave no discernible effects in the concentration and time ranges studied. However, blank experiments yielded relaxation effects on the temperature-jump apparatus with phenol red and polymyxin, as well as with phenol red and nickel. The former effect can be attributed to complexation of the polypeptide with the indicator, perhaps as in the case of bovine serum albumin with phenol red (15). Nickelous ion complexes with certain pH indicators (16, 17).

The acid dissociation constants of polymyxin B and the stability constant of the polymyxin–Ni(II) complex were determined by potentiometric titration using the Bjerrum technique (18, 19).

RESULTS

Equilibrium Studies—Polymyxin B has five titratable ammonium protons; these amino groups serve as metal binding sites. The number of ligand protons displaced during complexation with nickel cannot be determined clearly in a restricted pH titration range. Furthermore, formation of binuclear and higher order nickel complexes with polymyxin is possible.

Consequently, an unambiguous analysis of the titration data for the nickel–polymyxin system is difficult to achieve. Moreover, both nickel(II) and copper(II) ions, when complexed to di- and oligopeptides, labilize peptide protons (20–23). This effect also was observed in the stability constant determination of Cu(II)–polymyxin (24). Thus, any interpretation of the titration data must consider these factors as in the following treatment.

Under the conditions of excess polymyxin and $6.3 < pH < 7.1$, the initial assumption made was that only a 1:1 complex is formed in which nickel is bound either to H_3L^{3+} or H_2L^{2+}. This assumption is based on two considerations. It is very unlikely that H_5L^{5+} and H_4L^{4+} will bind strongly to nickelous ion on grounds of electrostatic repulsion. Model studies strongly suggest that ring strain and rotational barriers preclude a complexed species in which nickel is simultaneously attached to four or five γ-NH_2 groups.

For the 1:1 complex system, a plot of $\bar{n}/(1-\bar{n})$ *versus* free binding species concentration (where $\bar{n}$ is the average number of ligand molecules bound per metal ion) yields a slope equal to the stability constant. Attempts were also made to fit the data with higher order and binuclear

Table I—Equilibrium Data at 25° and 0.1 *M* Ionic Strength [a]

Acid Dissociation of Polymyxin B			
pKa_1	pKa_2	pKa_3	pKa_4
7.99 (±0.01)	8.71 (±0.02)	9.09 (±0.04)	9.43 (±0.03)

Acid Dissociation of Indicators [b]	
Indicator	K_{In}, *M*
Phenol red	1.26×10^{-8}
p-Nitrophenol	7.08×10^{-8}

[a] Stability constant of polymyxin B–Ni(II) complex ($\log K_1$) = 4.52 (±0.01). [b] I. M. Kolthoff, *J. Phys. Chem.*, **34**, 1466 (1930).

complexes. Within experimental error, the values of the equilibrium quotients corresponding to these complexes approach zero. The best fit of calculated *versus* observed values of $\bar{n}$ was obtained when the binding form of polymyxin was H_3L^{3+} and not H_4L^{4+} or H_2L^{2+}. A plot of $\bar{n}/(1-\bar{n})$ *versus* $[H_3L^{3+}]$ is shown in Fig. 2, from which $K_1 = (3.3 \pm 0.1) \times 10^4\ M^{-1}$.

The relatively simple system consisting of protonated ligand, free metal ion, and complex does not fit the data above pH 7.1. The assumption of higher order (*e.g.*, bis) complexes does not improve the fit. It is reasonable to assume that, as the pH is raised, new nickel(II) species will be present. However, inclusion of these species invalidates the Bjerrum method, necessitating that several assumptions be made to analyze the data.

As a consequence of the ligand's relatively high affinity for the metal ion and its excess concentration, hydrolyzed nickel(II) is not important when $\bar{n} = 1$. Data fitting was then attempted with binuclear and deprotonated complexes, singly and together. For a deprotonated complex, an adjustable parameter was introduced, representing the relative concentrations of deprotonated and protonated complex species. The agreement between observed and calculated $\bar{n}$ was not improved by inclusion of adjustable parameters, and quantitative equilibrium data for the system nickel(II)–polymyxin above pH 7.1 were not obtained.

Unlike the stability constant determination, the measurement of the acid dissociation constants of polymyxin was straightforward. In the pH titration range studied (6.0–10.0), four acid dissociation constants were determined, leading to formation of the fully deprotonated peptide. The results for the pKa and stability constant determinations are reported in Table I.

Kinetic Studies—The conditions chosen for the initial kinetic studies allow the least ambiguous calculation of equilibrium concentrations necessary for interpretation of the data. These calculations were performed with the Newton–Raphson iteration method. Less desirable, but useful, are experiments in which pseudo-first-order conditions prevail and the metal ion is in excess. The metal-ion concentration is approximately equal to the total stoichiometric amount, and the concentrations of other species are calculated accordingly.

For the stopped-flow data, evaluation was achieved by employing close-to-equilibrium analysis. Therefore, only the last portion of each reaction trace was used in determining the relaxation time, τ. Use was made of the stoichiometric equilibrium constants in Table I and the concentrations in Table II in calculating these relaxation times. The experimental values of τ (Table II) were obtained by averaging at least five experimental values (12, 14). The deviations were usually less than ±10% in these averaged relaxation times. The exponentiality of the last portion of the reaction was confirmed by semilogarithmic plots of concentration *versus* time. Long-term stability in the kinetic (and also the equilibrium) studies was established by the absence of baseline drift for times longer than the lapsed time between solution preparation and data collection.

The most favorable experimental conditions are slightly acid pH and excess ligand since the equilibrium parameters under these conditions are known. For these solutions, the reaction consistent with the stopped-flow data is shown in Scheme I.

$$\begin{array}{ccccc} Ni^{2+} + H_3L^{3+} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & NiH_3L^{5+} & & \\ K_{a0}\ \Big\downarrow & & & & \\ Ni^{2+} + H_4L^{4+} & \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} & NiH_3L^{5+} & + & H^+ \end{array}$$

Scheme I

Scheme I is characterized by a single relaxation time for which:

$$1/\tau S = k'_1 + k_1 R/S \qquad \text{(Eq. 1)}$$

Table II—Stopped-Flow Data at 25° and 0.1 *M* Ionic Strength [a]

$[Ni(II)]_0$ [b] $\times 10^3\ M$	$[Polymyxin]_0$ $\times 10^3\ M$	pH	τ, sec, Experimental	τ, sec, Calculated
0.513	1.08	7.10	3.7	4.2
0.720	1.07	7.18	3.3	3.3
1.04	0.935	7.00	3.4	3.4
1.03	2.00	7.00	3.4	3.3
0.260	0.659	6.98	6.2	6.1
0.781	2.10	6.96	4.0	4.0
0.260	1.21	6.71	7.7	6.8
0.520	0.972	6.71	5.4	6.0
0.208	0.755	6.72	7.0	7.1
0.520	0.915	6.48	7.1	7.0
1.04	1.02	6.48	5.7	5.7
0.513	1.03	6.45	6.9	6.8
3.00	0.254	7.90	0.29	0.34
5.00	0.267	7.85	0.25	0.26
10.0	0.321	7.60	0.21	0.19
3.00	0.203	7.58	0.49	0.52
7.00	0.287	7.45	0.23	0.31
5.00	0.282	7.45	0.33	0.41
10.0	0.287	7.44	0.24	0.24
20.0	0.373	7.34	0.22	0.15
5.00	0.287	7.33	0.44	0.51
10.0	0.279	7.32	0.32	0.29
20.0	0.239	7.26	0.23	0.19
7.00	0.455	7.11	0.52	0.54

[a] Using *p*-nitrophenol as indicator at $1.73 \times 10^{-5}\ M$ when Ni(II) is in excess and at $4.32 \times 10^{-5}\ M$ when polymyxin is in excess. [b] The subscript refers to total stoichiometric concentration.

where R and S are defined in the *Appendix*. These terms arise from coupling of Scheme I to the faster indicator and ligand protolytic equilibria. A plot of $1/\tau S$ against R/S yields $k_1 = (3.8 \pm 0.2) \times 10^3\ M^{-1}\ sec^{-1}$ (Fig. 3). The value of k'_1 is unobtainable from Fig. 3, however, and only upper and lower limits of $0.6 < k'_1 < 2.4\ M^{-1}\ sec^{-1}$ can be determined. The reverse rate constant k_{-1} could be calculated from the relation $k_{-1} = k_1K_1$; however, a direct experimental determination of this constant is preferable.

To determine k_{-1} directly, attack of H_4L^{4+} on Ni^{2+} was neglected. There remains only one reaction in Scheme I, in which H_3L^{3+} is the only attacking ligand species. The $1/\tau$ expression for this reaction is given in Eq. 2, where α arises from the faster protolytic equilibria:

$$1/\tau = k_{-1} + k_1([Ni^{2+}]\alpha + [H_3L^{3+}]) \qquad \text{(Eq. 2)}$$

and is also given in the *Appendix*. A plot of Eq. 2 yields $k_1 = (3.5 \pm 0.2) \times 10^3\ M^{-1}\ sec^{-1}$ and $k_{-1} = 1.2 \pm 0.1\ sec^{-1}$ (Fig. 4). The ratio of these rate constants, $2.9 \times 10^4\ M^{-1}$, is in good agreement with the value of $3.3 \times 10^4\ M^{-1}$ obtained for the stability constant by potentiometric titration. The two values of k_1 determined agree within experimental error.

Under the conditions of these experiments, polymyxin exists primarily as the fully protonated species, H_5L^{5+}. With ethylenediamine (25) and related systems (8, 26), protonated forms of the ligand react several orders of magnitude slower than the corresponding neutral species. Therefore, for ligand species with all amine binding sites protonated, the reaction rates should be even slower, if not negligible, within experimental error. Thus, protonated forms of polymyxin other than those included in Scheme I were assumed not to be important attacking forms of the ligand. As previously mentioned, polymyxin has multiple binding sites. Therefore, as in the equilibrium studies, an attempt was made to fit the data to a mechanism whereby an additional metal ion is consumed. This mechanism was inconsistent with the data in the acid pH region and with the ligand in excess.

Although the equilibrium data in slightly basic media could not be successfully determined, the lack of these data does not preclude a kinetic study in the same media. Under pseudo-first-order conditions with the metal in excess, the approximations $\Sigma[\text{ligand species}] \ll [Ni^{2+}] \sim [Ni^{2+}]_0$ are valid, and the interpretation is simplified. The last 12 experiments of Table II were run under these assumptions. Several reaction schemes were applied to these data; Scheme II yielded the best fit.

$$\begin{array}{ccccccc} Ni^{2+} + H_3L^{3+} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & NiH_3L^{5+} & & & & \\ & & \Big\downarrow K_{a3},\ K_{a4} & & & & \\ Ni^{2+} + NiHL^{3+} & + & 2H^+ & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & Ni_2HL^{5+} & + & 2H^+ \end{array}$$

Scheme II

Table III—Rate Constants for Ni(II)–Polymyxin B at 25° and 0.1 *M* Ionic Strength

Method	k_1 (M^{-1} sec^{-1})	k_1' (M^{-1} sec^{-1})	k_2 (M^{-1} sec^{-1})	k_{-1} (sec^{-1})
Stopped flow (excess ligand), M + L ⇄ ML	$3.5\ (\pm1.2) \times 10^3$	—	—	$1.2\ (\pm0.1) \times 10^{-1}$
Stopped flow (excess ligand), M + L = ML M + HL = ML + H	$3.8\ (\pm0.2) \times 10^3$	<2.4 >0.6	—	—
Stopped flow (excess metal)	$4.3\ (\pm0.2) \times 10^3$	—	$\leq 1 \times 10^4$ $\geq 3 \times 10^3$	—
Temperature jump (excess metal)	$\sim 6.0 \times 10^3$[a]	—	—	—

[a] Value represents a composite rate constant; see text.

The observed reciprocal relaxation time is related to the concentration variables according to:

$$(1/\tau)S' = k_2 + k_1R'/S' \qquad \text{(Eq. 3)}$$

where R' and S' are given in the *Appendix*. A plot of Eq. 3 yields $k_1 = (4.3 \pm 0.2) \times 10^3\ M^{-1}\ sec^{-1}$ (Fig. 5). The value of k_2 is unobtainable from Fig. 5, and only upper and lower limits of $1 \times 10^4 > k_2 > 3 \times 10^3\ M^{-1}\ sec^{-1}$ can be determined.

To calculate the concentrations of $NiHL^{3+}$ and Ni_2HL^{5+}, required in Eq. 3, it is necessary to know the values of the complex acid dissociation constants as well as the stability constant K_2 for bimetallic complex formation. Since these constants are not known, estimates were used as initial trial values in a computerized data-fitting routine. Subsequent variation of these parameters by a factor of 10 above and below the initial values resulted in a variation in the rate constants of ±20%, with little sensitivity toward the assumed value of $K_2 \simeq 4 \times 10^3\ M$.

Rate constants for polymyxin complexation with nickelous ion are summarized in Table III.

To confirm the veracity of this treatment, another method was used. Temperature-jump experiments were carried out on slightly alkaline solutions containing excess metal with phenol red as the indicator. Although the approximations required to apply Eq. 3 are not as valid as in the stopped-flow experiments, good agreement was obtained between observed and calculated values of the relaxation times. The value of the rate constant for the formation of the 1:1 complex, k_1, was $6 \times 10^3\ M^{-1}$ sec^{-1}, which is in reasonable accord with the value obtained by stopped flow.

DISCUSSION

Equilibrium Studies—In the pH 6.4–7.1 range, the potentiometric titration data indicate essentially a 1:1 complex between nickelous ion and polymyxin. Although it was determined that the ligand binding species is represented by H_3L^{3+}, it is impossible from the potentiometric data to determine directly which γ-NH_2 groups participate in complex formation. Model studies strongly suggest that complex formation between nickel and the γ_1- and γ_2-NH_2 groups on the cyclic portion of polymyxin has the minimum ring strain and rotational barrier, although other possible structures can be postulated.

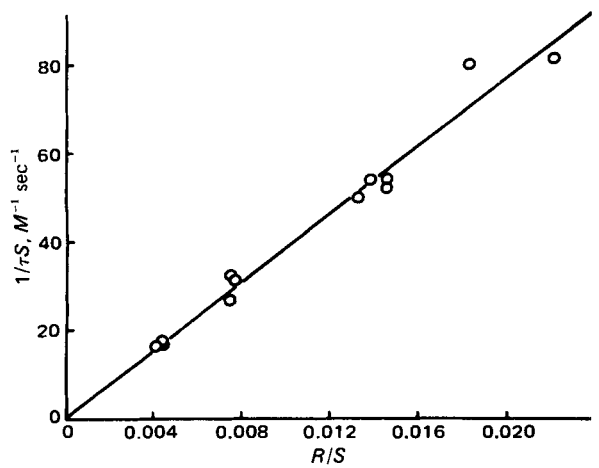

Figure 3—*Plot of* $1/\tau S$ versus R/S. *The straight line was obtained by least-squares data analysis, calculated from the concentrations and relaxation times given in Table II.*

Table IV—Rate Constant Ratios for Selected Ni(II)–Ligand Systems[a]

$Ligand_i/Ligand_j$	f	$f \times k_i/k_j$
$Malonate^{2-}$/H $malonate^-$	1/2	11
$Oxalate^{2-}$/H $oxalate^-$	1/2	8
$Cysteine^{2-}$/H $cysteine^-$	1	10
$H\ II^{3-}/H_2\ II^{2-}$	1	4
III^-/H III	1	13
IV/H IV^+	1/2	120
V/H V^+	1/2	980
$H\ VI^+/H_2\ VI^{2+}$	2/3	64
$H_2\ VII^{2+}/H_3\ VII^{3+}$	2/3	61
H_3L^{3+}/H_4L^{4+}	1/2	800[b]
H_3L^{3+}/H_4L^{4+}	1/2	3300[c]

[a] The statistical factor, f, takes into account the difference in the number of ligand donor atoms initially available for complex formation. Rate constants may be found in Ref. 31. Abbreviations used are: II, 4,5-dihydroxybenzene-1,3-disulfonic acid; III, pyridinedicarboxylic acid; IV, 2-aminomethylpyridine; V, ethylenediamine; VI, triethylenetetraamine; VII, tetraethylenepentaamine; and H_3L^{3+} and H_4L^{4+}, protonated polymyxin species. [b] Ratio corresponds to upper limit of k_1'. [c] Ratio corresponds to lower limit of k_1'.

The fact that no evidence was found for a binuclear complex in the nickel–polymyxin system below pH 7.1 is consistent with the results for the Cu(II)–polymyxin study (24), in which the data were successfully fit to a monocopper complex up to pH 8. Above pH 7.1, the equilibrium studies imply, and the stopped-flow studies confirm, that a binuclear species is present.

In the copper–polymyxin study, a $CuHL^{3+}$ species is postulated to exist, in which copper is attached to two γ-NH_2 groups and two peptide nitrogens. Such a complex species was not detected in the nickel–polymyxin system in near-neutral media. Under similar concentration conditions (excess polymyxin, basic pH), nickelous ion, unlike cupric ion, may prefer to form different order metallic complexes with polymyxin (27, 28). It is not likely that the data above pH 7.1 represent slow hydrolysis of peptide bonds as determined for certain oligopeptides (29), because the solutions were stable during the experiments (*vide supra*).

It is thus concluded that, below pH 7.1 and with the ligand in excess, polymyxin forms a 1:1 complex with nickelous ion in which the ligand binding species is H_3L^{3+}.

Kinetic Studies—The 1:1 complex NiH_3L^{5+} is formed by attack on nickel of polymyxin as either H_3L^{3+} or H_4L^{4+}. The positive charges of these ligand species are so high that the very fact of forming a complex is interesting, as is the magnitude of the formation rate constant. Most neutral ligands have rate constants in the range of $1.6–5.0 \times 10^3\ M^{-1}\ sec^{-1}$ for nickel complex formation (30, 31). One possible exception is ethylenediamine. For this ligand, $k_1 = 3.5 \times 10^5\ M^{-1}\ sec^{-1}$; a special complex formation mechanism has been proposed for such a comparatively large rate constant. To determine whether polymyxin has special kinetic properties, the "normal" mechanism of complex formation should first be considered.

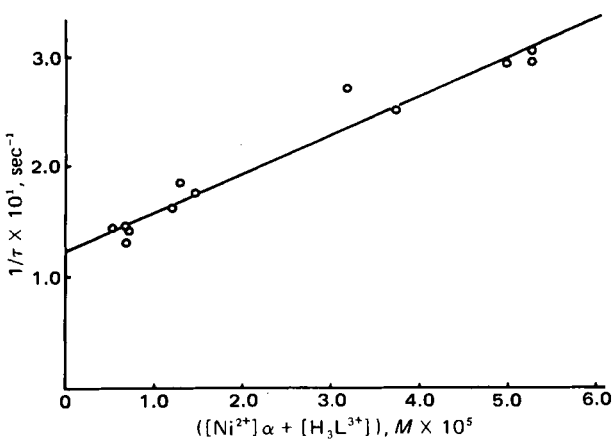

Figure 4—*Plot of* $1/\tau$ versus $[Ni^{2+}]\alpha + [H_3L^{3+}]$. *The straight line was obtained by least-squares data analysis.*

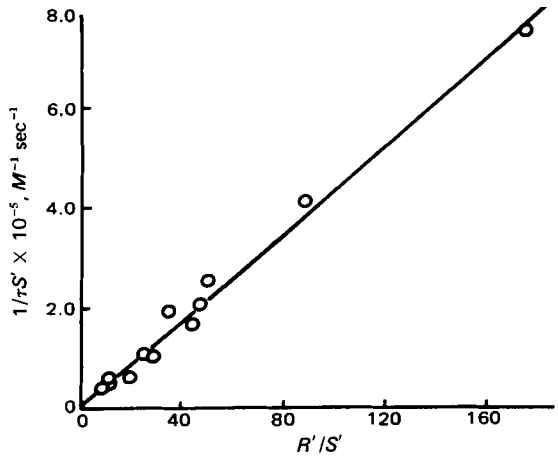

Figure 5—*Plot of* $1/\tau S'$ versus R'/S'. *The straight line was obtained by least-squares data analysis, calculated from the concentrations and relaxation times given in Table II.*

The normal (or eigen) substitution mechanism consists of rapid formation of an ion-pair between the aquometal ion and ligand followed by rate-determining water loss from the metal ion's inner coordination sphere (31). If there are no appreciable changes in the structures of the ion-pair or the solvent cage around the reactants, this mechanism predicts that the ratio of rate constants for two ligands having different charges by virtue of differing protonation should be approximately seven. Ratios for several ligands are listed in Table IV. Many systems have modest ratios in conformance with this mechanism. The rate constant ratios for methylaminopyridine, the polyamines, and polymyxin are considerably larger. This difference suggests anomalous complexation behavior.

For the polyamines, substitution was described in terms of an internal conjugate base mechanism (7), where hydrogen bonding between basic amine donor atoms and water molecules in the metal ion's inner coordination sphere results in enhanced formation rate constants. The clearest indicator of anomalous behavior is a larger than normal substitution rate constant, shown only by ethylenediamine (7). This result recently was reinterpreted (32). However, the new analysis leaves the result unchanged: the reaction between Ni^{2+} and ethylenediamine is anomalously rapid regardless of mechanistic assignment. Amino acids also often have rate constant ratios considerably different from those expected on electrostatic grounds. However, the apparent unreactivity of the zwitterionic form is responsible for this effect (33).

If the value of $k_1 = 3.8 \times 10^3\ M^{-1}\ sec^{-1}$ for complexation of H_3L^{3+} with Ni^{2+} corresponds to normal substitution, then the ligand acts as an electrically neutral species in which the ligand's metal binding site is uncharged. The ligand's formal charge of +3 must then be so remote from this active site that it does not influence the complex formation rate. Addition of one more proton to form H_4L^{4+} places two positive charges on each binding site of only one pair of potential binding groups. Ring closure requires proton loss, but this step would not be expected to be rate determining (7, 25). Hence, it is difficult to see why the reaction of H_4L^{4+} should be so slow compared with H_3L^{3+}.

To act as a neutral ligand, the effective charge differs from the formal charge through either remoteness from the metal binding site or screening. The polypeptide structure may form a barrier that partially shields the metal binding site from the positive charges. However, the high charge and rapid exchange of the protons with solvent, which ensures a random distribution of charge over the entire polymyxin molecule, would make that type of screening unlikely. Therefore, the polymyxin H_3L^{3+} species probably has a high complex formation rate constant because it follows the internal conjugate base mechanism.

In the polymyxin species H_4L^{4+}, an internal conjugate base effect is improbable since only one nitrogen donor is initially available for complexation with Ni(II). The limits set for the forward rate constant, $0.6 < k_1' < 2.4\ M^{-1}\ sec^{-1}$, suggest that H_4L^{4+} attacks nickelous ion as a relatively highly charged ligand. No evidence suggests that H_4L^{4+} attack on Ni(II) follows an abnormal substitution pathway. In fact, the experimentally determined range of k_1 suggests that this ligand species has an effective charge of approximately three based on the normal substitution mechanism.

At basic pH and excess Ni(II), a second complex between polymyxin B and nickelous ion is formed and is of the type Ni_2HL^{5+}. The attacking ligand species in this complexation reaction is $NiHL^{3+}$, in which two protons from the previously formed NiH_3L^{5+} complex have dissociated. If the range of k_2 ($3 \times 10^3 < k_2 < 1 \times 10^4\ M^{-1}\ sec^{-1}$) for complexation of this ligand species with Ni(II) corresponds to an effective ligand charge, q, of $0 < q < +1$, as would be predicted if the metal center in $NiHL^{3+}$ is sufficiently remote or screened from the complexing ligand site (*cf.*, Fig. 1), then the observed upper limit for k_2 is abnormally large, suggesting the presence of an internal conjugate base effect as discussed for the 1:1 complex.

The antibiotic activity of polymyxin B has been investigated (1–3). Metal-ion binding affects antibiotic activity in several ways; chelation may even enhance efficacy. An example is the use of metal-impregnated cellulose as a stratum for antibiotic attachment (34). Polymyxin attached to celluloses treated with various metal ions retained its antimicrobial activity. Since only a portion of the polymyxin binding sites would be attached to the cellulose-bound metal ion, this result cannot be used to infer whether the site of metal binding and the site or sites of antimicrobial activity are the same.

The present results show that polymyxin is rapidly reactive toward metal ions (relatively high k_1 and k_2) and that it can simultaneously bind two metal centers (K_2). Whether, as a class of compounds, the antibiotics exhibit all these characteristics has not been demonstrated; such reactivity patterns, however, if present, would help explain several features of antimicrobial activity such as the bactericidal ability of polymyxin while on insolubilized strata.

APPENDIX

Under the experimental conditions of slightly acid pH and excess ligand, where Scheme I prevails, the relaxation spectrum is calculated from the solution of a single linear differential rate equation. To obtain this differential equation in one variable, it is sufficient to apply mass balance on the ligand, metal, indicator, and proton together with the expanded protolytic equilibrium quotients for the ligand and indicator. Well-known procedures incorporating these 10 relations yield Eqs. 1 and 2. The previously undefined terms are:

$$R = [Ni^{2+}]\alpha + [H_3L^{3+}] + \frac{1}{K_1} \qquad \text{(Eq. A1)}$$

$$S = [Ni^{2+}]\alpha_2 + [H_4L^{4+}] + \frac{[NiH_3L^{5+}]\alpha_1 + [H^+]}{K_1K_{a2}} \qquad \text{(Eq. A2)}$$

$$\alpha = \left(\beta + \frac{[H_3L^{3+}]}{K_{a2}} + 4\frac{[H^+][H_3L^{3+}]}{K_aK_{a2}}\right)\Big/\sigma \qquad \text{(Eq. A3)}$$

$$\sigma = \left(\frac{[H_3L^{3+}]}{K_{a2}} + 2\frac{[H^+][H_3L^{3+}]}{K_{a1}K_{a2}}\right)\left(\frac{[H^+]}{K_{a2}} + 2\right) + \left(\beta - \frac{[H_3L^{3+}]}{K_{a2}}\right)\left(1 + \frac{[H^+]}{K_{a2}} + \frac{[H^+]^2}{K_{a1}K_{a2}}\right) \qquad \text{(Eq. A4)}$$

$$\beta = \frac{K_{In} + [H^+] + [In^-]}{K_{In} + [H^+]} \qquad \text{(Eq. A5)}$$

$$\alpha_1 = \left(\frac{[H^+]}{K_{a2}} + 2\frac{[H^+]^2}{K_{a1}K_{a2}}\right)\Big/\sigma \qquad \text{(Eq. A6)}$$

$$\alpha_2 = \left(\beta + 2\frac{[H^+][H_3L^{3+}]}{K_{a1}K_{a2}}\right)\Big/\rho \qquad \text{(Eq. A7)}$$

$$\rho = \left(\beta + 2\frac{[H_3L^{3+}]}{[H^+]}\right)\left(1 + \frac{[H^+]}{K_{a1}} + \frac{K_{a2}}{[H^+]}\right) + \left(\frac{[H^+][H_3L^{3+}]}{K_{a1}K_{a2}} - \frac{[H_3L^{3+}]}{[H^+]}\right)\left(1 + \frac{2K_{a2}}{[H^+]}\right) \qquad \text{(Eq. A8)}$$

Under conditions of slightly basic pH and excess metal ion, where reaction Scheme II prevails, the relaxation spectrum is calculated analogously from the solution of two linear and independent differential rate equations in two variables. Assuming separability of the two possible relaxation effects as described in the text, the previously undefined terms are:

$$R' = [Ni^{2+}]\alpha_3 + [H_3L^{3+}] + \alpha_4/K_1 \qquad \text{(Eq. A9)}$$

$$S' = [Ni^{2+}]\alpha_5 + [NiHL^{3+}] \qquad \text{(Eq. A10)}$$

$$\alpha_3 = \left(\beta + \gamma + \frac{[H_3L^{3+}]}{K_{a2}} + 4\frac{[H^+][H_3L^{3+}]}{K_{a1}K_{a2}}\right)\Big/\sigma' \qquad \text{(Eq. A11)}$$

$$\gamma = \frac{[NiH_3L^{5+}]K_{a3}}{[H^+]^2} \qquad \text{(Eq. A12)}$$

$$\sigma' = \left(\frac{[H_3L^{3+}]}{K_{a2}} + 2\frac{[H_3L^{3+}][H^+]}{K_{a1}K_{a2}}\right)\left(\frac{[H^+]}{K_{a2}} + 2\right) + \left(\beta + \gamma - \frac{[H_3L^{3+}]}{K_{a2}}\right)\left(1 + \frac{[H^+]}{K_{a2}} + \frac{[H^+]}{K_{a1}K_{a2}}\right) \quad \text{(Eq. A13)}$$

$$\alpha_4 = \gamma\left(\frac{[H^+]}{K_{a2}} + 2\frac{[H^+]^2}{K_{a1}K_{a2}}\right)\Big/\sigma' + 1 \quad \text{(Eq. A14)}$$

$$\alpha_5 = 2\frac{[NiH_3L^{5+}]}{[H^+]}\left(\frac{[H^+]}{K_{a2}} + 2\frac{[H^+]^2}{K_{a1}K_{a2}}\right)\Big/\sigma'\rho' - 1/\rho' \quad \text{(Eq. A15)}$$

$$\rho' = 1 + \frac{[H^+]}{K_{a4}} + \frac{[H^+]^2}{K_{a3}K_{a4}} \quad \text{(Eq. A16)}$$

In deriving Eqs. A9–A16, the reasonable assumptions were made that, under the experimental conditions, $K_{a3}/[H^+]$ and $K_{a3}K_{a4}/[H^+] \ll 1$.

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