

## CHAPTER 11

# Interactions Between Metal Ions and Capsular Polymers

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## 11.1 INTRODUCTION

Many different types of bacteria elaborate a capsule or slime layer as their outermost envelope component. Among its many functions, the capsule serves as a buffer between the environment and the cell. The capsule may prevent some substances such as antibiotics and biocides in the environment from contacting the cell surface, yet at the same time concentrate growth-limiting nutrients from the surrounding environment for subsequent transport into the cell.

Of the various dissolved substances that exist in aquatic habitats, metal ions are unique in that over a rather narrow concentration range, their status can change from an essential growth-promoting element to a toxin. Bacteria must therefore maintain tight control over the level of metal to which they are exposed in habitats with widely fluctuating dissolved metal concentrations.

One of the two main mechanisms of metal accumulation by bacteria involves nonspecific binding of the metal to the cell surface, capsule, and extracellular slime layer (1). Capsules possess features that suggest that they act as effective modulators of metal ion concentration at the cell surface, scavenging metals from solution when their concentrations are low and serving as impermeable barriers when metals exist at toxic levels in the surrounding environment.

Besides the influence exopolymer-metal interactions have on cell survival, the binding of metals by bacterial capsular material also has an impact on ecological and geochemical processes. Recent evidence suggests that the complexation of metal ions by capsular polymers may even contribute to corrosion reactions that have significant economic consequences. Before these broader implications of capsular-metal interactions can be clearly understood, more accurate information is needed on the thermodynamic and kinetic properties of the complexes that are formed.

The physical and chemical properties of bacterial capsule and slime polymers promote unique associations with metals. The tendency for many bacterial exopolymers to exist in a colloidal or gel-like state in the presence of metal ions results in the establishment of a multiple phase system. Consequently, classical approaches that describe equilibrium conditions for simple molecules or other polyelectrolytes in single-phase systems must be modified

in order to obtain useful thermodynamic stability constants that apply to the range of conditions over which bacterial-metal interactions occur.

In this chapter, interactions between metals and capsular polymers are described in terms of the chemical reactions that are involved as well as the consequences of such interactions to the bacterial cell and to the surrounding environment. Although the discussion focuses on the aquatic environment, many of the processes described apply to soil, plant, and animal systems as well. Finally, an approach is presented for the determination of "intrinsic" stability constants that takes into consideration the unique features of bacterial exopolymer-metal interactions.

## 11.2 THE BACTERIAL CAPSULE

The vast majority of bacteria observed in aquatic habitats, including the marine environment, possess an envelope structure characteristic of Gram-negative cells. The capsule, which is usually a polysaccharide with a repeating sequence of two to six sugar subunits (2), is anchored to the bacterial outer membrane. In some instances, the oligosaccharide side chain of lipopolysaccharide in the outer membrane is believed to contribute to the capsular structure (3). Some bacteria elaborate capsules composed of two chemically distinct polysaccharides (4, 5). A protein component is often recovered during isolation of capsule preparations (6-8). Whether the protein performs any function in this exocellular location is not well understood at this time. Other products of cell metabolism, excreted by the bacteria and trapped by the exopolymer matrix, may also contribute to the overall chemical properties of the capsule.

Exopolymers exhibit a variety of associations with the cell surface. Some bacteria elaborate capsular exopolymers that are firmly bound to the cell surface. This type of association often enables the cell to establish a stable orientation with respect to its environment. Cell-bound exopolymers may extend from 0.1 to 10  $\mu\text{m}$  from the cell surface into the surrounding environment, creating a buffer zone between the surface of the cell and the external environment (9, 10).

In some instances, the capsular polymers maintain a more transient association with the cell and take the form of what is commonly referred to as "slime." A portion or, in some cases, the bulk of the exopolymer sloughs into the surrounding medium. Different strains of the same bacterium may elaborate exopolymers that maintain different associations with the cell (11). There is some evidence from the medical field that overproduction of capsular polymer leads to sloughing. Production of slime has been shown to be controlled, to some extent, by the culture or environmental conditions (12). When both capsules and slimes are produced, the chemical compositions of their polysaccharides are usually similar (13, 14).

In aquatic and industrial fields, the term "slime" is often used to refer

to a slippery or visible growth of microorganisms on surfaces. In this context, slime includes the microorganisms, the exopolymers, and any adsorbed material from the aqueous phase.

The network of polymers that makes up the capsule or slime forms a colloid or gel phase, depending on the nature of the surrounding environment. The volume enclosed by the capsule, hereafter referred to as the gel phase, generally contains greater than 99% water by weight. Aggregation of the exopolymers may occur under certain conditions resulting in the formation of visible flocs. The tendency for bacterial exopolymers to form a colloid or gel phase around the cell, which often results in biofilm or floc formation, suggests that these biomolecules possess properties that are different from those of other naturally occurring polymers.

### 11.3 EFFECTS OF METALS ON EXOPOLYMER PRODUCTION

Metals have been shown to influence the production of capsule and slime exopolymers. Various ions are known to be required as cofactors in polysaccharide synthesis. Wilkinson and Stark (15) demonstrated in cultures of *Enterobacter aerogenes* that polysaccharide was stimulated by Mg, K, and Ca ions. Later, Corpe (6) reported that polysaccharide production in *Chromobacterium violaceum* was enhanced in the presence of Fe and Ca ions.

The amount of Cr in the culture medium affected the yield and form of exopolymer produced by a coryneform bacterium isolated from Cr-polluted marine sediments (16). Exopolysaccharide production increased as more Cr(III) was added to the medium.

The morphology of the capsule of *Azotobacter chroococcum* was also affected by metal availability in the growth medium (17). Under Fe- or Mo-sufficient conditions, the capsule was condensed, whereas under deficient conditions the capsule appeared more diffuse and extensive.

The composition of alginate produced by cells of *Azotobacter vinlandii* is affected by Ca availability. Removal of Ca from the growth medium leads to an increase in the relative concentration of mannuronic acid in the exopolymer (18, 19).

It is evident that no generalizations can be made with respect to the effects metals and other cations have on bacterial capsule production. This likely reflects basic physiological differences between bacterial types. It may also reflect differences in the types and concentrations of metals in the environment in which the bacteria exist.

### 11.4 PROTECTION FROM METAL TOXICITY

The role of capsular polymers in the protection of bacterial cells from the toxic effects of metals was demonstrated by Bitton and Friehofer (20). Sur-

vival of *Klebsiella aerogenes* in a 0.85% NaCl solution containing 10  $\mu\text{g}$  Cu or Cd  $\text{mL}^{-1}$  was significantly greater for cells that produced capsules compared to mutants that lost their capsule-producing ability. It was further shown that capsule isolated from the competent strain enhanced the survival of the noncapsulated cells when added to the metal-containing solution. The capsule bound 0.02 and 0.01  $\mu\text{g}$  Cu and Cd  $\text{mg}^{-1}$  polysaccharide.

In another study, nonmucoid variants of a coryneform bacterium isolated from Cr-contaminated marine sediments were less tolerant to 156  $\mu\text{g}$  Cr (III)  $\text{mL}^{-1}$  than a mucoid wild-type strain (21). The mucoid strain accumulated significantly more Cr (III) than the nonmucoid strain. Greater than 80% of the metal was in the exopolymer. At 91  $\mu\text{g}$  Cr (III)  $\text{mL}^{-1}$  external concentration, the slime polymers from coryneform and Enterobacteriaceae isolates contained 1000 and 1943  $\mu\text{g}$  Cr (III)  $\text{g}^{-1}$  dry weight polymer, respectively. It appears from these and other studies conducted to date that bacteria that produce exopolysaccharides tolerate higher concentrations of metal than strains that produce little or no capsule or slime. The tolerance likely occurs as a result of a reduction in free metal ion concentration at the cell surface, in turn a result of metal ion complexation by sites on the exopolymer molecules.

## 11.5 BIOACCUMULATION OF METALS VIA BACTERIAL EXOPOLYMERS

Bacteria that exist in sediments attached to particles or as a biofilm on submerged surfaces depend on capsule and slime exopolymers to maintain their sessile nature. Consequently, the exopolymers are optimally positioned to interact with metal ions that approach these surfaces. Complexation of metals by biofilms has been suggested to play an important role in controlling toxic metal concentrations in the water phase (22).

Montgomery and Price (23) indicated that the largest net uptake of metals from a source of sewage pollution occurred in the fouling community of a model turtle grass mangrove ecosystem. In other studies, bacterial epiphytes were shown to be a major factor in contributing to the total Cr, Cu, Fe, Pb, and Zn of aquatic plants (24). Binding of Mo by extracellular polysaccharides produced by the epiphytic bacteria resulted in a reduction in the amount of that entering the plant (25).

Because sessile bacteria contribute a significant portion of the total living biomass in sediments and, in addition, are high in nutritive value, they are a preferred food source for many benthic organisms. Harvey and Luoma (26) presented evidence that bacteria contribute to the nutrition of the estuarine clam *Macoma balthica*. The clam did not appear to utilize exopolymer isolated from a slime-producing strain of *Pseudomonas atlantica*, however. Exopolymers did increase the biological availability of particle-bound Ag, Cd, and Zn when the metals were present at low concentrations

(27). At higher metal concentrations, the clams avoided exopolymer-adsorbed particles.

Patrick and Loutit (28) demonstrated that metals, initially concentrated by bacteria and passed on through oligochaete worms, eventually accumulated in fish. More recently, it was shown that the Cr content of the snail *Amphibola*, which was fed Cr-complexed bacterial polysaccharide, contained threefold higher levels of the metal than snails fed polysaccharide containing very low levels of Cr (29). Examination of body parts demonstrated that 61% of the Cr was associated with internal tissues and only 39% was adsorbed to the shell of the snail. It was further shown that polysaccharide with bound Cr was degraded more slowly than Cr-free polysaccharide by the heterotrophic sediment bacteria. It is evident from these studies that detritivores are capable of accumulating exopolymer-bound metals during their ingestion of the bacteria. The capsular and slime exopolymers excreted by sessile bacteria therefore provide a means of metal entry into the aquatic food chain.

#### 11.6 PROPERTIES OF CAPSULAR POLYMERS RELEVANT TO METAL INTERACTIONS

That capsular polymers are most readily visualized by electron microscopy after staining with cationic dyes such as ruthenium red leads to the suggestion that they are acidic in nature (30). Chemical characterization of capsular and slime polysaccharide subunits from bacteria isolated from a variety of natural habitats indicates that they contain uronic acids and other substituted sugars that possess acidic functional groups. The density of acidic residues on the polymer chain may vary in different bacterial species. Some bacteria produce capsular polymers that contain only neutral sugar subunits whereas a few bacteria excrete capsules or slime composed entirely of uronic acids. The capsules of most bacteria examined to date contain from 5 to 25% uronic acids (31). Ketal-linked pyruvylated sugars also contribute acidic functional groups to capsular and slime polysaccharides (32-34). Unlike the ester-linked residues, ketals confer a negative charge on the polysaccharide similar to that of a uronic acid. Sialic acids, which refer to a series of substituted neuraminic acids, are found in the capsules of several bacterial genera (35, 36). These also contain free carboxyl groups which confer a net negative charge to the polymer. Although a variety of other functional groups have been identified in bacterial capsules and slimes, apart from hydroxyl residues, there is little information on their interactions with metal ions.

#### 11.7 MECHANISMS OF METAL ION BINDING TO BACTERIAL EXOPOLYSACCHARIDES

Although different classes of biological molecules may be associated with bacterial exopolymers, the metal binding reactions considered here are re-

stricted to those involving the polysaccharide component. Metals tend to form bonds with electron-donating groups in order to fill their outer electron shell. The most effective electron donor group associated with acidic capsule and slime polysaccharides is the carboxyl residue. Lone-pair electrons on carboxyl groups interact with the charge-compensating metal ions. Weak electron donors are also present on acidic and neutral polysaccharides in the form of oxygen atoms associated with the ether bond and hydroxyl residues on the sugar subunits (37).

On the basis of Rendelman's (38) interpretation of ion interactions with polysaccharides, metal binding by uncharged polysaccharides occurs as a result of coordination between the metal cation and oxyanion and hydroxyl groups on the donor molecule. The affinity exhibited by uncharged polysaccharides generally decreases with increasing ionic radius of metals.

The general metal-molecule interaction is an acid-base reaction:



where the acid is represented by  $H^{+}$ , the metal ion by  $M^{n+}$ , and the base by  $L^{-1}$ . The binding of Cu ion to the capsule of a freshwater sediment bacterium (FRI) was shown to result in the displacement of protons that caused a shift in the  $pK_a$  of the capsule from 4.90 to 4.05 (39). An observed decrease in the conditional stability constant for the Cu-capsule complex with decreased pH also suggests that there was competition between Cu ions and protons at the site of metal binding. Although no uronic acids were detected in the capsular material, the  $pK_a$  value suggests the presence of carboxyl groups, possibly in the form of ketal-linked pyruvate residues (40). The release of protons by other acidic polysaccharides during exposure to increased concentrations of copper has also been reported (41).

Steiner (42) described four classes of ion binding by extracellular polymers based on the shape of the adsorption isotherms. The two most important classes were those that involved salt bridges with carboxyl groups on acidic polymers and those that involved weak electrostatic bonds with hydroxyl groups on neutral polymers. The "S-type" isotherm produced by complexes between metal ions and the capsule of *Zoogloea ramigera* 115 was proposed to involve primarily hydroxyl groups of the glucose subunits even though the polysaccharide contains free carboxyl groups on the ketal-linked pyruvate residues (43). This type of metal binding is believed to be the most important mechanism of metal removal in activated sludge.

Metal interactions with charged polysaccharides are controlled to some extent by linear charge density. In some cases, the affinity of an acidic polysaccharide for a cation increases with increasing charge density (44). Metal-polymer interactions are often stronger when the polymer exists as a gel than when dissolved in solution. This is likely due to an increase in charge density in the former. Such evidence supports the concept of "salt bridging," in which a polyvalent metal ion bonds to two anionic groups on

separate polymer chains. The recent studies of Manzini et al. (45), which suggest that more than two uronate groups are complexed with each Cu ion, supports the contention that metal ions participate in intermolecular bonding. "Steric fit" thus becomes important and supports the evidence that the ionic radius of a metal frequently determines whether complexation will occur.

In general, carboxylated polysaccharides exhibit preferential binding to cations with large ionic radii. With few exceptions, carboxylated polyanions exhibit a higher selectivity for transition metals than the alkaline earth metals. In some instances, however, the affinity of Mg fluctuates relative to other cations in different anionic polysaccharides. In addition, selectivity coefficients may be different, depending on whether the polymer exists in solution or in a gel state (46). Affinity of alginic acid for the transition metals decreases in the order  $\text{Cu} > \text{Ni}$ ,  $\text{Co} > \text{Zn} > \text{Mn}$ , which roughly follows the Irving-Williams order of decreasing complex stability.

Polyuronides (alginates and pectic substances) have been recognized for their metal-binding ability for over 100 years. Consequently, much of what is known about complexes formed between acidic polysaccharides and metal ions is based on studies with these substances. As early as 1825, Braconnot suggested that pectins might be used to treat heavy metal poisoning in humans owing to the insoluble complex that is formed (47).

Cozzi et al. (48) proposed that the stability and selectivity of complexes between alkaline earth ions and alginates is influenced by hydroxyl groups. It was shown that two hydroxyl groups on the uronate subunit are more stabilizing than one. The differences in affinity among alkaline earth cations such as Ca for polymannuronic, polygalacturonic, and polyguluronic acid regions of alginic acid were suggested to be due to variations in the positioning of the hydroxyl groups on the monomer subunits (49). The spacing of coordinating oxygen atoms in the polyanion therefore has a major influence on which cations will bind as well as their stability. In general, the more coordination bonds that are formed between the metal and the "ligand" or donor molecule, the greater the stability of the complex. The formation of stable complexes of metal ions with organic ligands usually involved both electrovalent (nonionic) and coordinate covalent bonding.

An "egg-box" model has been proposed for the structure of alginic acid in which a series of cavities that offer four oxygens for coordination with Ca ions exists in regions containing repeating sequences of guluronic acid (47, 50). Cooperative binding of the guluronic acid-rich regions with functional groups on other nearby polymer chains is necessary for the proposed configuration. It was further demonstrated that with polygalacturonic acid, fewer cavities are possible, most of which offered only three oxygen atoms for coordination, and with polymannuronic acid, only shallow cavities are formed. Blocks of polyguluronic acid have greater selectivity toward and stability with Ca and Sr than do repeating sequences of polygalacturonic and polymannuronic acids. Bacterial alginates exhibit a wide variation in

the relative contribution of guluronic acid and mannuronic acid residues in the polymer molecule. It may be concluded from the preceding discussion that the mechanisms of cation binding by alginic acid and possibly other bacterial exopolysaccharides are different from those described for ion binding to classical polyelectrolytes.

Copper ions in solution displace Ca ions bound to alginic acid. A high selectivity for Cu over Ca is a characteristic feature of carboxylated polyanions, in general. The binding mechanism that is responsible for the higher affinity for Cu than Ca appears to be independent of free hydroxyl groups in the polymer and of the steric arrangement of the carboxyl groups (49).

Metal ions in solution are always fully coordinated or solvated by water molecules. When a metal ion becomes complexed by an organic ligand there is a displacement and reordering of water molecules: the number of water molecules displaced depends on the dimension and charge of the metal ion and on the size of the coordinated electron-donating group of the ligand. It was proposed that Cu ions have a larger ordering effect on the water molecules than Ca ions, and that the displacement of Ca by Cu on the polymer should be favored by the relatively large positive entropic change (49).

Order within the liquid subphase may also depend on the chemical properties of the capsule. In the case of M41 capsular polysaccharide of *Escherichia coli*, only the polyanions are ordered and the cations and water molecules behave like a liquid filling the spaces between polymer molecules (32). This is in contrast to alginic acid and hyaluronic acid where cations and most water molecules have well-defined positions and may determine gross configuration of the polymer chains fibers.

More recently, Manzini et al. (45) emphasized the importance of carboxyl groups in cupric ion binding. They suggested that an intimate interaction exists between copper ions and carboxyl groups on acidic polysaccharides. Cupric ion binding to a carboxyl group is electrovalent. In the case of polyuronates, the mode and extent of the interaction are believed to depend on several factors: the nature of the component sugars and their relative distribution in the chain, the magnitude of the overall electrostatic field, and the ratios of copper to polymer and of copper to simple supporting electrolyte.

Molybdenum binding by extracellular slimes of rhizosphere bacteria was investigated by Tan and Loutit (51). They determined that Mo was bound mainly by a passive process to the uronic acid-containing portion of the slime layer surrounding a *Pseudomonas* sp. Greater than 90% of the Mo associated with the cells was bound to the slime. The slime polysaccharide from *P. aeruginosa* was reported to bind Mo through the glucuronic acid subunit (52). Copper is also bound to this subunit as well. Glucuronic acid was found to contribute 32% of the total slime weight. Complexation occurred through the two oxygen atoms of the carboxyl group and the oxygens on the hydroxyl groups of C-3 and C-4 of the uronic acid molecule. As much as 97% of the total Mo concentrated by the cells was bound to the slime.

In summary, oxygen atoms of carboxyl and hydroxyl groups on the uronic acid subunits participate in the binding of metal ions by acidic capsular and slime polysaccharides. Oxygen atoms of hydroxyl groups on neighboring neutral sugars also contribute to the coordinate binding of metal ions that promotes the formation of stable complexes.

### 11.8 SELECTIVITY AND RELATIVE AFFINITY OF BACTERIAL EXOPOLYMERS FOR METAL IONS

Early investigations on the interactions of metal ions with bacterial exopolymers arose from studies on the effects of metal wastes on sewage treatment microorganisms. The floc-forming strain 115 of *Z. ramigera* was shown to remove significantly more dissolved Co, Cu, Fe, and Ni from solution than a strain that did not produce a gelatinous matrix around the cells (53). Young cultures of the floc-forming strain exhibited a lower affinity for Zn than older cultures that contained more exopolymer. Zinc binding by isolated exopolymer material was later confirmed.

Further studies with the floc-forming *Zoogloea* sp. suggested that uptake of metal ions was nonspecific, although no selectivity coefficients were provided (54). About 25% of the floc weight could be attributed to bound ions. The capacity of the floc to bind iron ions was enhanced by the presence of anions such as phosphate. Purified preparations of the exopolymer bound 3.9 mg (70  $\mu\text{mol}$ ) Fe, 3.5 mg (140  $\mu\text{mol}$ ) Mg, and 7.6 mg (190  $\mu\text{mol}$ ) Ca per 50 mg dry weight polymer. Cobalt, Al, Mn, and Ni were bound to a lesser extent. The relative affinity of the cell-floc material for metal ions was determined to be  $\text{Fe} > \text{Cu} > \text{Co} > \text{Ni}$  (55). Because the exopolymers bind large quantities of water, Dugan (54) proposed that metal binding occurred through an exchange with polymer-bound water molecules and that the removal of water resulted in the observed flocculation that occurred in the presence of metals.

The combined polysaccharide species purified from the crude floc produced by cells of *Z. ramigera* 115 was reported to bind 0.25  $\mu\text{mol Fe}^{3+} \text{ mg}^{-1}$  polysaccharide (56). In view of the Fe binding capacity based on total polymer weight (1.4  $\mu\text{mol mg}^{-1}$ ) reported by Dugan (54) for this same bacterial strain, it appears that constituents other than polysaccharide are responsible for the bulk of the metal complexing ability of this capsular material.

More recent studies have demonstrated that flocs of *Z. ramigera* 115 absorbed a maximum of 3 mmol Cu  $\text{g}^{-1}$  dry weight between pH 6 and 7 and a maximum of 1.8 mmol Cd  $\text{g}^{-1}$  dry weight at pH 8 (57). In contrast to most polyanions, the Cu and Cd binding capacity of the floc material was not affected by changes in ionic strength. Repeated flocculation of a solution containing either copper or uranium with flocs of the exopolymer-producing bacterium resulted in a reduction in the total dissolved concentration of each metal to less than 0.1 and 0.0005 g  $\text{L}^{-1}$ , respectively. Essentially all of the

Cu and Cd complexed to the flocs was released by acidification at pH 4 and pH 3, respectively. These results suggest that the metals were complexed to carboxyl groups on the floc exopolymer via ionic bonding. No attempt was made to identify the site of metal interaction, however.

During toxicity studies of metals on marine bacteria, Corpe (7) found that extracellular polymers elaborated by cells of *P. atlantica* exhibited selectivity for Mg and Cu over Ca, Zn, Pb, Co, and Ni. No binding constants were reported for any of the polymer-metal complexes that formed. It was reported, however, that cells cultured in the presence of  $5 \times 10^{-5} M$  copper contained no more of the metal than exopolymers from cells cultured under conditions in which no copper was added (besides that present in the nutrients added for growth). On the basis of these results, Corpe concluded that the concentration of metal by bacteria is not influenced by the amount of free metal in the surrounding medium. However, when the added copper concentration was increased to  $4 \times 10^{-4} M$ , the cell wall and extracellular polymer fractions contained significantly higher concentrations of the metal than wall and exopolymer preparations obtained from cells receiving no copper supplement. Others have demonstrated that the amount of metals bound by extracellular polymers increases with increasing free metal ion concentration (58). Thus there is some evidence to suggest that the amount of metal bound by these envelope components is controlled by the surrounding metal concentration.

In another study, metal uptake was compared between a capsule-producing and noncapsulated strain of *K. aerogenes* (59). Of the metals tested (Cu, Cd, Ni, Mn, Co), only Ni was accumulated to the same extent by both strains. All the other metals were concentrated to a greater extent by the capsule-producing strain. Six to 10 times more Cu and Cd were concentrated by the capsular polymers than was taken up by the cells themselves. In continuous cultures, a shift from cellular uptake to extracellular binding of Cu, Cd, and Mn was observed as the dilution rate was decreased. Metal removal from solution by the encapsulated strain was of the order  $Cu > Cd > Co > Mn > Ni$ . This affinity series is similar to that of *Z. ramigera* 115 flocs and to laboratory-scale activated sludge biomass.

The extraction of extracellular polymers from *K. aerogenes* reduced the capacity of the cells to adsorb Cd, Mn, Co, and Ni (58). Freundlich isotherms indicated that the relative affinities of metals for binding sites on the extracellular polymer followed the series  $Cd > Co > Ni > Mn$ . At  $0.1 \text{ mg L}^{-1}$  free Co, Cd, or Ni, the polymer was close to saturation at approximately 1 mg metal bound per gram of polymer.

The majority of studies conducted to date, therefore, demonstrate that bacterial exopolymers exhibit an affinity and selectivity for metal ions. Unfortunately, the type of quantitative information needed for comparison of the affinities of different microbial exopolymers for various metals is not often provided. Consequently, parameters such as binding strength, com-

petition, and binding site density are largely unknown for complexes formed between bacterial exopolymers and metals.

### 11.9 CONDITIONAL STABILITY CONSTANTS FOR CAPSULE-METAL COMPLEXES

The stabilities of metal complexes in solution are measured by equilibrium constants for their formation. Although stability constants have been determined for over 1500 organic compounds for a variety of metals, only a few have been reported to date for bacterial exopolysaccharide-metal complexes. Rudd et al. (60) obtained  $\log K_i$  values of 7.69, 5.16, 5.48, and 5.49 for Cu, Cd, Co, and Ni complexes, respectively, with *K. aerogenes* Type 64 capsule at the same polymer concentration at pH 6.3 over a metal concentration range of 0.2  $\mu\text{M}$  to 0.1 mM. The number of binding sites per molecule ( $1.7 \times 10^6$  MW) ranged from 51 for Cd to 7 for Cu. The complexation capacity ( $\text{mol g}^{-1}$  dry wt) was  $4 \times 10^{-6}$  and  $3 \times 10^{-5}$  for Cu and Cd, respectively. Only one type of binding site was available to each metal. They found an indirect correlation between the stability constant and the number of binding sites on the polymer molecule. When metals were added in combination to *K. aerogenes* capsule, only Co was reduced in overall binding. This suggests some specificity of binding sites. Other evidence suggested that only two distinct types of binding sites were available on the polymer, however. The capsule was reported to contain glucose, mannose, rhamnose, and glucuronic acid along with acetate and pyruvate residues.

The conditional stability constant for a complex formed between Cu ions and a capsular polysaccharide component from a freshwater sediment bacterium was found to be comparable to that of a complex formed between the metal and a humic acid preparation (39). Protein as well as polysaccharide fractions of the capsular material participated in Cu binding. A highly purified polysaccharide fraction exhibited a maximum binding capacity of 253 nmol Cu  $\text{mg}^{-1}$  carbohydrate. Evaluation of infrared spectra of the complex formed between Cu and the polysaccharide capsular component suggested that the interaction occurred at a carboxyl residue. Experiments conducted at Cu concentrations, pH, and temperatures recorded in sediments from which the bacterium was isolated indicated that the exopolymers were capable of binding Cu under natural conditions. In view of these results, it is likely that bacterial exopolymers compete with other metal-complexing agents for metal ions in natural aquatic systems.

### 11.10 INFLUENCE OF METAL IONS ON THE PHYSICAL STATE OF EXOPOLYMERS

Interactions with certain metal ions cause many capsular and slime exopolymers to flocculate. Bridging between bacterial exopolymers and metals

either as free cations or in the form of organic complexes or inorganic hydroxides promotes flocculation and rapid settlement of the bacteria and metal from solution (61). At very high metal concentrations (0.2 M) the extracellular polymer from one strain of *P. atlantica* formed water-insoluble precipitates with Fe, Cu, and Pb (7). Binding of metals causes some slime polymers to condense into gels. Brown and Lester (43) stated that in the presence of Cu(II) ions, all carboxylated polymers form gels. Cations such as Ca may also cause gel formation as in the case of alginic acid.

Coprecipitation was reported when metals were mixed with the purified polysaccharide fraction from the *Zoogloea* floc. Maximum polymer precipitation occurred in the presence of a 0.5 mM Fe(III) solution (56). Ferrous iron was about 50% as effective in this regard as Fe(III). Because binding of metal ions to the exopolymer promoted flocculation, it was proposed that the reaction could provide a means of separating the metal ions in a highly concentrated form from acid mine water (54).

Rudd et al. (60) compared the metal-binding capacity of colloidal and soluble forms of capsular polymer recovered from *K. aerogenes*. By a subtraction method, they determined that approximately five times more Ni complexed with the soluble form than with the colloidal form. More Cu, Cd, Mn, and Co were also complexed with the former. Accurate estimation of the metal-binding capacity of polymers that exist in a colloid or gel phase, however, requires a more rigorous approach than any used to date.

### 11.11 PROBLEMS DETERMINING EFFECTIVE CONCENTRATION OF POLYMER-BOUND METALS

Of all the problems associated with determining the interactions between microorganisms and metals, the inability to determine actual effective metal concentration at the site of complex formation is the most significant (62). Determination of thermodynamic properties such as stability constants and binding site density depend on accurate estimations of the concentrations or activities of interacting chemical species at the site of complex formation. In the case of capsular polymers, a variety of factors complicates efforts to obtain accurate estimates of the amount of metal that is bound. It has been demonstrated, for example, that the concentration of counterions in the vicinity of negatively charged reaction sites of polyanions is higher than their concentration in the bulk solution (63). When this electrostatic field component is ignored, metal-binding constants of polyanions such as microbial capsules and humic substances tend to vary with pH and ionic strength of the surrounding medium (39, 64).

A feature exhibited by many bacterial capsules that hinders estimation of metal concentrations at binding sites is their tendency to exist as highly hydrated gels. In this respect, capsular polymers are similar to ion exchange resins and when hydrated form two separate phases: a gel phase composed

of a polymer subphase containing metal-binding sites that are surrounded by and in contact with a liquid subphase and a bulk liquid phase. Marinsky (65) showed that by assuming the existence of a hypothetical semipermeable membrane between the gel phase and the bulk liquid phase, a difference in water activity leads to an osmotic pressure that contributes to the free energy of ionization even when there is no acting electrostatic field. Just as there are differences in water activity, so there are differences in metal ion and other counterion activities between the two phases. The formation of colloids, gels, and insoluble precipitates with increased metal binding by capsular polymers suggests that volume changes must also be taken into account when estimating metal concentrations at the site of interaction.

Thus in addition to the molecular structure and density of the ligand's charged groups and the size, structure, and hydration state of the metal, which must be considered when evaluating the thermodynamic properties of most complexes formed between organic compounds and metals, it is necessary to take into account electrostatic field and osmotic pressure effects as well as volume changes when determining metal-binding properties of bacterial capsule and slime polymers. In view of these circumstances, activity values based on valid activity coefficients should be employed instead of concentration measurements when describing thermodynamic constants of complexes between metals and bacterial exopolymers.

#### 11.12 DETERMINATION OF INTRINSIC STABILITY CONSTANTS FOR EXOPOLYMER-METAL COMPLEXES

Characterization and comparison of complexes formed between metals and various ligands that exist in aquatic systems are most effectively achieved through thermodynamic expressions such as stability constants and binding site density determinations. These expressions are commonly obtained from Scatchard, Langmuir, and Freundlich plots based on measurements of the quantity of free metal in solution, the ligand concentration, and the quantity of metal bound to the ligand under equilibrium conditions (41, 58). The constants derived from these various plots are typically "conditional" constants in that they reflect the characteristics of the metal-ligand interaction for those conditions under which the empirical data were obtained.

To be of value in assessing interactions between metal ions in the environment and surface components of microbial cells under the variety of conditions to which natural aquatic populations and even laboratory cultures are exposed, "intrinsic" binding constants should be obtained that are independent of environmental variables. Intrinsic binding constants may be obtained if all of the factors that influence free energy change at the site of ligand-metal interaction are taken into account.

From the information presented in the preceding sections, it is evident that the number of independent variables that affect metal-polymer inter-

actions is very high. Nevertheless, it is possible to determine the contributions these factors make to the free energy of complex formation. The remainder of this chapter describes an experimental and mathematical approach that can be used to obtain intrinsic binding constants and binding site density for bacterial exopolymer-metal complexes.

### 11.12.1 Acid Dissociation Constant

The first step in determining conditional or intrinsic metal binding constants for anionic capsular polymers involves the estimation of the number of moles of acidic groups (potential metal-binding sites) on the polymer molecule ( $A_t$ ). This is achieved by potentiometric titration of the capsular polymer with base. Acidic polymers should be in their fully protonated form at the beginning of the titration because the amount of base consumed is used to estimate the number of acidic groups on the polymer molecule. An independent method of estimating the concentration of acidic functional groups on the polymer should be carried out and compared with the titration data. For example, a uronic acid assay (66) could be used if this class of subunits was known to be the sole contributor of acidic groups on the polymer. If the source of acidic groups is not known, a tritium exchange approach could be applied (67). Although it is desirable to have the polymer completely hydrated at the beginning of the titration, many capsular polymers are sparingly soluble in water, particularly when all the acidic groups are protonated. As long as the base is accessible to all of the reaction sites on the polymer by the end of the titration, the total number of groups can be obtained.

Titration is performed at different ionic strengths ( $I$ ) using a salt in which the anion does not form complexes with the metal cation being studied. Sodium nitrate and sodium perchlorate salts are often used to control ionic strength in solutions in which complexes between copper and organic molecules are to be characterized. At low  $I$ , equilibration times are sometimes very long, particularly near the end point of the reaction. Electrode sensitivity also tends to decrease at low  $I$ . Equilibration times may be prolonged even more if the polymer forms a viscous gel at the relatively high concentrations desired for titration. Nevertheless, it is important to perform the titration under these conditions if accurate  $pK_a$  values are to be obtained.

The number of moles of total acidic groups neutralized at any point in the titration,  $A^-$ , is calculated from the expression:

$$A^- = \frac{10^{-pH} V_s}{\gamma_{H^+}} + m_{b,ac} \quad (1)$$

where  $V_s$  is the volume of the bulk liquid phase,  $\gamma_{H^+}$  is the activity coefficient of hydrogen ions in the bulk liquid phase, and  $m_{b,ac}$  is the number of moles of base added at any point in the titration of the polymer. The end point of

the reaction is defined as the point at which a plot of pH versus  $m_{b,ac}$  shows a maximum slope. The degree of polymer ionization,  $\alpha$ , can be estimated at any point in the titration by the expression

$$\alpha = \frac{A^-}{A_t}$$

or

$$\alpha = \frac{A^-}{m_{b,ep}}$$

(2)

where  $A_t$  is number of moles of titratable groups on the polymer and is equal to the number of moles of base added to reach the end point in the titration,  $m_{b,ep}$ .

Initially, an apparent dissociation constant,  $K_{HA}^{app}$ , of the polymer is calculated on the basis of the measured activities of species in the bulk liquid phase according to the expression

$$\begin{aligned} K_{HA}^{app} &= \frac{(H^+)(A^-)}{(HA)} \\ &= \frac{(H^+)\alpha}{1 - \alpha} \end{aligned} \quad (3)$$

and

$$pK_{HA}^{app} = -\log K_{HA}^{app}$$

or

$$= \text{pH} - \log \frac{\alpha}{1 - \alpha} \quad (4)$$

The parentheses above and hereafter refer to activity, which is the product of the molar concentration and activity coefficient. Activity reflects "effective concentration" of all interacting species that are used to determine thermodynamic equilibrium constants. After the titration is performed at several different ionic strengths, a plot of  $-\log K_{HA}^{app}$  versus  $\alpha$  should yield a different slope for each  $I$  (Fig. 11.1).  $I$  should be set high enough in one of the titrations to yield a slope of zero and the resulting  $pK$  value used later as a first approximation of the intrinsic dissociation constant,  $K_{HA}^{int}$ .

From Figure 11.1, it is evident that the difference between  $K_{HA}^{int}$  and  $K_{HA}^{app}$  varies with the degree of neutralization and with ionic strength. In thermodynamic terms, this deviation (conventionally denoted as  $\Delta pK = pK_{HA}^{app} - pK_{HA}^{int}$ ) reflects the net change in free energy of ionization

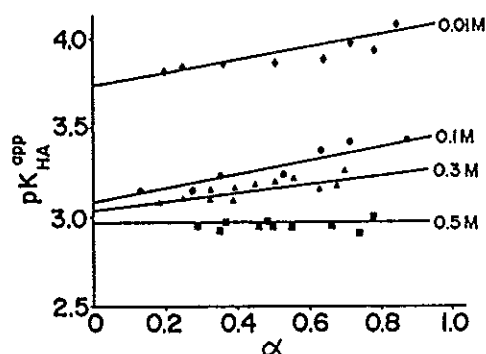


Figure 11.1 Plots of apparent  $pK_{HA}$  versus degree of ionization of alginic acid in aqueous solutions of different concentrations of  $NaNO_3$  as obtained from base titration experiments at  $25^\circ C$ .

contributed by the electrostatic field around the polymer molecule and that contributed by the difference in water activity across the hypothetical membrane which separates the gel phase from the bulk liquid phase (65).

The variation in  $\Delta pK$  at different  $\alpha$  and  $I$  also reflects a change in gel phase volume or, more specifically, a change in the polymer subphase volume, where the ionization reactions occur. The polymer subphase volume may be accurately obtained using an iterative procedure that is applied to satisfy three simultaneous algebraic equations that calculate the number of moles of counterion bound electrostatically in the polymer subphase, ionic strength in the polymer subphase, and partitioning of the counterion activity between the polymer subphase and bulk liquid phase. Calculation of the intrinsic acid dissociation constant of capsular polymers depends on an accurate estimation of the polymer subphase volume at different  $\alpha$ .

One of the algebraic equations used to obtain the polymer subphase volume is based on the behavior of  $Na^+$  formed during the titration of a salt-free solution of the capsular polymer with  $NaOH$ . Under these conditions, the activity of the  $Na^+$  (counterion) in the bulk liquid phase (measured with a sodium electrode) can be used to calculate the sodium activity coefficient,  $\gamma_{Na^+}$ , from the expression

$$\gamma_{Na^+} = \frac{(Na^+)_{bp}}{C_{Na^+}}$$

where  $(Na^+)_{bp}$  is the measured sodium ion activity in the bulk phase and  $C_{Na^+}$  is the concentration of sodium ions present in the system (i.e., total number of moles of  $NaOH$  added/volume of the solution). In this case, the number of moles of  $NaOH$  added to the solution is approximately equal to  $A^-$ . In addition,  $\phi_{P,Na}$ , which is the practical osmotic coefficient of the free

sodium ion in a salt-free solution containing the sodium form of the polymer, can be substituted for  $\gamma_{\text{Na}^+}$  because the osmotic pressure is governed by the free, unbound  $\text{Na}^+$  in a salt-free polyelectrolyte solution (68).  $\phi_{\text{P,Na}}$  can therefore be determined from the expression

$$\phi_{\text{P,Na}} = \frac{[(\text{Na}^+)_{\text{bp}}] [V_t]}{A_{\text{ionized}}^-}$$

where  $V_t$  is the total volume of the polymer suspension and the brackets here and in subsequent equations denote a grouping of mathematical terms.

The number of moles of sodium ion that are electrostatically bound to the polymer during titration with NaOH is obtained by subtracting the Na in the bulk phase from the total sodium in the system as described below

$$\overrightarrow{Na}^+ = [1 - \phi_{\text{P,Na}}] A^- \quad (5)$$

where the arrow bar represents the gel polymer subphase. At each step of the titration, the change in  $\phi_{\text{P,Na}}$  can be determined for each value of  $\alpha$ .

Just as Equation 5 provides a means to calculate the sodium activity in the polymer subphase from sodium ion measurements in the bulk liquid phase of an electrolyte-free system, it may also be used when  $\text{NaNO}_3$  is added to the polymer suspension to adjust the ionic strength. According to the Boltzmann distribution theory, the electric potential reduces to a very low value in the gel liquid subphase and essentially to zero in the bulk liquid phase regardless of whether an electrolyte is present (68).

With  $A^-$  and  $(\overrightarrow{Na}^+)$  calculated from Equations 1 and 5 and  $V_p$  assigned some initial value that is less than the total solution volume, the first estimation of the polymer subphase ionic strength is obtained by the expression

$$\overrightarrow{I} = \frac{[\overrightarrow{Na}^+ + A^-]}{2V_p} \quad (6)$$

where  $V_p$  is the volume of the polymer subphase. If the estimation of  $V_p$  is correct, the activity of sodium in the polymer subphase,  $(\text{Na}^+)$ , will equal the sodium activity in the bulk liquid phase multiplied by the deviation factor,  $10^{\Delta pK}$ , described earlier according to the expression

$$(\overrightarrow{Na}^+) = (\text{Na}^+) 10^{\Delta pK} \quad (7)$$

where

$$(\overrightarrow{Na}^+) = \frac{\overrightarrow{Na}^+ [\overrightarrow{\gamma}_{\text{Na}^+}] \overrightarrow{I}}{V_p} \quad (8)$$

and

$$(\text{Na}^+) = C_{\text{Na}^+} [\gamma_{\text{Na}^+}]_I$$

and

$$\Delta pK = pK_{\text{HA}}^{\text{app}} - pK_{\text{HA}}^{\text{int}}$$

The activity coefficient of sodium in the polymer subphase,  $\gamma_{\text{Na}^+}$ , is obtained from tabulated data (69) or from the expression

$$[\gamma_{\text{Na}^+}]_I = \frac{[\gamma_{\pm \text{NaCl}}]^2 \bar{I}}{[\gamma_{\pm \text{KCl}}] \bar{I}} \quad (9)$$

where  $\gamma_{\pm \text{NaCl}}$  and  $\gamma_{\pm \text{KCl}}$  are the mean activity coefficients of NaCl and KCl (70) at the ionic strength,  $\bar{I}$ , obtained from Equation 6. If Equation 7 cannot be satisfied, a new estimate of  $V_p$  is obtained from the expression

$$V_p = \frac{[\vec{N}a^+] [\vec{\gamma}_{\text{Na}^+}]_I}{[C_{\text{Na}^+} \gamma_{\text{Na}^+}]_I 10^{\Delta pK}} \quad (10)$$

which is obtained by substitution and rearrangement of Equation 8. The new estimate of  $V_p$  is then substituted in Equation 6. Equations 6, 9, and 10 are iterated to generate second, third, . . . ,  $n$ th improved estimations of  $V_p$  until two successive trials provide values that are within acceptable limits.

Once an accurate polymer subphase volume is obtained at each  $\alpha$  and  $I$ , the activity of sodium ion in the gel phase is calculated from Equation 8. Then from the Donnan equilibrium relationship (71, 72),

$$p\vec{H} = pH + p\vec{N}a - pNa$$

the hydrogen activity of the bulk liquid phase in Equation 4 is replaced by the actual hydrogen activity of the polymer subphase. The  $pK_{\text{HA}}$  thus obtained should represent the  $pK_{\text{HA}}^{\text{int}}$  at every point in the titration. In other words,

$$pK_{\text{HA}}^{\text{int}} = pK_{\text{HA}}^{\text{app}} + p\vec{N}a - pNa$$

The scheme presented above allows the ratio of sodium ion activity in the gel polymer subphase to sodium ion activity in the gel liquid subphase or bulk liquid phase,  $(\vec{N}a^+)/(\text{Na}^+)$ , to increase with decreasing ionic strength, a feature that cannot be taken into account by Manning's approach (73). In studies with ion exchange resins and alginate gels, Marinsky (71) was unable to predict the hydrogen ion activity in the gel polymer subphase

from pH measurements in the bulk liquid phase without such a partition factor. Thus the scheme presented above takes into account any free energy change that occurs as a result of the transfer of counterions from the bulk liquid phase to the reaction sites on the capsular polymer molecules. Once the intrinsic acid dissociation constant for the capsular polymer has been determined, intrinsic stability constants for interactions between metal cations and the polymer molecule can be obtained by the approach described below. This same procedure can be used to describe interactions between metals and other polymers that exist as gels in their normal state of hydration.

### 11.12.2 Intrinsic Stability Constants

The interaction between a bivalent metal and a capsular polymer may involve the formation of one or more types of complexes, each with a characteristic intrinsic stability constant. The approach described below determines how many different complexes are formed and the intrinsic stability constant for each. Initially, it is assumed that a single monodentate complex is formed. If the conditions for this type of complex are met, then a single constant is derived. If the conditions are not met, then the data are subjected to an evaluation using expressions that assume the formation of a bidentate complex. It is also possible to evaluate a metal-polymer interaction that involves both types of complexes. The approach identifies the relative contribution of each type of complex and their respective stability constants. The method also determines the number of metal binding sites on the polymer molecule at different  $\alpha$  and  $I$ .

An exopolymer suspension is prepared at the desired ionic strength and an estimation of the total titratable groups is estimated by uronic acid or tritium exchange assay as described above. Aliquots of a metal salt solution are added stepwise to the polymer suspension and the pH and metal ion activity monitored simultaneously until the equivalents of total metal ion added reaches a value that is no more than and preferably less than 5% of the total titratable groups present. The polymer suspension is then titrated with NaOH adjusted to the ionic strength of the polymer solution and the pH and metal ion activity are monitored with pH and ion specific electrodes until the end point is reached. The degree of polymer ionization,  $\alpha$ , is calculated from the expression

$$\alpha = \frac{A_t - HA - 2\vec{M}_{sb}}{A_t} \quad (11)$$

where  $\vec{M}_{sb}$  is the number of moles of bivalent metal ion bound to the polymer,  $HA$  is the number of moles of total undissociated functional groups on the polymer, and  $A_t$  is the number of moles of total titratable functional groups on the polymer as described previously.

$\vec{M}_{sb}$  is determined as the total number of bivalent metal ions,  $M_t^{2+}$ , added minus the metal ions in the bulk liquid phase according to the expression

$$\vec{M}_{sb} = M_t^{2+} - \frac{(M^{2+})V_s}{[\gamma_{M^{2+}}]_l} \quad (12)$$

where  $\gamma_{M^{2+}}$  is the activity coefficient of the free bivalent metal ion in the bulk liquid phase. The latter is determined from the expression

$$[\gamma_{M^{2+}}]_l = \frac{[\gamma_{\pm MCl_2}]^3}{[\gamma_{\pm KCl}]^2} \quad (13)$$

where the activity coefficients of the  $MCl_2$  and  $KCl$  at a specific ionic strength can be obtained from published tables in the literature (70).

$HA$  in Equation 11 is calculated as the total titratable functional groups on the polymer minus those groups neutralized by the added base and those groups ionized as a result of proton displacement by metal ions according to the expression

$$HA = A_t - \left[ m_{b,ac} + \frac{10^{-pH} V_s}{\gamma_{H^+}} \right] \quad (14)$$

where  $m_{b,ac}$  refers to moles of base added during the titration.

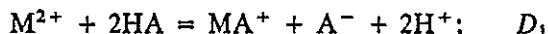
**11.12.2.1 Monodentate Complexes.** When a monodentate complex is formed between a divalent metal and a polyanion the equilibrium reaction at the binding site may be defined as



where  $\beta_{MA^+}^{int}$  is the intrinsic stability constant of the monodentate complex. Coupling this reaction with the acid dissociation reaction



yields



where

$$D_1 = \frac{(\vec{MA}^+)(\vec{H}^+)^2(\vec{A}^-)}{(\vec{M}^{2+})(\vec{HA})^2} \quad (15)$$

$\vec{MA}^+$  refers to the bivalent metal ions that lose their hydration water and form complexes with specific functional groups on the polymer, and  $M^{2+}$  refers to the electrostatically bound bivalent metal ions in the polymer subphase.  $D_1$  can also be expressed as

$$D_1 = \frac{\beta_{MA^+}^{int}}{[\beta_{HA}^{int}]^2} \quad (16)$$

$\beta_{HA}^{int}$  is included in Equation 16 to account for effects due to changes in pH and  $I$ . In Equation 15,  $(\vec{H}^+)^2/(\vec{M}^{2+})$  can be replaced by  $(H^+)^2/(M^{2+})$  on the basis of the Donnan equilibrium expression described earlier. Because the polymer subphase volume cancels from the three terms  $(\vec{MA}^+)$ ,  $(\vec{A}^-)$ , and  $(\vec{HA})^2$ ,  $D_1$  can be described in a form that contains the measurable terms

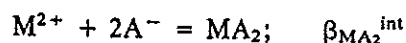
$$D_1 = \frac{(H^+)^2}{(M^{2+})} \times \frac{[\vec{MA}^+][A^-]}{[HA]^2} \quad (17)$$

where  $(H^+)$  and  $(M^{2+})$  are measured electrochemically.  $\vec{MA}^+$  is the total number of moles of monodentate complex formed, which is assumed to equal the moles of metal that are bound to reaction sites on the polymer,  $\vec{M}_{sb}$ , because most of the bivalent ions entering the polymer subphase are likely to be bound to ionized acid functional groups on the polymer. The total number of dissociated groups that are unoccupied,  $A^-$ , is defined by

$$A^- = \left[ m_{b,ac} + \frac{10^{-pH} V_s}{\gamma_{H^+}} \right] - MA^+$$

If only monodentate complexes are formed,  $D_1$  will be constant at every value of  $A^-/V_p$  in the titration.  $A^-/V_p$  is an expression of  $\alpha$  that has been adjusted for any volume changes that may have occurred in the polymer subphase.

**11.12.2.2 Bidentate Complexes.** A variation of  $D_1$  with the amount of base, metal ion, or polymer added to the system indicates that all or part of the polymer-bound bivalent metal ions form a bidentate complex which is described by the equilibrium reaction



and the overall reaction is described as



where

$$D_2 = \frac{\beta_{MA_2}^{int}}{[\beta_{HA}^{int}]^2} \quad (18)$$

or after substitution and rearrangement,  $D_2$  can be described by

$$D_2 = \frac{(H^+)^2}{(M^{2+})} V_p \frac{\vec{MA}_2}{[HA]^2} \quad (19)$$

which, with the exception of  $V_p$ , consist of terms that can be measured. In Equation 19,  $\vec{MA}_2$  is assumed to be equal to  $\vec{M}_{sb}$ .

When metal is introduced to the system,  $V_p$  not only varies with pH and ionic strength, as demonstrated previously in the evaluation of the acid dissociation constant, but also with the amount of metal ion bound to the polymer. In the case of monodentate complex, the  $V_p$  term cancels out of Equation 17 for the determination of  $D_1$ . This is not the case, however, in Equation 19 for the determination of  $D_2$ . Before describing the iterative procedure to determine  $V_p$ , it is necessary to consider the situation in which both monodentate and bidentate complexes contribute to a polymer-metal interaction, because it is unlikely that bidentate complexes are responsible for all of the interaction that occurs in aquatic environments or under the titration conditions described above, that is, when the equivalents of total metal ion present are equal to or less than 5% of the total titratable groups on the polymer (72).

**11.12.2.3 Monodentate and Bidentate Complexes.** Where both monodentate and bidentate complexes contribute to the interaction between a capsular polymer and a bivalent metal ion,  $\vec{MA}^+$  in Equation 17 is replaced by  $\vec{M}_{sb}$ , yielding

$$D_1' = \frac{(H^+)^2}{(M^{2+})} \frac{[\vec{M}_{sb}][A^-]}{[HA]^2} \quad (20)$$

where  $\vec{M}_{sb}$  is defined as  $[\vec{MA}^+ + \vec{MA}_2]$ . Substituting Equations 17 and 19 into Equation 20 yields

$$D_1' = D_1 + \left[ D_2 \frac{A^-}{V_p} \right] \quad (21)$$

Similarly, by replacing  $\vec{MA}_2$  with  $\vec{M}_{sb}$ ,  $D_2$  may be redefined as  $D_2'$ , where

$$D_2' = \frac{(H^+)^2 [\vec{M}_{sb}] V_p}{(M^{2+}) [HA]^2} \quad (22)$$

or

$$D_2' = D_2 + \left[ D_1 \frac{V_p}{A^-} \right] \quad (23)$$

where  $\vec{M}_{sb}$  and  $HA$  in Equation 22 are determined from Equations 12 and 14, respectively.

Ideally,  $D_1$  and  $D_2$  should be obtained from the slope and intercept of plots of  $D_1'$  versus  $A^-/V_p$  and  $D_2'$  versus  $V_p/A^-$ . Then substituting the values of  $D_1$  and  $D_2$  determined above and the experimentally obtained  $\beta_{HA}^{int}$  (which is the reciprocal of the intrinsic acid dissociation constant determined previously) into Equations 16 and 18, the intrinsic stability constants for the monodentate complex,  $\beta_{MA^+}^{int}$ , and bidentate complex,  $\beta_{MA_2}^{int}$ , are obtained.

**11.12.2.4 Determination of the Relative Contribution of Monodentate and Bidentate Complexes.** By limiting the equivalents of metal added to the system to less than 5% of the total moles of titratable functional groups present, the likelihood of monodentate complex formation is maximized. This is necessary in order to minimize the possibility of error when Equation 20 is used to determine  $D_1$  (which assumes that the bulk of the interaction is due to monodentate complex formation) for cases in which bidentate complexes contribute significantly to the polymers interaction with a metal.

Once  $D_1$  and  $D_2$  have been calculated, the extent to which the bidentate complex  $MA_2$  contributes to the polymer-metal interaction can be determined in a subsequent titration that places no limits on the amount of metal present. This is achieved by simultaneously solving Equations 17 and 19, where

$$\vec{M}_{sb} = \vec{MA}^+ + \vec{MA}_2 \quad (24)$$

and  $\vec{M}_{sb}$  is obtained by subtracting the moles of free metal in the bulk liquid phase (measured electrochemically) from the total moles of metal added to the system.

It is also possible to determine the relationship between the actual number of binding sites that are occupied by metal ions and the potential number of metal binding sites on the polymer. Assuming that the moles of potential metal binding sites are equivalent to the moles of total titratable functional groups  $A_1$ , substitution of Equation 14 in Equation 20 or 22 reveals that the portion of available sites that are occupied by metal ions (defined as  $\vec{M}_{sb}/A_1$ ) is influenced by the stability constant and  $A_1$  in addition to pH and metal

concentration. The average number of binding sites occupied per polymer molecule,  $\vec{M}_{p_{sb}}$ , can be defined as

$$\vec{M}_{p_{sb}} = \frac{\vec{M}_{sb}}{P}$$

where  $P$  is the moles of polymer added to the titration solution. The binding site density can thus be determined for each type of complex that is formed at any pH or  $I$ .

**11.12.2.5 Determination of Polymer Subphase Volume in the Presence of Metal Ions.** In the case of capsular polymers, accurate estimation of the amount of metal that is contained in the polymer subphase volume is complicated by the fact that the polymer volume changes with environmental conditions. Nevertheless, this difficulty can be overcome by modifying the iterative procedure that was applied to the solution of the three simultaneous equations described earlier. The equations used to estimate the effective polymer subphase volume when the polymer is titrated in the presence of metal ions are derived from those employed previously for the determination of the intrinsic acid dissociation constant. The equations are modified in order to account for the fact that both monovalent and divalent ions are present and because the latter not only interact electrostatically with the polymer but also form complexes with specific functional groups on the polymer molecule. Equation 6 is thus modified to

$$\vec{I} = \frac{4\vec{M}^{2+} + \vec{Na}^+ + A^- + \vec{MA}^+}{2V_p} \quad (25)$$

Assuming that the majority of metal-polymer complexes formed are monodentate under the initial conditions of the titration as discussed above, the last two terms of the numerator,  $[A^- + \vec{MA}^+]$ , may be replaced by  $[A_t - HA]$  in Equation 14.

The moles of monovalent (sodium) ion,  $\vec{Na}^+$ , that interact electrostatically with the polymer, which was previously defined by Equation 5, is redefined as

$$\vec{Na}^+ = [A_t - HA - 2\vec{M}_{sb}] [1 - \phi_{P,Na}]$$

for bivalent metal ions that have a tendency to form strong complexes with the polymer. By substitution, then,

$$\vec{Na}^+ = [m_{b,ac} + \frac{10^{-pH}}{\gamma_{H^+}} - 2\vec{M}_{sb}] [1 - \phi_{P,Na}] \quad (26)$$

For a bivalent metal that forms weak complexes with the polymer, the term  $2\bar{M}_{sb}$  must be modified to  $2\bar{M}_{sb}[1 + \phi_{P,M}]$  to include the electrostatically bound component, which is negligible for strongly complexing metals.  $\phi_{P,M}$  is the practical osmotic coefficient of the bivalent metal ion form of the polymer. Similar to  $\phi_{P,Na}$ ,  $\phi_{P,M}$  is determined by titration of electrolyte-free polymer with a metal hydroxide while measuring the activity of free metal ion. Dividing this activity by the moles of metal hydroxide added provides an estimate of  $\phi_{P,M}$ .

Because the terms  $\bar{M}^{2+}$  and  $V_p$  are not known, their values must be estimated in Equation 25, then using two nested iterative loops, more precise values are obtained as follows: If the estimated  $V_p$  is correct, Equation 7 will be satisfied. If Equation 7 is not satisfied, Equation 10 is used to obtain an improved estimate of  $V_p$  by the iterative procedure described previously except that Equation 25 is used in place of Equation 6. Each time an improved estimate of  $V_p$  is generated, the algorithm enters an outer loop to determine whether the value of  $\bar{M}^{2+}$  generated by the equation

$$\bar{M}^{2+} = \frac{[C_{M^{2+}} \gamma_{M^{2+}} 10^{2\Delta PK}] V_p}{[\bar{\gamma}_{M^{2+}}] \bar{r}} \quad (27)$$

equals the estimated value used in Equation 25, where

$$\gamma_{M^{2+}} = \frac{[\gamma_{\pm MCl_2}]^3 \bar{r}}{[\gamma_{\pm KCl}]^2 \bar{r}} \quad (28)$$

The new values of  $V_p$  and  $\bar{M}^{2+}$  generated in Equations 10 and 28, respectively, are substituted in Equation 25 and the iterative process continued until the values of  $V_p$  and  $\bar{M}^{2+}$  satisfy Equations 10 and 28. A computer program has been developed that accepts the measured titration data and, using the equations described above, provides the intrinsic binding constants for each type of metal-capsular polymer complex that is formed.

The importance of taking into account changes in ionic strength, polymer subphase volume, and competition of  $H^+$  and  $M^{2+}$  for binding sites when describing the interactions between metal ions and bacterial exopolymers may be illustrated by the interaction between alginic acid and cupric ions in Figures 11.2–11.4 below. A plot of  $D_1'$  versus  $A^-/V_p$  (Fig. 11.2) provides a series of data points that scatter around a  $D_1$  value of  $1 \times 10^{-3}$ . The slope of the line does not permit an evaluation of  $D_2$ . However, a plot of  $D_2'$  versus  $V_p/A^-$  (Fig. 11.3) estimates  $D_2$  to be  $8 \times 10^{-5}$ . The slope of the line in Figure 11.3 provides an additional estimate of  $D_1$  and verifies the previous estimate of  $1 \times 10^{-3}$  obtained from Figure 11.2. From these extrapolated values of  $D_1$  and  $D_2$  and a calculated  $\beta_{HA}$  value of  $1.26 \times 10^{-3}$ , intrinsic binding constants of 631 and 50.1 were obtained for  $\beta_{MA^+}$  and  $\beta_{MA_2}$ , respectively.

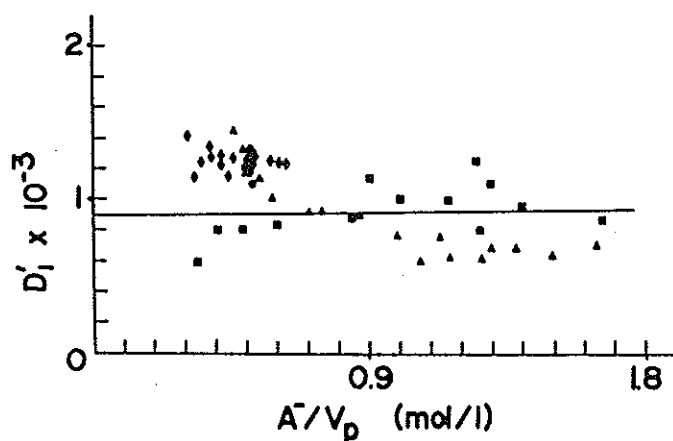


Figure 11.2 Plot of  $D'_1$  versus  $A^-/V_p$  (moles of free functional groups per liter of polymer subphase volume). Reaction mixture contains 0.0083 g alginic acid in 10 mL 0.1 M  $\text{NaNO}_3(\text{aq})$ ,  $\bullet$ ; 0.0083 g alginic acid in 10 mL 0.01 M  $\text{NaNO}_3(\text{aq})$ ,  $\blacktriangle$ ; 0.0400 g alginic acid in 10 mL 0.1 M  $\text{NaNO}_3(\text{aq})$ ,  $\blacklozenge$ ; and 0.0400 g alginic acid in 10 mL 0.01 M  $\text{NaNO}_3(\text{aq})$ ,  $\blacksquare$ .

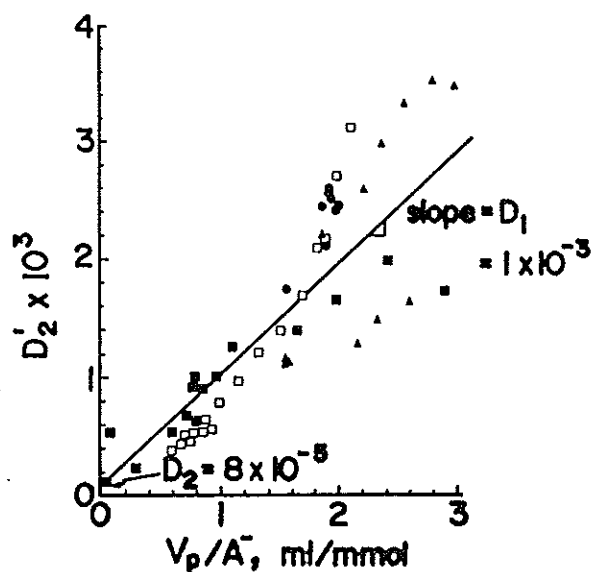


Figure 11.3 Plot of  $D'_2$  versus  $V_p/A^-$ . Reaction mixture contains: 0.0883 g alginic acid in 10 mL 0.1 M  $\text{NaNO}_3$ ,  $\bullet$ ; 0.0083 g alginic acid in 10 mL 0.01 M  $\text{NaNO}_3$ ,  $\blacktriangle$ ; 0.040 g alginic acid in 10 mL 0.1 M  $\text{NaNO}_3$ ,  $\square$ ; and 0.040 g alginic acid in 10 mL 0.01 M  $\text{NaNO}_3$ ,  $\blacksquare$ .

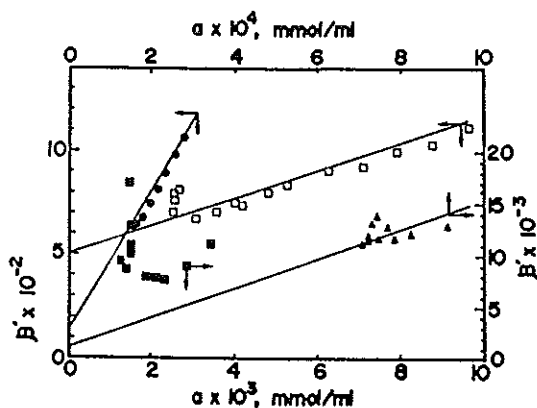


Figure 11.4 Plot of  $\beta_1'$  versus  $a$  (moles of free functional groups/liter of solution volume). Conformational changes in and electric field effects around the polymer molecules are not taken into account by this expression. Reaction mixture contains 8.3 mg alginic acid in 10 mL 0.1 M  $\text{NaNO}_3(\text{aq})$ ,  $\bullet$ ; 8.3 mg alginic acid in 10 mL 0.01 M  $\text{NaNO}_3(\text{aq})$ ,  $\blacktriangle$ ; 40 mg alginic acid in 10 mL 0.1 M  $\text{NaNO}_3(\text{aq})$ ,  $\square$ ; 40 mg alginic acid in 10 mL 0.01 M  $\text{NaNO}_3(\text{aq})$ ,  $\blacksquare$ . Arrows indicate appropriate axis.

If pH effects and conformational changes are not taken into account, a plot of  $\beta_1'$  versus  $a$ , where  $a$  is the monomeric concentration of ionized ligand and  $\beta_1'$ , which is based on bulk liquid phase volume  $V_s$ , is defined as

$$\beta_1' = \frac{(M_{sb})}{(A^-)(M^{2+})} = \beta_1 + \beta_2 a$$

where

$$a = \frac{A^-}{V_s}$$

$$\beta_1 = \frac{(MA^+_{sb})}{(A^-)(M^{2+})} = \frac{[MA_{sb}]}{[A^-](M^{2+})}$$

$$\beta_2 = \frac{(MA_{2, sb})}{(A^-)^2 (M^{2+})} = \frac{[MA_{2, sb}] V_t}{[A^-]^2 (M^{2+})}$$

The values of  $\beta_1'$  scatter over two orders of magnitude (Fig. 11.4) and, although a rough linear relationship may exist for values obtained from a particular set of conditions, no general correlation can be obtained if different experimental conditions are imposed. Comparison of Figures 11.2–11.4 dem-

onstrates how data may be misinterpreted if critical features of the polymer-metal interaction are overlooked.

In summary, accurate assessment of the interactions between metal ions and bacterial capsules or other cell wall components requires knowledge of the effective concentration of the metal and other counterions at the site of complex formation. In the case of capsules, it is necessary to consider the possibility that more than one phase exists and that variation in pH, ionic strength, and metal concentration induce volume changes in the polymer subphase where the interactions take place. By taking into account these features of bacterial capsules and slimes, it is possible to obtain accurate estimates of the thermodynamic constants. The constants may then be used to predict how these microbial products compete with other metal-complexing agents in the bacterial cell and in the surrounding environment under the variety of conditions in which metal binding takes place. This approach should be useful in assessing the relative importance of microbial exopolymers in controlling the movement and fate of metals in natural systems.

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