Two-phase model for describing the interactions between copper ions and exopolymers from *Alteromonas atlantica*

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Interactions between copper ions and exopolymer from the marine film-forming bacterium *Alteromonas atlantica* were evaluated by a two-phase model that treats the polymer as if it exists in a phase separate from the bulk solution. The model takes into account electrostatic interactions and molecular volume changes within the polymer phase to determine the copper activity in the domain where copper interacts with the ligands on the polymer molecule(s). The volume of the polymer phase varied with pH, ionic strength, and copper ion concentration. Exopolymer recovered from chemostat cultures grown at different dilution rates exhibited unique interligand distances, number of ionizable ligands, and molecular volumes. The variations in physical properties, in part, reflected differences in polymer chemistry. The exopolymer contained a lower density of ionizable groups and a smaller molecular volume per number of ionizable groups than alginic acid. The numerical procedure yielded a stability constant of 1×10^5 L/mol for a type I complex between copper ion and exopolymer produced at a dilution rate of 0.02 h^{-1} that was valid over a range of hydrogen ion concentrations and ionic strengths. The approach provided useful insight on how environmental variables affect the physicochemical properties of microbial exopolymers.

Key words: exopolysaccharide, metal ions, chemostat culture.

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Les interactions entre les ions cuivre et l'exopolymère présent dans le biofilm formé par une bactérie marine, *Alteromonas atlantica*, ont été évaluées selon un modèle en deux phases qui traite le polymère comme s'il se trouvait dans une phase séparée de la solution brute. Ce modèle tient compte des interactions électrostatiques et des changements du volume moléculaire dans la phase du polymère séparé dans le but d'évaluer l'activité du cuivre dans le domaine où le cuivre interagit avec les ligands sur la (les) molécule(s) du polymère. Le volume de cette phase de polymère isolé varie avec le pH, la force ionique et la concentration des ions cuivre. L'exopolymère recueilli de cultures en chémostat à différents niveaux de dilution est unique en ce qui a trait aux distances entre les ligands, le nombre de ligands ionisables et les volumes moléculaires. Les variations dans les propriétés physiques reflètent des différences dans la chimie du polymère. Comparé à l'acide alginique, l'exopolymère contient une plus faible densité de groupements ionisables et un volume moléculaire plus petit par nombre de groupements ionisables. La méthode numérique permet d'obtenir une constante de stabilité de 1×10^5 L/mol pour un complexe de type I fait de l'ion cuivre et de l'exopolymère produit à un niveau de dilution de 0,02 h^{-1}. Cette concentration est valide à l'intérieur d'une gamme de concentrations d'ions hydrogène et de forces ioniques. Cette approche apporte une perspective utile pour comprendre comment les variables environnementales peuvent affecter les propriétés physico-chimiques des exopolymères d'origine microbienne.

Mots clés : exopolysaccharide, ions métal, culture en chémostat.

[Traduit par la rédaction]

Introduction

Many exopolysaccharides produced by microorganisms in aquatic habitats are polyanions containing weakly acidic, ionizable carboxyl groups (Sutherland 1980). These polymers exist primarily as colloids at the concentrations found in most natural aquatic environments and have a tendency to react strongly with counterions, particularly group IIA elements such as Ca^{2+} and transition metal ions such as Cu^{2+}.

A two-phase model was recently described that characterized the interactions between cupric ions and alginic acid, an anionic polysaccharide with physicochemical properties representative of many bacterial exopolymers. The model described quantitatively the relationship among polymer concentration, the amount of metal bound, and the amount of metal in solution for dilute concentrations of alginic acid and cupric ions under different environmental conditions (Jang *et al.* 1990). Unlike the conventional approach in which solution pH and ionic strength are fixed in metal adsorption experiments and conditional stability constants are determined by the Langmuir, Freundlich, or Scatchard plots, the two-phase model accounts for deviations from "ideality" contributed by the variable electric field that is established at the surface of the polymer and the uncertainty of the effective concentrations of bound and unbound ligands along the polymer molecule in order to obtain intrinsic stability constants for the different types of complexes formed. Intrinsic stability constants have the advantage of being relatively independent of environmental variables, which are not always defined in natural systems. These constants are needed to predict the distribution of metal

species among various compartments of a dynamic ecosystem.

This paper uses the two-phase model to evaluate the interactions between cupric ions and exopolymer isolated from the marine, film-forming bacterium *Alteromonas atlantica*, grown at different dilution rates in a chemostat. Using this approach, it was possible to demonstrate how these important biological molecules respond to environmental change. An intrinsic stability constant was obtained that described the interaction between the exopolymer and copper ions.

Theory

When polyanionic macromolecules such as acidic polysaccharides are suspended in aqueous solutions, they form a gel or colloid phase. The gel phase containing the colloidal polymer is separated from the bulk aqueous phase by a hypothetical semipermeable membrane, which is permeable to counterions but not to polymer molecules. Within the gel phase exists a polymer subphase, which includes the polymer molecules and a small aqueous region around the polymer molecules, where strong electrostatic attractive forces exist (Jang *et al.* 1989*a*). The polymer subphase contains the ligands or acid (carboxyl) groups, and their concentration is best defined as moles of acid groups per unit volume of the polymer subphase (i.e., the region in which the acid groups are restricted). The moles of acid groups are determined by base titration, and the polymer subphase volume is determined by a recently described iterative procedure based on Donnan equilibrium, the osmotic properties of a polyelectrolyte solution, and base titration data (Jang *et al.* 1989*a*).

Effect of pH on binding of metal ions to acidic polysaccharides

Metal ions compete with protons for acidic sites on anionic polysaccharides. As standard base is added to an acidic polysaccharide solution containing trace quantities of a metal ion, more ionizable sites become available for metal binding and a stronger electric field develops around the polymer molecule. Metal binding "capacity" thus becomes a variable in an environment of changing pH. Furthermore, a stability constant that describes the binding of a metal ion to acidic polysaccharides should be defined on the basis of the concentration of dissociated, unoccupied, ionized ligands (A^-) on the polymer molecule.

Effect of ionic strength on binding of metal ions to acidic polysaccharides

The ligands on the polymer molecule are accessible to various hydrated counterions (H^+, Na^+, and M^{2+}, where M^{2+} refers to a divalent metal ion) in the bulk aqueous phase. At low ionic strength I (0.01 M $NaNO_3$), the electric field is not effectively screened by the low concentrations of neutral electrolytes. Consequently, the partition coefficient of a counterion (ratio of activity of counterion in the gel phase to that in the bulk aqueous phase) may be significantly greater than unity. The stability constant that describes the binding of a metal ion to an acidic polysaccharide molecule must, therefore, be defined on the basis of the activity of the metal, $(\overrightarrow{M^{2+}})$, in the polymer subphase volume, V_p, where the arrow over this and subsequent symbols refers to the polymer subphase. $(\overrightarrow{M^{2+}})$ can be estimated from the measured free metal ion activity in the bulk phase, (M^{2+}), by using the Donnan equilibrium relationship

$$[1] \quad (\overrightarrow{M^{2+}}) = (M^{2+})10^{2\Delta pK}$$

where the parentheses, ( ), refer to activity (activity coefficient × molal concentration) in this and subsequent equations (for Cu^{2+}, the molal single-ion activity coefficient, γ_{Cu}^{2+}, is 0.224 and 0.501 at ionic strengths of 0.1 M (0.105 *m*) and 0.01 M (0.01 *m*) $NaNO_3$, respectively), and

$$[2] \quad \Delta pK = pK_{HA}^{app} - pK_{HA}^{int}$$

where app is apparent and int is intrinsic, and where pK_{HA}^{int} is determined by base titration of metal-free polymer-containing solution and pK_{HA}^{app} is calculated by titration of polymer containing a trace amount of bound metal according to the expression

$$[3] \quad pK_{HA}^{app} = pH - \log\{<A^->/<HA>\}$$

where angle brackets, < >, refer to the number of moles of a chemical species in the titration volume and HA is the protonated ligands.

In equations 1–3 it is assumed that (*i*) the partition coefficient of a counterion is solely controlled by the electrostatic field around the polymer chain(s) and the ionic strength of the bulk solution and (*ii*) the value of pK_{HA}^{int} is not affected by the degree of ionization (fraction of ligands ionized) or by the binding of copper to the polymer. In other words, the free energy associated with the acid dissociation reaction remains constant for a polymer during the binding process. The Donnan model and its use in the development of the concepts under discussion have been presented in greater detail in earlier publications (Marinsky *et al.* 1983).

Determination of polymer subphase volume

Previous work has demonstrated that polymer subphase volume, V_p, of a dilute suspension of acidic polysaccharide (rigorously defined as the mass of the polymer and water in kilograms, excluding electrolytes, on a molal basis) accounts for only a small fraction of the total volume (Jang *et al.* 1989*a*). Even at low concentrations of polymer and metal ions, it is more accurate to express the mass-action stability constants on the basis of the volume to which the ligands (bound and unbound) are restricted rather than on the total solution volume, which implies (incorrectly) that the ligands are distributed uniformly throughout the solution.

The polymer subphase volume, V_p, is obtained at different degrees of ionization, α, and ionic strength, *I*, by a numerical iterative procedure that tests different values of polymer subphase activity, $(\overrightarrow{Na^+})$, under different trial values of V_p until the Donnan equilibrium relationship for Na^+ is satisfied.

$$[4] \quad \overrightarrow{pH} - pH = \overrightarrow{pNa^+} - pNa^+$$

The moles of electrostatically bound Na^+ in the polymer subphase can be estimated from the practical osmotic coefficient, $\phi_{p,Na}$, of Na^+ in a solution of charged polymer in the sodium form by $<Na^+> = (1 - \phi_{p,Na})<A^->$. $\phi_{p,Na}$ is determined by the charge density, ξ, on the polymer molecule, which can be calculated from the effective interligand distance, *b*, and the fraction of total ligands, A_t, that are ionized (defined as α). Effective interligand distance, *b*, is calculated from the measured value of γ_{Na}, the activity coefficient of free Na^+ of a polymer with a degree of ionization α in a salt-free solution using Manning's (1969) counterion condensation theory.

[5] $\xi = -2 \ln \gamma_{Na}$ for $\xi < 1$

[6] $\xi = 0.607(\gamma_{Na})^{-1}$ for $\xi > 1$

where

[7] $$\xi = \frac{e^2}{4\pi\epsilon_0\epsilon_r kT}\frac{\alpha}{b}$$

where e is the charge of a monovalent species (1.6×10^{-19} C), ϵ_0 is the permittivity in vacuo (8.854×10^{-12} F/m), ϵ_r is the relative permittivity (78.5 for water at 25°C), k is the Boltzmann constant (1.38×10^{-23} J/K), α is the fraction of ligands that are ionized (1.0 for a completely ionized polymer), and T is the absolute temperature in Kelvin. According to the theory, the critical value $\xi = 1$ corresponds to the charge spacing $(b/\alpha) = 7.135 \times 10^{-10}$ m (7.135 Å). Once b is determined for a completely ionized polymer, the theoretical osmotic coefficient of the dilute solution of a partially ionized polymer can be predicted by

[8] $\phi_{p,Na} = 1 - 0.5\xi$ for $\xi < 1$

[9] $\phi_{p,Na} = (2\xi)^{-1}$ for $\xi > 1$

A detailed description and verification of this procedure can be found elsewhere (Jang *et al.* 1989*a*, 1989*b*).

Taking into account electrostatic interactions, polymer subphase volume, combining acid dissociation constants with metal binding reactions, and the effect of competition from hydrogen ions for metal-binding sites, pseudotype I and pseudotype II complex forming distribution constants, D_1^{poly} and D_{2ph}^{poly}, respectively, are defined by

[10] $$D_1^{poly} = \frac{(H^+)^2}{(M^{2+})}\frac{<M_{sb}><A^->}{<HA>^2} = D_1 + D_{2ph}\frac{<A^->\overrightarrow{(H^+)}}{V_p}$$

[11] $$D_{2ph}^{poly} = \frac{\overrightarrow{(H^+)}}{(M^{2+})}\frac{<M_{sb}>V_p}{<HA>^2} = D_{2ph} + D_1\frac{V_p}{<A^->\overrightarrow{(H^+)}}$$

where $<M_{sb}>$ is the number of moles of metal ions bound to ligands such that

[12] $<M_{sb}> = <MA^+> + <MA^+ - HA>$

where $<MA^+>$ is the number of moles of metal ion bound to a single ligand and $<MA^+ - HA>$ is the number of moles of metal ion bound to two ligands,

[13] $\overrightarrow{(H^+)} = (H^+)10^{\Delta pK}$

D_1 describes

[14] $M^{2+} + 2HA \rightleftharpoons MA^+ + 2H^+ + A^-$

and D_{2ph} describes

[15] $M^{2+} + 2HA \rightleftharpoons <MA^+ - HA> + H^+$

In equations 14 and 15, the distribution constant D_1 refers to the complex formed between a bivalent metal ion and an ionized carboxyl group, whereas the distribution constant D_{2ph} refers to the complex formed between a bivalent metal ion and two ionized carboxyl groups with the electron-donating oxygen atoms on the proximal hydroxyl groups serving to stabilize the complex. Both types of complex could coexist. A plot of the pseudotype I or the pseudotype II distribution coefficients according to [10] and [11] can reveal the values of D_1 and D_{2ph}. The stability constants for type I and type II complexes are then determined by

[16] $\beta_1 = D_1\{\beta_{HA}^{int}\}^2$

and

[17] $\beta_{2ph} = D_{2ph}\{\beta_{HA}^{int}\}^2$

where β_{HA}^{int} refers to the intrinsic stability constant of the acid dissociation reaction of the polymer. Details on the derivation of the above equations have been described elsewhere (Jang *et al.* 1990). Briefly, D values describe the chemical equilibrium of the *overall* binding process (dehydrogenation followed by binding of the metal ion to the polymer). Therefore, multiplying D by $\{\beta_{HA}^{int}\}^2$ (dividing D by $\{K_{HA}^{int}\}^2$) yields the stability constants for metal binding.

Methods and materials

Organism and crude exopolymer recovery

Alteromonas atlantica (previously *Pseudomonas atlantica*), strain T_6C, was cultured in a chemostat in medium composed of 2.5% Rila salts (Rila Products, Teaneck, N.J.), 0.5% (w/v) proteose peptone (Difco), and 1% (w/v) galactose, in double-distilled water (dd-water), pH 7.5, at 25°C at various dilution rates. After two turnovers the cells were sedimented by continuous-flow centrifugation at 16 000 × *g* and the menstruum was treated with three volumes of cold (4°C) isopropyl alcohol to precipitate cell-free exopolymer. The precipitated exopolymer was dissolved in dd-water and dialyzed against the same before lyophilizing to dryness.

Exopolymer purification

Crude exopolymer was purified by the method described by Read and Costerton (1987). The exopolymer was first treated with RNase and DNase at 37°C, then with protease overnight at the same temperature. The digested exopolymer was then dialyzed against dd-water and lyophilized to dryness.

Preparation of exopolymer for titration

Lyophilized exopolymer (100–200 mg dry weight) was suspended in 75 mL dd-water and dissolved by adjusting the pH to 8.6 with NaOH. The polymer solution was back-titrated to pH 2.5 with HCl and then dialyzed against dd-water with several water changes. Each polymer solution was distributed as 5-mL volumes in acid-washed polypropylene, screw-cap vials and stored at −80°C. Just prior to titration, the 5-mL aliquots of polymer solution were thawed and transferred to an acid-washed polypropylene titration vial, the ionic strength was adjusted with sodium nitrate, and the final volume was adjusted to 20 mL with dd-water. The vials were sealed with a lid that contained inlets for a temperature probe, working electrodes, reference electrode, and nitrogen purge line. The nitrogen was bubbled through a water trap prior to introduction to the titration vial to minimize sample evaporation. The solution was equilibrated at 25°C in a water bath prior to titration.

Preparation of NaOH

The NaOH solutions were made with dd-water that was boiled to remove CO_2 and standardized against primary standard, potassium acid phthalate, using phenolphthalein as the indicator.

Titration procedure

First titration; in absence of copper

All base additions and pH measurements were made under a nitrogen atmosphere to prevent interference of CO_2 from the air. The polymer samples were titrated with either 0.01 or 0.1 M NaOH, using a 1-μL fixed-volume pipettor (Oxford). During the course of the titration, the pH values were plotted against the volume of

TABLE 1. Chemical composition of crude exopolymer from *A. atlantica* grown in a chemostat at different dilution rates

Dilution rate (h^{-1})	Neutral hexose ($\mu g/mg$)*	Uronic acid ($\mu g/mg$)*	Protein ($\mu g/mg$)*	Protein/ neutral hexose
0.005	531 ± 91	36.5 ± 0.4	261 ± 11	0.49
0.01	384 ± 10	57.0 ± 0.4	210 ± 8.4	0.55
0.02	412 ± 32	65.7 ± 4.0	162 ± 4.1	0.39

*Mean ± standard deviation, n = 3.

NaOH added, and the inflection point on the curve was taken as the end point of the titration.

Second titration; determination of sodium activity coefficient

A polymer solution containing no added sodium nitrate was titrated as above. At the end point of titration, the polymer was completely ionized and the moles of total sodium ions added (as NaOH) just balanced the negatively charged ionized ligands. The activity coefficient γ_{Na} at $\alpha = 1.0$ is calculated as the concentration of free sodium ions divided by the overall concentration of sodium ions. A sodium ion electrode was used to measure the free sodium ion concentration at the end point of titration.

Third titration; in presence of copper

The polymer solution was titrated with $CuSO_4$ until the total moles of $CuSO_4$ added was 5–7% of the total moles of ligands present (determined from base titration). The pH and free-Cu^{2+} concentration were monitored alternatively at each addition of $CuSO_4$. The polymer was then titrated with NaOH until the pH reached 6.5. Under these conditions, pH increased while free Cu^{2+} decreased, since the moles of ionized ligands available for binding copper increased with added base. At pH > 6.5 the free Cu^{2+} does not reflect the amount of unbound Cu^{2+} as a result of the formation of $Cu(OH)_2$.

Determination of initial and final total copper concentration

The amount of bound copper that was associated with the polymer before titration (initial copper) was determined by graphite furnace atomic absorption spectrometry following digestion with nitric and hydrochloric acid at 100°C. Total copper concentration at the end point of polymer titration following addition of $CuSO_4$ was determined by the same method, and the amount of bound copper at the end point was determined by subtraction of free copper (determined by copper ion electrode) from measured total copper. The amount of initial copper was very small compared with the amount of copper added to the titration solution. The moles of initial copper was included in the total copper in the calculation of bound copper at the end point.

Instrumentation

A pH/ion meter (Accumet 825 MP, Fisher Scientific) controlled by a five-position switch box (model 753, Fisher Scientific) connected to a combination glass electrode (AccuPhast 13-639-280, Fisher Scientific), a cupric ion electrode (94-29, Orion) a sodium ion electrode (ISM-146-Na, Lazar Laboratory), and a thermister probe were used to record pH, Cu^{2+}, Na^+, and temperature, respectively.

Chemical assays

Protein was determined by the method of Lowrey *et al.* (1951), using bovine serum albumin as a standard. Neutral hexose was determined by the phenol – sulfuric acid method of Dubois *et al.* (1956), using glucose as a standard. Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen (1973), using glucuronic acid as a standard.

Results

Exopolymer composition

The chemical composition of crude exopolymer produced by cells of *A. atlantica* grown in a chemostat varied with dilution rate. As dilution rate, d, increased from 0.005 to 0.02 h^{-1}, protein content decreased and uronic acid content increased (Table 1). The lowest protein – neutral hexose ratio was obtained at $d = 0.02\ h^{-1}$.

Acid dissociation constant

The acid dissociation constant of crude exopolymer produced at different nutrient loading rates was evaluated at different degrees of ionization, α (fraction of ionized ligands), and ionic strength, I. Titration of exopolymer in the absence of Cu^{2+} with NaOH yielded different apparent acid dissociation constants pK_{HA}^{app} at different α. The pK_{HA}^{app} of exopolymers produced at $d = 0.005$, 0.01, and 0.02 h^{-1} generally increased with increasing α at any value of I (Figs. 1*a*–1*c*). The pK_{HA}^{app} appeared to approach a constant value at lower values of α for exopolymer produced at $d = 0.02\ h^{-1}$ (Fig. 1*a*). pK_{HA}^{app} values at any particular value of α varied with I for crude exopolymer produced at all three dilution rates (Figs. 1*a*–1*c*) and for purified exopolymer (Fig. 1*d*). pK_{HA}^{app} generally decreased with increasing I. pK_{HA}^{app} values of crude exopolymers produced at $d = 0.01$ and $0.005\ h^{-5}$ tended to diverge at different I at low values of α, a trend that was not apparent with crude exopolymer produced at $d = 0.02\ h^{-1}$. A best-fit, linear extrapolation of the data produced intrinsic pK_{HA} (pK_{HA}^{int}) values of 3.0, 3.5, and 4.5 for crude exopolymer produced at dilution rates of 0.005, 0.01, and 0.02 h^{-1}, respectively, and 3.4 for the purified exopolymer ($d = 0.005\ h^{-1}$) (Table 2). Comparing Figs. 1*c* and 1*d*, one finds that the pK_{HA}^{int} of the exopolymer ($d = 0.005\ h^{-1}$) changed from 3.0 to 3.4 after protein removal. The pK_{HA}^{app} values (not shown) were also affected by the removal of protein.

Interligand distance

The interligand distance, b, for the completely ionized sodium form of exopolymer preparations from *A. atlantica* in a salt-free solution was calculated using Manning's counterion condensation theory (1969) and measured values of the activity coefficient of free Na^+. Values for b ranged from 3.15 to 6.75 Å (1 Å = 0.1 nm) (Table 2). Ligand concentration ranged from 4.44×10^{-3} to 1.58×10^{-3} mol titratable groups/g dry weight exopolymer (Table 2).

Polymer subphase volume

Exopolymer subphase volume varied with degree of ionization. The volume per mole of total titratable ligand (V_p/A_t) of exopolymer produced at all dilution rates tested showed the greatest variations with α at the lowest ionic strengths (Figs. 2*a*–2*c*). Exopolymer produced at $d = 0.02\ h^{-1}$ exhibited a maximum V_p/A_t at $\alpha = 0.6$ over a range of I (Fig. 2*a*). V_p/A_t of exopolymer produced at $d = 0.01\ h^{-1}$ also varied with α (Fig. 2*b*). Furthermore, the maximum volume occurred at different α depending on I. At $I = 0.05$ M, exopolymer volume exhibited a maximum at $\alpha = 0.50$–0.55, while at $I = 0.1$ M, the maximum

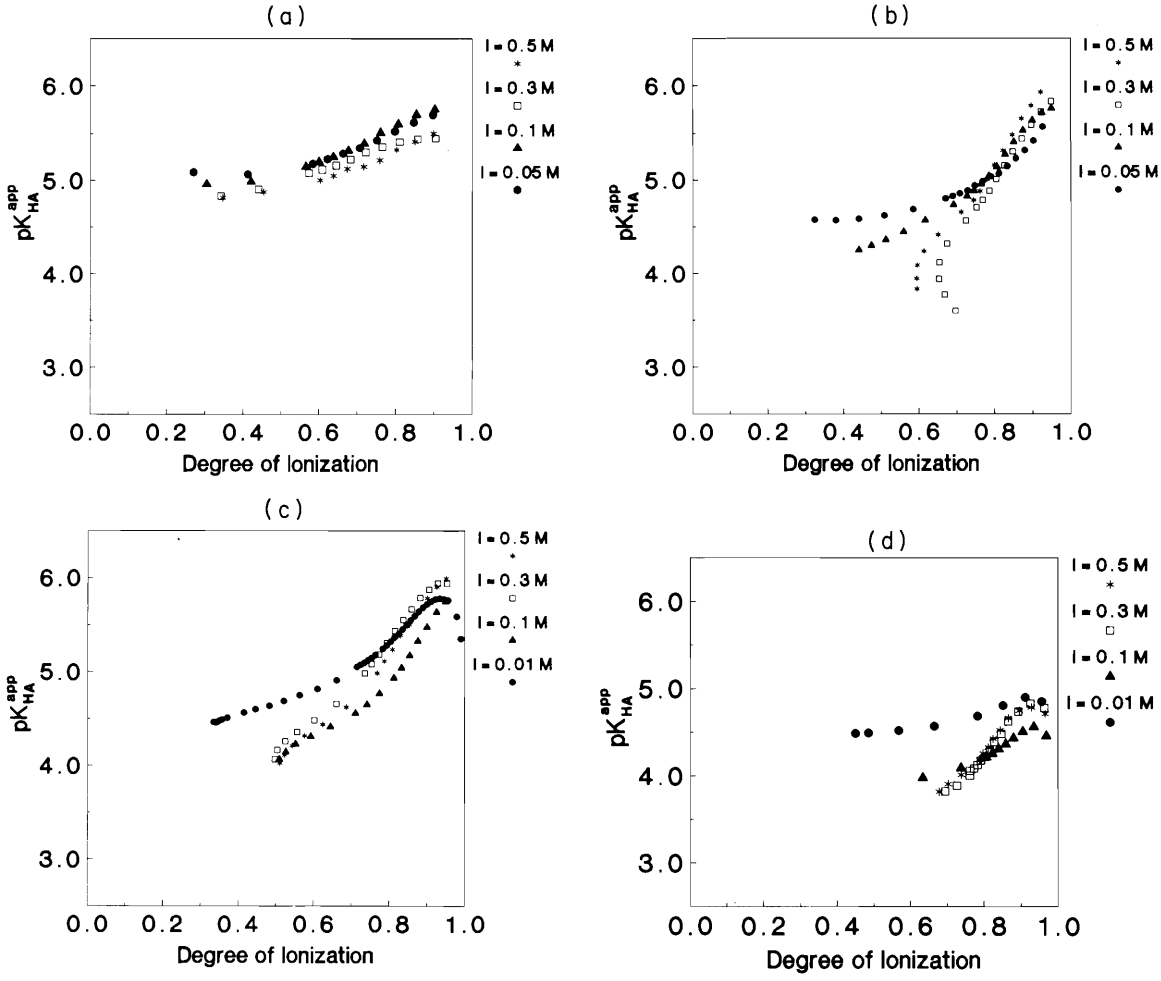

FIG. 1. Effect of degree of ionization and bulk aqueous phase ionic strength on apparent acid dissociation constant of crude exopolymer recovered from cultures of *A. atlantica* grown at dilution rates of (*a*) 0.02 h^{-1}, (*b*) 0.01 h^{-1}, and (*c*) 0.005 h^{-1} and (*d*) enzyme-purified exopolymer recovered from cells cultured at $d = 0.005\ h^{-1}$.

TABLE 2. Characteristics of exopolymers recovered from cultures of *A. atlantica* grown in a chemostat at different dilution rates

Polymer preparation	b (Å)	pK_{HA}^{int}	Mole titratable group/g dry mass	Type of Cu-polymer complex	β_{1p} (L/mol)
$d = 0.005\ h^{-1}$					
Unpurified	5.87	3.0	4.44×10^{-3}	—*	
Purified	4.05	3.4		—*	
$d = 0.01\ h^{-1}$	6.75	3.5	3.50×10^{-3}	—*	
$d = 0.02\ h^{-1}$	3.15	4.5	1.58×10^{-3}	I	1.0×10^{5}†

*Model did not provide a distribution constant that was independent of α and I.
†Stability constant was calculated as follows:

$$\beta_{1p} = D_{1p}\{\beta_{HA}^{int}\}^2$$
$$= \{1 \times 10^{-4}\}\{10^{4.5}\}^2$$
$$= 1.0 \times 10^5\ \text{L/mol}$$

volume occurred at $\alpha = 0.7$. In addition, the values of V_p/A_t for exopolymer produced at $d = 0.01\ h^{-1}$ were approximately 20% of those observed for exopolymer produced at $d = 0.02\ h^{-1}$ when compared at the same values of I (Fig. 2*b*). Values of V_p/A_t for exopolymer produced at $d = 0.005\ h^{-1}$ were similar to those of exopolymers produced at $d = 0.01\ h^{-1}$ at low I (Fig. 2*c*).

Normalized exopolymer subphase volume, when titrated in the presence of Cu^{2+} ions at a concentration equivalent to 5% of the total titratable ligands, A_t, on the exopolymer, displayed a response to α and I generally similar to that observed in the absence of Cu^{2+} for exopolymer produced at $d = 0.02\ h^{-1}$ (Fig. 3*a*). For example, the maximum normalized volume achieved in the presence of Cu^{2+} at comparable I was similar to that observed in the absence of Cu^{2+}. However, unlike V_p/A_t of exopolymer titrated in the absence of Cu^{2+}, which decreased from a maximum value as α increased from 0.6 to 0.8, V_p/A_t of

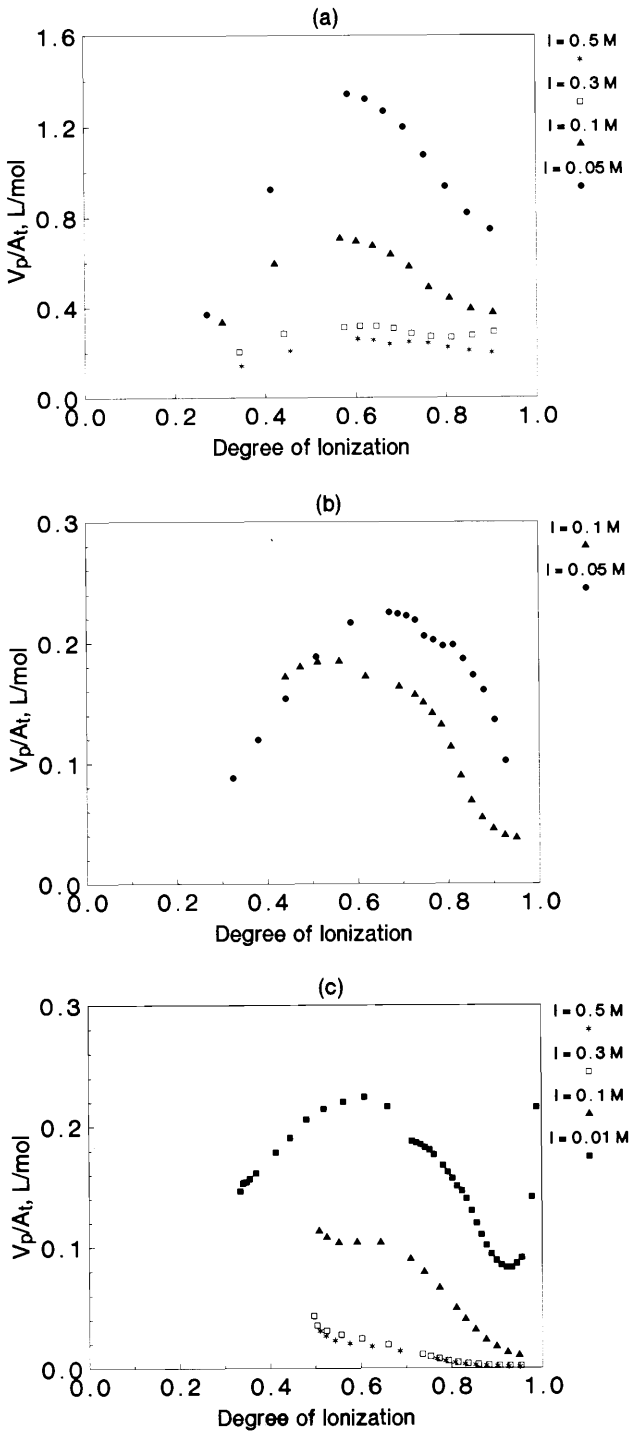

FIG. 2. Effect of degree of ionization and bulk aqueous phase ionic strength in the absence of added copper on polymer subphase volume of crude exopolymer recovered from cultures of *A. atlantica* grown at dilution rates of (*a*) 0.02, (*b*) 0.01, and (*c*) 0.005 h^{-1}.

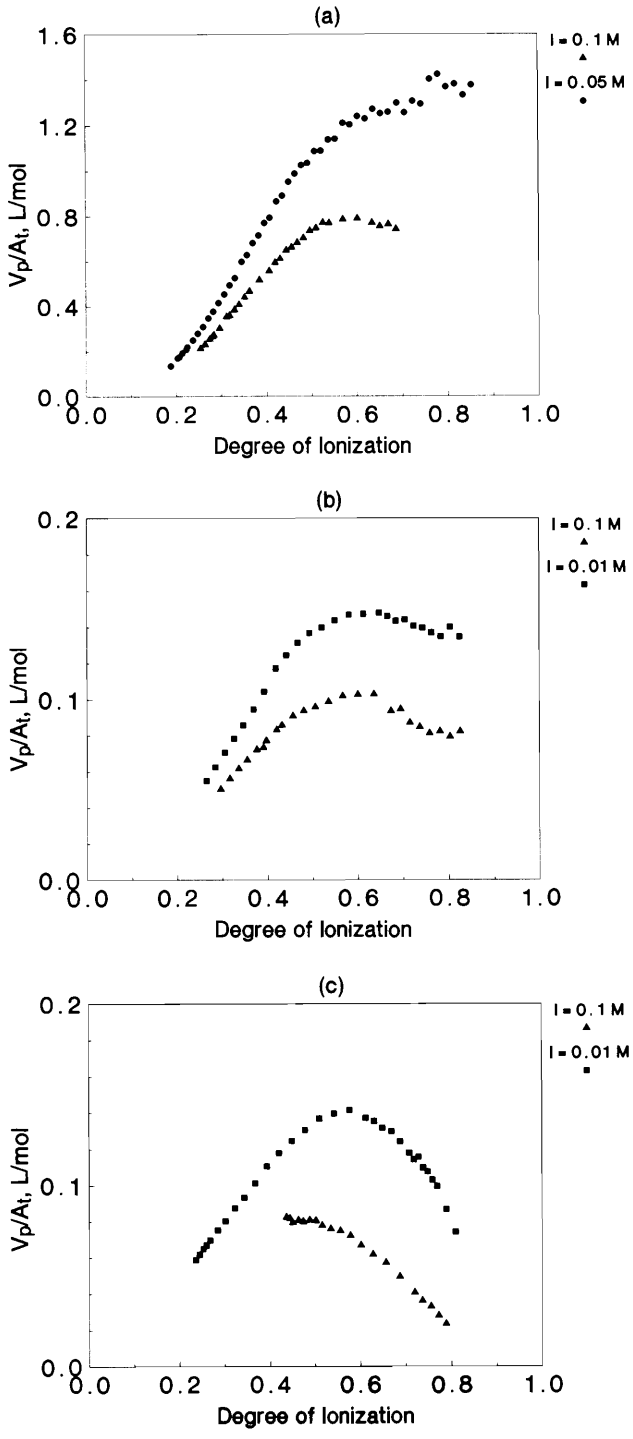

FIG. 3. Effect of degree of ionization and bulk aqueous phase ionic strength in the presence of added copper on polymer subphase volume of crude exopolymer recovered from cultures of *A. atlantica* grown at dilution rates of (*a*) 0.02, (*b*) 0.01, and (*c*) 0.005 h^{-1}.

exopolymer titrated in the presence of Cu^{2+} continued to increase slightly over this range of α.

V_p/A_t of exopolymer produced at d = 0.01 h^{-1}, when compared at the same I (0.1 M), responded differently to α, depending upon whether trace amounts of copper were present (Figs. 2*b* and 3*b*). Between α = 0.7 and 0.8, the normalized volume decreased more dramatically in the absence than in the presence of trace copper. In addition, the maximum V_p/A_t in the presence of copper was only 56% of that in the absence of copper. In both the presence and absence of copper, however, the maximum V_p/A_t was obtained between α 0.5 and 0.6. The maximum V_p/A_t of this exopolymer preparation, when titrated in the presence of copper, was only 26% of that of exopolymer produced at d = 0.02 h^{-1} at comparable I (0.1 M).

The response of normalized polymer subphase volume to

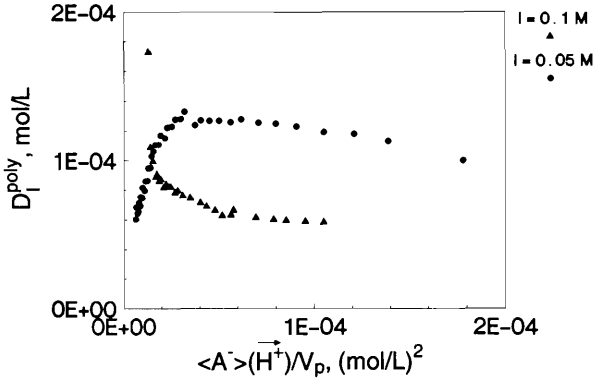

FIG. 4. Effect of competition from hydrogen ions on the distribution coefficient for pseudotype I complex between copper ions and ligand binding sites on crude exopolymer from *A. atlantica* grown at a dilution rate of 0.02 h^{-1}.

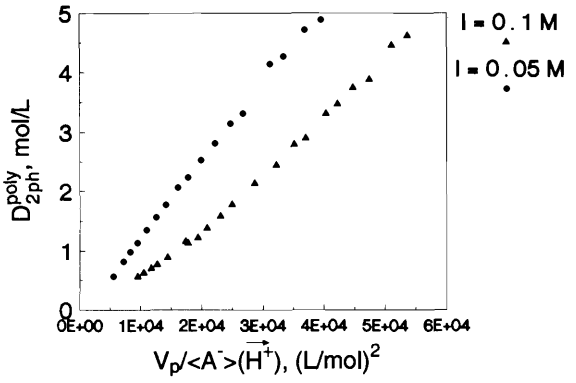

FIG. 5. Effect of competition from hydrogen ions on the distribution coefficient for pseudotype II complex between copper ions and ligand binding sites on crude exopolymer from *A. atlantica* grown at a dilution rate of 0.02 h^{-1}.

α for exopolymer produced at $d = 0.005$ h^{-1} in the presence and absence of copper varied depending on ionic strength. At low I (0.01 M), the response was similar with a maximum V_p/A_t achieved at approximately $\alpha = 0.6$, decreasing at lower values of α regardless of whether copper was present (Figs. 2*c* and 3*c*). However, the maximum volume in the presence of copper was only 62% of that in the absence of copper. At higher I (0.1 M), V_p/A_t continued to increase as a decreased from 0.8 to 0.4 (Figs. 2*c* and 3*c*). Like exopolymer produced at $d = 0.01$ h^{-1}, the maximum V_p/A_t for exopolymer produced at $d = 0.005$ h^{-1}, when titrated in the presence of copper ion, was less (56%) than that observed in the absence of copper. The maximum normalized polymer subphase volume of exopolymer produced at the lowest dilution rate was only 10% of that of exopolymer produced at $d = 0.02$ h^{-1} when copper ions were present (Figs. 3*a* and 3*c*). In summary, the polymer subphase volume appeared to vary with degree of ionization of the polymer, ionic strength, copper concentration, and polymer composition.

Intrinsic stability constant

The data described above were evaluated by a mathematical model developed to provide an intrinsic stability constant(s) that describes the interaction between cupric ions and ligands on the exopolymer. Of the exopolymers produced at the different dilution rates, only the exopolymer produced at $d = 0.02$ h^{-1} yielded a distribution constant, D_1^{poly}, for the stability of a pseudotype I complex that was relatively independent of a and I (Fig. 4). The values of D_1^{poly} ranged from 0.5 to 2.5 $\times$ 10^{-4} mol/L, with a mean value of 1 $\times$ 10^{-4} mol/L, when the data were presented as D_1^{poly} versus $<A^->(\vec{H^+})/V_p$. When the experimental data were presented as D_2^{poly} versus $V_p/<A^->(\vec{H^+})$, a straight line with a slope of 1 $\times$ 10^{-4} ($=D_1$) and an intercept of zero ($=D_{2ph}$) was obtained (Fig. 5). An intercept that approaches zero in this case suggests that type II complex is not significant relative to type I complex as described by D_1. Multiplying the mean D_1 obtained from Fig. 4 (which corresponded closely with the D_1 estimated from the slope of the line of D_{2pH}^{poly} versus $V_p/<A^->(\vec{H^+})$ in Fig. 5) by $\{\beta_{HA}^{int}\}^2$ yielded the intrinsic stability constant, (β_{1p}), of 1 $\times$ 10^5 L/mol for type I complex (Table 2).

Discussion

Variation in apparent pK_{HA} with degree of ionization of exopolymer from the marine film-forming bacterium *A. atlantica* suggests that electrostatic interactions occur in the vicinity of the ionizable groups on the exopolymer molecules. This variation, defined as ΔpK, describes the extent to which the apparent acid dissociation constant (pK_{HA}^{app}) deviates from the intrinsic acid dissociation constant (pK_{HA}^{int}). Unlike alginic acid, an anionic polysaccharide from algae, in which the ΔpK decreased with increasing ionic strength (Marinsky *et al.* 1983, Geesey and Jang 1989, Jang *et al.* 1989*a*), the variation in pK with α was only mildly affected by the ionic strength of the bulk solution. These results suggest that although *A. atlantica* exopolymer does exhibit the physical properties of a flexible polyelectrolyte like sphagnum peat, it does not display the strong gel-like behavior of alginic acid (Marinsky *et al.* 1983).

The differences in electrical properties of the exopolymers from *A. atlantica* grown at different dilution rates may be related to the charge distribution of density in the polymeric material. Calculated average interligand distances were greater in material recovered at $d = 0.005$ and 0.01 h^{-1} than that at $d = 0.02$ h^{-1}. The differences in charge density appear to be related, at least in part, to the degree of protein contamination of the exopolymer preparations. The average interligand distance varied indirectly with the protein–carbohydrate ratio of the exopolymer material (Table 1). When protein was selectively reduced in the exopolymer produced at $d = 0.005$ h^{-1} by protease treatment, the average charge density of the remaining polymeric material increased (b decreased from 5.87 to 4.05 Å). The nature of the protein associated with the exopolymer has not been determined, although previous studies have shown that it contributes to the binding of cupric ions in other bacteria (Mittelman and Geesey 1985). Although the model developed in this work does not differentiate the binding ligands of proteins and polysaccharides, the different values of pK_{HA} obtained with the crude and purified exopolymer do reflect the effect of protein on the microenvironment and morphology of the exopolymer of *A. atlantica*.

A best-fit line through the titration data which produced a slope of zero when plotted as pK_{HA} vs. α (typically obtained at high ionic strength) was shown to provide a reasonable estimation of pK_{HA}^{int} for alginic acid (Jang *et al.*

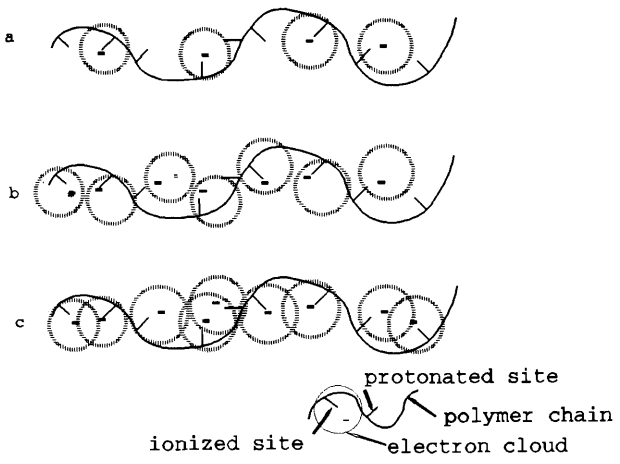

FIG. 6. Proposed influence of degree of ionization on polymer subphase volume normalized with respect to total ligands of crude exopolymer from *A. atlantica*.

1989*a*; Geesey and Jang 1989). Although the titration data from exopolymers of *A. atlantica* did not describe a best-fit line with a slope of zero, extrapolation of the data obtained at the highest I tested (0.5 M $NaNO_3$) yielded a pK_{HA}^{int} for exopolymer produced at 0.02 h^{-1} that was higher than those obtained at $d = 0.01$ and 0.005 h^{-1}. That the exopolymers obtained from cells grown at different dilution rates exhibited different pK_{HA}^{int} values is consistent with the evidence presented above demonstrating the chemical differences among the exopolymers produced under the different nutrient loading rates.

That the electric field from the charged ligands on the exopolymer was not solely responsible for the deviation of the acid dissociation constant with α and I was indicated by the fact that the base titration data, when plotted as pK_{HA} vs. α at different I, did not converge to a common value at $\alpha = 0$. These results indicate that the ligands and counterions are not distributed uniformly throughout the solution containing the polymer. Instead, the data suggest that exopolymer of *A. atlantica*, like alginic acid and humic acids, exists in a colloid phase that is separate from that of the bulk aqueous solution and that the concentration of ligands and counterions used for the determination of the acid dissociation constant should be based on the polymer subphase volume (Marinsky 1976; Marinsky and Reddy 1984; Jang *et al.* 1989*a*).

The polymer subphase volume (V_p), when determined by the iterative procedure described by Jang *et al.* (1989*a*), varied with α and I. Whereas V_p/A_t for alginic acid achieved a maximum of 5.5 mL/mmol ionizable ligand at $\alpha = 0.8$ or greater, a maximum normalized polymer subphase volume of 1.4 mL/mmol ionizable ligand was obtained when $\alpha = 0.6$ and $I = 0.01$ M ($NaNO_3$) for exopolymer produced at $d = 0.02$ h^{-1}. The corresponding V_p may be calculated as 1.4 mL/mmol ligand × 1.58 mmol ligand/g polymer × 0.0137 g polymer = 0.03 mL or 0.3% of the solution volume. Thus, the density of ligands, protonated or ionized, based on the polymer subphase volume should be several orders of magnitude larger than the ligand density based on bulk solution volume.

The increase in V_p of exopolymer produced at all three dilution rates at low ionic strength as α increased from 0 to 0.6 conforms to Manning's (1969) limiting law. At low α, only a small fraction of ligands are ionized, and the average distance between any two adjacent charged groups is relatively large. Therefore, the electron cloud around each charged group behaves independently of the other (Fig. 6*a*). As α increases, both the strength of the electric field and the volume of the liquid affected by this electric field increase in magnitude until the critical charge density ($\xi = 1$) is reached, when the electron clouds begin to overlap and discrete regions converge on each other (Fig. 6*b*). Further increase in α results in an increase in charge density and overall subphase volume but in a decrease in subphase volume per mole of ligand are a result of the increased overlap of electron clouds (Fig. 6*c*). When the average interligand distance, b, was divided by α where the local maximum V_p occurs (0.6), the result was very close to 7.135 Å, which is the "Bjerrum length" for monovalent ions in the classical theory of simple electrolyte solution and, in this case, equivalent to the critical charge spacing, $\xi = 1$, as defined in [7].

The decrease in polymer subphase volume at a particular α and I when copper was added to the exopolymer suspension is consistent with results obtained from alginic acid (Jang *et al.* 1989*a*). The number of moles of electrostatically bound cupric ions was less than 1% of the site-bound cupric ions as determined by the iterative procedure. Condensation of the polymer under these conditions is thought to be due to intramolecular and intermolecular cross-linking of ligands by the divalent cupric ions. In the case of alginic acid, it has been demonstrated that each cupric ion binds two carboxyl oxygen atoms in the polymer (Paoletti *et al.* 1981). There is also evidence that hydroxyl oxygen atoms on the alginic acid polymer coordinate cupric ions (Cozzi *et al.* 1969). Whether similar interactions exist between cupric ions and *A. atlantica* exopolymer remains to be determined.

Estimation of polymer subphase volume by the iterative approach provides a means of relating polymer morphology to chemical composition. The exopolymer produced at $d = 0.005$ and 0.01 h^{-1} contained fewer anionic uronic acids than exopolymer produced at $d = 0.02$ h^{-1}. This reduction in ligand concentration was reflected by a larger interligand distance. The increased distance between ligand groups on the polymer molecule should result in a reduction in the overall repulsive forces between ligands with a concomitant decrease in the sensitivity of polymer subphase volume to degree of polymer ionization. That the maximum subphase volumes of exopolymers produced at $d = 0.005$ and 0.01 h^{-1} were significantly less than that of exopolymer produced at $d = 0.2$ h^{-1} supports this theory. Similarly, alginic acid, which has a ligand concentration many times greater than that of *A. atlantica* exopolymer produced at $d = 0.02$ h^{-1}, exhibited a maximum $V_p/\langle A_t \rangle$ that was approximately 4.5 times larger than that of the latter (Jang *et al.* 1989*a*). Thus, volume estimates based on the iterative procedure are consistent with existing theory of charged polymer behavior in aqueous environments. The iterative procedure thus provides a relatively easy means to estimate the volume of microbial exopolymers under various environmental conditions.

In view of the evidence that polymer subphase volume within the colloid domain varies with pH, ionic strength, and the amount of metal bound, intrinsic metal binding constants of the exopolymer preparations should be defined

according to the counterion activity and the ligand density in the polymer subphase. When these constraints were applied to the titration data of *A. atlantica* exopolymer, only the data from exopolymer produced at $d = 0.02\ h^{-1}$ yielded a distribution constant, D_I^{poly}, for type I complex that was independent of α and I. That similar values were obtained for D_I^{poly} from two different plots of the data suggests that the estimate for the distribution constant is reasonable. Why titration data from exopolymer produced at the other dilution rates did not yield a distribution constant that was independent of pH and ionic strength remains to be determined. Further modification of the model may be required to accommodate the chemical properties of these exopolymers.

The value of 1×10^5 L/mol calculated for the intrinsic stability constant of the type I complex formed between cupric ions and the ligands on the exopolymer produced at $d = 0.02\ h^{-1}$ is two orders of magnitude greater than that calculated for alginic acid (1.5×10^3 L/mol), using the same model described here (Jang *et al.* 1989*a*). In both cases, type I complex was found to be more important than type II complex. It has been previously confirmed by Marinsky *et al.* (1983) that the pK_{HA}^{int} value of alginic acid determined by the two-phase approach compared well with the pK_{HA} of the monomeric-subunit mannuronic acid. The data suggest that the interaction between exopolymer of *A. atlantica* and cupric ions is much more stable than that between cupric ions and alginic acid and support the view that some microbial exopolymers possess high affinity binding sites for cupric ions that can influence the partitioning of this metal ion in environments where these bacteria flourish (Mittelman and Geesey 1985).

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