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BIOLOGICAL CONTROL OF TRACE METAL EQUILIBRIA IN SURFACE WATERS

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It has long been known that aquatic organisms can release organic compounds to their environment. While there are organics present from the decomposition of animals and, through excretion, as waste products from animals (Ferrante, 1976), most of the organics released to the aquatic environment are from plants (Fogg, 1951; Fogg, 1963; Gessner, 1965; Hellebust, 1965; Fogg, 1966; Forsberg and Taube, 1967; Khailov and Burlakova, 1969; Sieburth, 1969; Anderson and Zeutschel, 1970; Khailov and Finenko, 1970; Zajic, 1970; Berman, 1976). The compounds released by phytoplankton which form complexes with metals include amino acids, polypeptides, proteins, porphyrins, pterins, and purines (Khailov, 1964). The littoral marine alga, Fucus vesiculosus, releases as much as 40% of the carbon which it fixes. Khailov and Burlakova (1969) found as much as 39% of brown algal (and 38% of red algal) production was released as soluble extracellular products. Phytoplankton can release as much as 50% of their carbon which has been photosynthetically fixed (Fogg, 1951; Berman, 1976). Fogg and Westlake (1955) speculated that the polypeptides extensively released by blue-green algae may form complexes with metals and have important effects on ecology. Most of the organic complexing capacity of sea water is due to autochthonous production of organic compounds (Davey et al., 1973) by phytoplankton (Duursma, 1963; Anderson and Zeutschel, 1970; Thomas, 1971; Daumas, 1976). In a study of copper release from the thalli of benthic red algae, Seeliger and Edwards (1979) found that 22% of the copper released from living thalli was bound to dissolved organic matter, and 80-90% of the copper released from decomposing thalli was associated with dissolved organic compounds.

Hellebust (1965) determined the composition and quality of ex-

creted photoassimilated carbon for 22 species of marine phytoplankton. In this study, compounds were characterized by solubility in extractants with particular attention paid to the quantity of glycolic acid excreted. This study also determined the proportion of charged and uncharged compounds in the excreted organics. The compounds in the neutral fraction were further characterized by chromatography. The isolated compounds included mannitol, aspartic acid, arabinose, glutamic acid, lysine, glycerol, glucose, proline, and a number of unidentified compounds which yielded a number of amino acids upon hydrolysis. The extracellular products of Cricosphaera elongata have been characterized by ultrafiltration (Gnassia-Barelli et al., 1978).

Early studies of the possible interactions of metals with extracellular products were circumstantial because researchers were unable to isolate metal-organic complexes from water or to show a change in the form of trace metals attributable to association with extracellular products. As early as 1939, Harvey had found that diatom growth could be stimulated by soil extracts. Johnson (1955) isolated organic constituents of sea water but was unable to characterize qualitatively the compounds. He was, however, able to show that the isolates were bioactive. The isolated compounds caused both increases and decreases in the growth rate of phytoplankton. In 1964, Johnson conducted studies with the synthetic chelating agent EDTA and concluded that, by analogy, natural marine phytoplankton populations required a concentration of natural chelating agents equivalent to an EDTA concentration of $10^{-8}M$. From these studies, Johnson (1964) concluded that powerful natural chelators were present in sea water in small concentrations.

Extracellular products from aquatic organisms have been much studied and have been implicated in many important ecological processes, especially those with nutritive and antibiotic properties (Lucas, 1946). It is often difficult to separate these effects from those of changes in trace metal equilibria in aquatic systems. Analysis is complicated further by the circumstantial nature of some of the evidence for biological control of trace metal equilibria.

Biological control of the speciation of trace metals has been implicated in decreasing the inhibitory effect of some poisonous metals such as copper (Steemann-Nielsen and Wium-Andersen, 1970; Gächter et al., 1973; Gächter et al., 1978; Gächter and Máreš, 1979; Davies and Sleep, 1980) and in enhancing the availability of nutrients such as iron, a nutrient which is very insoluble under most conditions in the photic zone of aquatic environments and, thus, by being in short supply is potentially limiting to primary productivity (Giesy, 1976). The inhibitory effects of copper on phytoplankton, such as lengthened lag period and reduced growth rate (Guy and Kean, 1980), have been attributed to the free (Cu^{++}) form of copper (Sunda and Guillard, 1976; Sunda et al., 1978; Sunda and Gillespie,

1979; Jackson and Morgan, 1980).

Reduced productivity in waters of marine upwellings has been observed even though the nitrogen and phosphorus concentrations in these waters were greater than the surrounding surface waters (Barber and Ryther, 1969). These latter authors observed that the lag phase was elongated. This fact, coupled with the observation that copper concentrations in these waters were high (and that after phytoplankton had grown in the water, the effect was reduced), led to the conclusion that the phytoplankters were releasing a substance which was reducing the inhibitory effects of copper. To test the hypothesis that the observed effects were due to some organic compounds in the water, investigators added EDTA, glycine, and an uncharacterized zooplankton extract to culture media (Barber and Ryther, 1969; Barber, 1973; Huntsman and Barber, 1975). All three of these organics enhanced the growth rate of phytoplankton. It was concluded that the marine upwelling water was rich in free Cu^{++} inhibitory to phytoplankton, and that the water needed to be conditioned by the extracellular products of algae.

Studies of the extracellular products of Chlorococcum ellipsoideum showed that these substances stimulated growth of Chlamydomonas globosa (Kroes, 1972). The extracellular products were presumed to be polysaccharides and proteins which elicited responses similar to those of EDTA. The author suggested that the observed response may be due to keeping iron in solution or chelating other metals. When Huntsman and Barber (1975) separated, by a filter membrane, the extracellular products of a dense marine phytoplankton population from a less dense population of mixed phytoplankton in upwelling water, the lag phase of the less dense population was decreased and carbon fixation was increased relative to populations which were not grown in proximity to the phytoplankton filtrate. The effects observed for the phytoplankton filtrate were similar to those observed for additions of EDTA.

In a laboratory culture study, Hardstedt-Romeo and Gnassia-Barelli (1980) found that natural phytoplankton exudates of Cricosphaera elongata decreased copper and cadmium accumulation by this marine phytoplankter. The polymeric phenols released as extracellular products from the marine brown algae, Ascophyllum nodosum (L.) and Fucus vesiculosus (L.), decreased the toxicity of Zn to both Skeletonema costatum (Greve) and Phaeodactylum tricorutum (Ragan et al., 1980). In these studies, in the presence of 0.5 mg Zn/l, all concentrations of polymeric phenols between 100 and 2000 $\mu\text{g/l}$ increased the rate of cell division. This effect was not observed in the absence of Zn additions. At concentrations of polyphenols greater than 2000 $\mu\text{g/l}$, the rates of cell division were decreased relative to controls. This effect may have been due to the available zinc concentration being decreased below the concentration which is limiting for growth. Low-molecular-weight extracellular products

(<500 MW) of Cricosphaera elongata did not reduce copper toxicity; however, extracellular products with molecular weights > 500 MW decreased copper toxicity to this species (Gnassia-Barelli et al., 1978). The greatest decrease in toxicity was observed in the presence of extracellular products ranging from 500 to 1000. Similar reductions in copper toxicity were observed when C. elongata was grown in sea water containing extracellular products from Prorocentrum micans, Dunaliella primolecta, and Chaetoceros lauderi.

Khailov (1964) found metal-binding extracellular products were excreted from the marine phytoplankters, Dunaliella salina and Pontosphaera huxley, and three marine macrophytic algae (Fucus serratus, Ascophilum nodosum, and Rhodomenia palmota). In a study of eight species of marine phytoplankton, Swallow et al. (1978) found that only one species, Gloeocystis gigas, produced enough extracellular product to reduce the cupric ion activity when the total copper concentration was $10^{-6}M$. These researchers concluded that this copper binding was simply the result of the mucilaginous extracellular products of this species. The other eight species studied all created copious quantities of mucilaginous material and none of them affected the speciation of copper. A large proportion of the soluble organic nitrogen in aqueous systems is proteinaceous and this material can be important in the complexation of heavy metals (Tuschall and Brezonik, 1980). These authors reported stability constants for copper-organic complexes ranging from 1.6×10^6 to 7.9×10^6 at a pH of 7.5 and from 3.2×10^6 to 1.3×10^7 at a pH of 4.5.

These field and culture studies were only circumstantial evidence that complex formation with copper was responsible for the observed reduction in inhibition. It still remained to isolate a copper-organic complex in natural waters or demonstrate a reduction in copper activity due to actual extracellular products. Many studies on the effects of synthetic chelating agents on phytoplankton have been conducted. Avakyan and Rabotnova (1971) found that natural products, such as oxalic, pyruvic, malic, tartaric, and citric acids, which are known to chelate copper, also reduce the toxicity of copper to the yeast Candida utilis. Slowey et al. (1967) found indirect evidence of copper-organic complexes in sea water by extracting the phospholipids, amino lipids, and porphyrin lipids into chloroform and measuring the copper content of this fraction of the Hirsch-Ahrens separation method.

Further indirect evidence of copper-organic complexes has been found by measuring free copper before and after oxidation. After oxidation of the organic compounds, the total measurable copper concentration increased (Corcoran and Williams, 1964; Williams, 1969; Foster and Morris, 1971). Rona et al. (1962) found that zinc and manganese in sea water were in a filterable, yet undialyzable, form and suggested that this was probably due to formation of an organic complex. This may not necessarily be true since colloidal inorganic

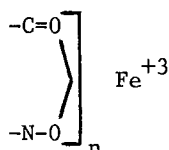
forms of zinc and manganese could do the same thing. Burton (1966) observed that vanadium in sea water was not precipitated with $\text{Fe}(\text{OH})_3$ and suggested that the vanadium may be complexed by organic matter. Albert (1950) inferred, from potentiometric studies, that copper and zinc are bound by amino acids. Fukai and Huynh (1975) found a high-molecular-weight fraction of dissolved organic matter in sea water, one which contained zinc and could not be measured potentiometrically or colorimetrically until the organic matter was oxidized with persulfate. Khallov (1964) and Koshy et al. (1969) studied the changes of color of ferric iron in solution and inferred that complexes were being formed. Similar results were found by Laevestu and Thompson (1958) when they oxidized sea water and observed an increase in measurable iron which they attributed to the iron being bound to organic complexes.

Studies of boron in sea water have also indicated that boron is released from organic complexes after oxidation (Gast and Thompson, 1958; Noakes and Hood, 1961). These authors suggest that the boron is bound to polyhydroxy organic compounds. However, Williams and Strack (1966) concluded that there was insufficient organic matter available in sea water to complex boron if stability constants for a boron-mannitol complex are assumed. Sillén (1961) and Goldberg (1965) have noted that, on a theoretical basis, concentrations of organic compounds at 10^{-4} to 10^{-5} M, in sea water, can form strong complexes with trace metals at low concentrations (10^{-6} to 10^{-7} M).

Besides the reductions in toxicity of trace metals which have been attributed to extracellular products, a number of authors have attributed nutrient availability to algal extracellular products. Early workers were not sure why algae released extracellular products; however, it was thought that the release of polypeptides may be due to cell wall formation processes. Fogg (1951) noted that the concentration of extracellular polypeptides increased when cells were iron deficient. Fogg (1951) suggested that iron was required for respiration and that energy was required to keep polypeptides inside living cells. Thus, iron limitation would result in increased release of polypeptides. Lange (1974) and Hunter (1972) suggested that the extracellular products of blue-green algae were as effective as EDTA in keeping iron and trace metal nutrients from precipitating at pH's above 8, and suggested that this could be ecologically important in maintaining the availability of trace metal nutrients. Lange (1974) did not characterize the extracellular products but speculated that they were probably polysaccharides. Spencer et al. (1972) studied the properties of extracellular products and their interaction with iron by infrared spectra, and suggested that the extracellular products were chelators specific for ferric-iron. Estep et al. (1975) investigated the growth-promoting activity of extracts from marine algal mats and sea grass on the siderochrome auxotroph Arthrobacter JG-9. Growth promotion of this particular species indicates the presence of secondary hydroxamic acids which are specific iron

chelators. These investigations found that the stimulatory compound was resistant to autoclaving and was not desferal; it was suggested that the compound was a secondary hydroxamate.

Neilands (1967) described hydroxamic acids and their role in iron metabolism by microorganisms. The hydroxamic acids are oxidized peptides with the general structure R-CON(OH). These compounds are formed by yeasts, bacteria, fungi and higher plants, and they act as growth factors, antibiotic antagonists, tumor inhibitors, or cell division factors. The complexes with ferric iron are of the form:



where n can range from 1 to 3. The 1:1 structure forms at low pH and is transformed to the 3:1 complex as the pH approaches neutrality. These compounds are considered to be iron-transfer agents, not electron transfer agents such as the hemes.

Blue-green algae have been found to excrete hydroxamate chelators which can enhance the growth of *Anabaena* species or inhibit growth of other species or both (Murphy et al., 1976). Murphy tested seven species of blue-green algae and 10 species of green algae for hydroxamate chelating activity and found only three species of blue-green algae (*Microcystis aeruginosa*, *Phormidium autumnale*, and *Anabaena flos-aquae*) which showed such activity. Murphy was also able to isolate three species of *Pseudomonas* and one species of *Aerobacter* which excreted hydroxamates. Some species of *Scenedesmus* are able to excrete polypeptides which solubilize iron, but these compounds do not form very strong complexes relative to the siderochromes of blue-green algae.

Murphy et al. (1976) suggest that the blue-green algae can assimilate iron-hydroxamate siderophore complexes, while eucaryotic algae cannot. This is ecologically important because it gives the blue-green algae a competitive advantage under low iron conditions. McKnight and Morel (1980) found that *Anabaena cylindrica* released strong copper complexing agents in response to iron-limited conditions. Under iron-rich conditions, only weak copper-organic complexes were formed. Competition studies between copper and iron indicated that the extracellular products were siderophores, especially trihydroxamates. They also found that only siderophores free of iron will bind copper, a fact which is in keeping with the great difference in formation constants for copper and iron complexes. The complex formation of copper with *A. flos-aquae* exudate was indistinguishable from that of the copper-desferal (CIBA-Geigy) complex, which is a hydroxamate sideramine.

The coordination numbers for copper (+4) and iron (+6) will result in different geometrics for the copper- and iron-siderophore chelates. The iron will form an octahedral geometry while the copper will form a square planar complex (Emery, 1971; as cited by McKnight and Morel, 1980). McKnight and Morel (1980) state that it is unlikely that the copper-siderophore complex would be assimilated by blue-green algal cells. Studies of copper toxicity to A. flos-aquae indicated that hydroxamate was not released from cells when they were copper stressed. It was found that the hydroxamates will chelate copper but form a less stable complex than with iron, and it was concluded that, in the system studied by the latter authors, the release of hydroxamate siderophores was in response to low iron concentrations, not copper toxicity.

Few of the culture or field studies have actually identified the extracellular products responsible for chelating metals and even fewer have quantified the amount released or the stoichiometric relations and affinity strengths. In the following section, I present information on the characterization of extracellular products which have been isolated.

Khailov (1964) found that the extracellular products of Fucus serratus, which formed complexes with copper, could be separated into four fractions on a column of Dowex 50-2B. Khailov (1964) noted that hydrogen ions were released upon titration with copper and suggested the formation of copper-organic complexes by hydrogen bonding. Dialysis studies indicated that most of the copper binding was due to compounds other than proteins or polypeptides. Sunda and Gillespie (1979), using a ^{14}C -glucose assimilation technique, determined the reactive chelating concentration in sea water to be approximately $0.05 \mu\text{M}$ EDTA equivalents and the formation constant of the copper-organic complex to be $\leq 10^{10}$. Based on these values and the concentrations and stability constants for the inorganic constituents of sea water, Sunda and Gillespie (1979) calculated the cupric ion activity to be $\leq 10^{-11}\text{M}$ when the total copper concentration was $1.4 \times 10^{-2}\text{M}$ ($0.9 \mu\text{g Cu/l}$). These studies were conducted in the Newport River Estuary (USA) and the organics were probably best characterized as humic-type substances rather than as extracellular products.

Johnson (1964) concluded from experiments where he compared the effect of EDTA on the growth of Skeletonema costatus that, by analogy, natural populations in sea water would require 10^{-8}M EDTA equivalents to chelate existing trace metals. Thus, he concluded that the natural chelators exist in small concentrations and form strong complexes with trace metals. The speculation of Johnson (1964) is in good agreement with the results of Sunda and Gillespie (1979).

The copper complexing capacities and formation constants for the extracellular products of Anabaena cylindrica, Navicula pelliculosa, and Scenedesmus quadricauda have been determined in culture (Van den

Berg et al., 1979) (Table 1). Using these stability constants, the free Cu^{++} concentration was predicted and was found not to be significantly ($\alpha = 0.05$) different from the measured cupric ion concentration. Stolzberg and Rosin (1977) observed a copper binding capacity of $4 \times 10^{-7}\text{M}$ in continuous cultures of the marine diatom Skeletonema costatum which were not stressed by copper. The conditional formation constant for the copper-organic complexes of Anabaena flos-aquae and Anaceptis nidulans have been determined to be 10^8 and 10^{10} , respectively (McKnight and Morel, 1979).

Anderegg et al. (1963) reported stability constants for hydroxamate complexes with lead, nickel, cobalt, and aluminum to be greater than 10^8 . The formation constants for the iron and copper siderophore complexes, determined by McKnight and Morel (1980) were 2×10^{10} and 3.7×10^7 , respectively. Hydroxamate concentrations of 5×10^{-6} to $5 \times 10^{-5}\text{M}$ were found in cultures with chlorophyll concentrations similar to those observed in field conditions. McKnight and Morel (1980) speculated that the iron concentration which limits growth of nitrogen-fixing, blue-green algae would be approximately 10^{-7}M , and that, at iron concentrations in this range, these algae would excrete hydroxamates at concentrations between 10^{-7} and 10^{-5}M . These authors concluded that the copper-siderophore complex would be the major copper species in blooms of nitrogen-fixing, blue-green algae where iron is the micronutrient which is limiting primary production.

Gnassia-Barelli et al. (1978) measured the complexing capacity for copper of several ultrafilter fractions of extracellular products of Cricosphaera elongata. Unfortunately, they report their results

Table 1. Concentrations of extracellular products and conditional stability constants for copper-organic complexes of 10-day-old algal cultures and two natural waters (Van den Berg et al., 1979).

	Ligands produced ($\mu\text{M/L}$)	Ligands per mg dry weight ($\mu\text{M/mg}$)	Conditional stability constants (pH)	
			7.6	8.0
<u>A. cylindrica</u>	6.73	0.110	7.7+0.1	8.1+0.2
<u>N. pelliculosa</u>	2.86	0.036	8.1+0.2	8.5+0.4
<u>S. quadricauda</u>	0.66	0.010	8.6+0.2	9.0+0.3
Bay of Quinte	—	—	7.6+0.2	8.0+0.2
Lake Ontario	—	—	8.7+0.5	9.1+0.5

as mg Cu/l or percent of total carbon bound in each fraction. Therefore, we do not know the binding capacity on a weight basis for the extracellular products. The authors do, however, report that 60% of the copper is bound by that fraction of the extracellular products having a molecular weight between 500 and 10,000. By comparing the copper threshold toxicity of sea water in the presence and absence of extracellular products, Gnassia-Barelli et al. (1978) concluded that approximately 50% of the copper was complexed to organic extracellular products when they were present. Unfortunately, this does not allow much generality in calculating the speciation of copper under different situations.

Gillespie and Vaccaro (1978) measured the relative copper binding capacities of organic carbon in sea water by measuring the assimilation of ^{14}C -glucose by marine bacteria when copper was added. The binding capacities of water from Saanich Inlet and Vinyard Sound (British Columbia and the Sargasso Sea) were found to be 0.0030, 0.0035, and 0.0060 $\mu\text{g Cu}/\mu\text{g DOC}$, respectively. This technique assumes that only free Cu is able to inhibit assimilation of the glucose substrate.

In conclusion, it can be said that those specific extracellular products of aquatic organisms which can form complexes with trace metals have not been well characterized. When they have been identified they have not generally been quantified in a manner such that the molar concentrations, required for thermodynamic speciation models, are available. The extracellular products released by phytoplankton have been the most studied in relation to possible metal chelating properties. The metal chelating properties of the extracellular products of macrophytic algae also have been investigated, but to a lesser extent. The quantity and quality of extracellular products are dependent upon species (Briand et al., 1978), growth phase (McKnight and Morel, 1979), light conditions and nutrient concentrations.

If all of the possible organic extracellular products are quantified, the problem exists that stoichiometric and thermodynamic constants are known for some of the compounds but not all of them. In fact, the formation constants, when known, may be conditional upon pH and ligand-metal ratios, so that competitive distribution equations cannot be written to account for pH and competing cations. In some cases there will be a number of possible organic ligands such as amino acids, polysaccharides, polypeptides, or simple organic acids, but no single compound which is very important in overall speciation. Also, there may be compounds such as humic-like compounds, which are a poorly-characterized continuous spectrum of polymeric compounds, for which no single simple formation constant is available.

While, because of theoretical considerations, it would be nice to describe all of the metal-ligand interactions individually and

solve all of the competitive interactions simultaneously, for practical reasons this may be impossible. This may be so because: there are many compounds with very similar formation constants; there is an error associated with the conditional stability constants; there is an error associated with measuring the total concentration of each individual ligand; or there is a lack of information about the stoichiometric and thermodynamic interactions of primary and competing trace metals. Even if all of the required information were available, it might be desirable to make simplifications to decrease the errors introduced by solving so many simultaneous equations.

Sposito (1981) has suggested that we use a quasiparticle model to represent a simplification of the complex situation of metal-organic interactions in surface waters. A quasiparticle model is a mathematical description of an aqueous solution, in which the actual assembly of organic compounds is replaced by an assembly of hypothetical identical macromolecules, whose mole mass is the number-average mole mass of the actual mixture and whose metal-complexation reactions closely mimic those of the real system (Sposito, 1981). An example of this conceptualization is the determining of overall conditional stoichiometric and formation constants by Scatchard analyses. Certain classes of metal-organic complexes may have similar stability constants and all of the sites which can be measured on polymeric molecules may not be available for binding metals. For these reasons, overall conditional stability constants and maximum binding capacities can be measured for organic ligands by the Scatchard analysis. Numerical solution of the partial derivatives which describe the hyperbolic Scatchard function results in overall conditional stability constants which can be used in quasiparticulate models (Giesy, 1980) to predict the forms of trace metals in solution. Giesy and Alberts (1981) have presented a discussion of humic compounds as quasiparticles which includes a discussion of functional group analysis and the problems of determining the molar concentrations of heterogeneous mixtures of organic ligands. They show that all of the measurable functional groups, carboxylic and phenolic, are not available for binding to metals such as copper, cadmium and lead.

It is often difficult to isolate the organic ligands from the inorganic ligands in natural waters. Sorption onto macroreticular resins, such as XAD-4, results in changes in the nominal molecular size distribution due to selective adsorption of the larger organics to the resin. Ultrafiltration may leave as much as 50% of the organic carbon in solution; this is due to the fact that often this much of the organic carbon in surface waters is less than the nominal 500 molecular weight cutoff of the smallest pore-size ultrafilters.

Because of the problems associated with isolation and quantification of organic ligands from surface waters, it is useful to measure the overall conditional stability constants in water before and after

photo-oxidation. In this way, the relative effects of organic and inorganic ligands can be separated. This technique suffers from the problem of release of cations and particulates upon oxidation of the organics with which they were associated.

In summary, the quasiparticulate model is not a perfect, or even theoretically-appealing, alternative to the complete description of the metal-ligand interactions, but may be the only pragmatic method of describing complex natural systems in a manner which allows the prediction of free metal concentrations. From this synoptic review it can be seen that much more information will be required if we are going to be able to quantify the effects of aquatic organisms on trace metal equilibria.

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DISCUSSION: J.P. GIESY

P. VALENTA

In our voltammetric measurement of the complexation of Cd with humic acids in sea water (not yet published), we could state that there is only one kind of site available for cadmium. Can you comment on this and cadmium complexation in general?

J.P. GIESY

We have observed cadmium to have a greater affinity for some humic materials than even copper but the total number of sites available is generally less. Also, there are seasonal changes in the number of types of sites. During some times of the year we can measure only one type of organic-cadmium binding site. Thus our data are not in disagreement with your findings.

Y.K. CHAU

Do you take into account the hydrolysis constants, solubility product constants, etc., in your model for the calculation of species?

J.P. GIESY

Yes the model includes solubility products and redox reactions; they don't happen to be very important in this system because of pH and redox considerations but are considered. Also, let me say that all solubility products and stability constants, as well as the activities of the constituents, are corrected for ionic strength.