

CHAPTER 44

The Evaluation of Monocopper (II) Citrate as a Potential Antimicrobial Metal Complex

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Monocopper (II) citrate is one of a group of metal complexes which are claimed by a U.S. patent to be effective antimicrobials. Presumably, this antimicrobial effect is manifest at alkaline pH where dissociation of the compound is minimal. Monocopper (II) citrate was evaluated in both neutral and alkaline trypticase soy broth and synthetic mineral salts media and found to be effective in inhibiting the growth of *Pseudomonas aeruginosa* over a short time period at alkaline pH. In deteriorated metalworking fluid, one area of practical application, cell counts were depressed for a short period.

INTRODUCTION

Antimicrobial agents which depend upon formation of a stable complex with certain metals (e.g., Cu, Zn, Fe) for their toxic activity have been well known since the work of Adrian Albert on 8-hydroxyquinoline (Albert et al. 1947; Rubbo et al. 1950). Examples of such compounds which are used widely in both industry and agriculture are 8-hydroxyquinoline, pyridinethiol-N-oxide (Rossmoore 1979), and the dithiocarbamates. Since the organic ligand in these cases is nontoxic in the absence of the complexing metal ion, a cooperative effect between the metal and the ligand is postulated to account for the antimicrobial activity (Albert 1973). The precise manner in which ligand-metal complexes act is unknown, but based on ample evidence, the metal-catalyzed oxidation of various cell components seems an attractive hypothesis (Sijpesteijn and Janssen 1959; Grisebach 1956; Van der Kerk 1977).

Numerous reports in the literature reflect the growing awareness of the involvement of complexation in biocide activity. Potentiation of effect when an agent is delivered as the metal complex as opposed to the free ligand is reported for certain amidine antifungals (Srivastra 1981); thiocarbamide compounds (Satpathy and Mishra 1981), and 8-hydroxyquinoline and its derivatives (Mason 1948; Tiwari and Mishra 1981). A correlation between complexation ability and the antibacterial effect of heterocyclic thiones also has been reported (Foye and Lo 1972). In addition, the action of the antibacterial compound, phanquone, is said to depend upon its ability to chelate metals (Husseini and Stretton 1980).

Recently, a U.S. patent claims that various metal ions complexed in a 1:1 ratio with polyfunctional organic acid ligands are effective antimicrobials (Maurer and Shringapurey 1977). Monocopper (II) citrate (MCC), the object of this study, is one example of such a complex. Other ligands claimed effective are the amino-, sulfhydryl-, and phosphinol polycarboxylic acids. In addition to copper, Zn, Ni, Cr, Bi, Hg, and Ag also are claimed effective. The rationale behind the mode of action of these 1:1 complexes is a pH-dependent dissociation of free metal ions. The complex form is stable at high pH but becomes unstable and will dissociate as the pH is lowered.

Presumably, following uptake by microbial cells growing in alkaline conditions, a release of toxic metal ions occurs at the physiological pH.

The reported solubility and stability of such complexes at high pH may make them suitable for use against the microbial flora of metal-working fluid emulsions (MWF). For practical as well as theoretical reasons, an evaluation of MCC was undertaken to assess its effects on the growth of *Pseudomonas aeruginosa*, a common contaminant of MWF, in both neutral and alkaline environments.

MATERIALS AND METHODS

The strain of *Ps. aeruginosa* used in this study was obtained as an isolate from an industrial MWF and was maintained on trypticase soy agar (TSA) slants with monthly transfer for several months prior to its use. Monocopper (II) citrate (MCC) was available as a commercial, concentrated aqueous solution. Analysis via thiosulfate titration (Hammock and Swift 1949) revealed a concentration of 1 mol/liter of copper ion. All concentrations are subsequently stated in ppm of Cu. The MCC solution was filter-sterilized through an 0.22 μm membrane filter (Millipore). Evaluation of MCC was made in complex and synthetic media as well as in a contaminated sample of MWF. Each experiment was conducted in duplicate, and the specific procedures are outlined below.

Complex Media-Trypticase Soy Broth

Trypticase soy broth (TSB) (BBL) was autoclaved and adjusted aseptically to pH 7.0 or 9.2. Each test flask contained 100 ml of TSB broth and was inoculated to give an initial density of 10^6 cells/ml. The MCC was added aseptically to each test flask prior to inoculation. The flasks were incubated in a New Brunswick shaker (Model #G25) at 30 C and 180 rpm, and growth was measured by turbidity increase using a Klett colorimeter (Model #800-3) with 540 nm filter. The concentrations of MCC used were: 0, 6.5, 65, and 325 ppm at pH 7.0; and 0, 65, 160, 325, 480, and 650 ppm at pH 9.2.

Synthetic Media at pH 7.0

A mineral salts base (MSB) medium (Palleroni and Duodoroff 1972) containing the following was used: Na-K phosphate buffer (0.033 M, pH 7.0), NH_4Cl (0.1% w/v), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05% w/v), ferric ammonium citrate (0.005% w/v), and CaCl_2 (0.0005% w/v). Sodium citrate (0.1% w/v) was used as a source of carbon. The medium was dispensed (100 ml/flask) into 250-ml Klett flasks and sterilized by autoclaving. The MCC was added aseptically and each flask was inoculated with a culture grown in the same medium (pH 7.0) to give an initial density of 10^6 cells/ml. Incubation and growth measurement were as for TSB flasks. The concentrations of MCC used were: 0, 65, and 325 ppm. In one experiment, MCC (33 ppm) was used as the sole carbon source in this synthetic medium.

Synthetic Media at pH 8.8

To obtain alkaline conditions without the precipitation of metal phosphates, β -glycerophosphate (Fischer) was substituted for the phosphate buffer in the above medium and the pH adjusted to 8.8 with KOH. To simulate the conditions of TSB, where amino acids and peptides are present as complexing ligands, β -alanine (0.1% w/v)

was used as a carbon source. Sodium lactate (0.1% w/v) also was added to promote rapid growth. The inoculum was conditioned by growing in this same medium at pH 8.8. The medium was filter-sterilized through an 0.22 μm filter (Millipore). Initial density was 10^6 cells/ml. Growth was assessed visually by turbidity.

Soluble Oil Emulsion

A sample of contaminated, soluble oil, metal-working fluid at a pH of 8.5 was used. The major bacterial contaminant was an uncharacterized *Pseudomonas* sp. The metal-working fluid was dispensed into sterile flasks (50 ml/flask) and shaken at 25 C and 180 rpm. Viability assessment was made by standard plate counts, and MCC was added immediately after determination of the initial cell number. The counts were done using the pour-plate method with TSA, with peptone broth (1.0%) at pH 7.0 as a diluent.

RESULTS AND DISCUSSION

Monocopper (II) citrate did not inhibit growth at any of the concentrations employed at pH 7.0 in TSB. When compared to the control, there was no increase in the lag phase in any test flask, and the increases in turbidity were the same in all flasks. Growth began following a lag of 2.2 and was observed for 10 h. This result was not unexpected considering the presumed mode of action of MCC. The complexed form is comparatively unstable at neutral pH and consequently would be present largely as dissociated citrate and free copper ions, neither of which alone is inhibitory to *Ps. aeruginosa* at the levels used.

The results for treatment of cultures with MCC in TSB at pH 9.2 are shown in Table 1. At relatively high concentrations, inhibition of growth occurred. However, this inhibition was only transient since turbidity developed even in 650 ppm after standing for 48 h at room temperature. During growth, a pH drop of one-half to three-quarters of a unit occurred in all flasks, presumably due to the production of organic acids by the bacterial cells. The long lag phase seen in the control and other flasks probably indicates the effects of the high pH alone on the growth since the inoculum was grown at pH 7.0 and not adapted to the high pH. It is also probable that the initial cell density had been depressed below the calculated inoculum as a result of this pH shock. Yet the results are not changed by these conditions. Monocopper (II) citrate can effect a short-term inhibition of growth at high pH.

Because of possible copper-exchange reactions between the citrate ligand and the amino acids or peptides in the TSB, synthetic media containing only inorganic salts were used, with Na citrate as the carbon source. Again, at pH 7.0 no inhibition occurred with the concentrations used. Growth began after a 1.5 h lag and was the same in

TABLE 1. Turbidity (Klett units) of cultures grown in TSB at pH 9.2, the effect of monocopper (II) citrate on growth

Time (h)	Control	Concentration of MCC (ppm)				
		65	160	325	480	650
0	0	0	0	0	0	0
5	3	1	4	0	0	0
17	403	310	156	70	0	0
22	503	530	486	430	3	0

TABLE 2. *Effect of monocopper (II) citrate on cell growth in synthetic media at pH 8.8*

Time (h)	Concentration of MCC (ppm)			
	3.0	6.5	16	33
10	+	-	-	-
17	++	-	-	-
24	++	+	-	-
27	++	+	-	-
30	++	++	+	-
38	++	++	+	-
45	++	++	++	-
48	++	++	++	+

+ = slight turbidity.

++ = heavy turbidity.

- = no turbidity.

both control and test flasks. However, in the case where MCC was used as a sole source of carbon, growth of the cells commenced after a lag of 20 h. A precipitate formed in this flask and, after analysis for metal ions, was found to contain largely copper (II) ions.

Table 2 shows the results for MSB buffered with glycerophosphate at pH 8.8. Comparatively low concentrations of MCC inhibited growth. As was the case with cells in alkaline TSB, growth resulted in a pH decrease and the inhibition was only short term. In spite of the presence of β -alanine, which also can complex with copper ions, inhibition occurred at concentrations 100 times lower than those effective in TSB. Also, cells grown in this low phosphate medium are necessarily induced for the enzyme alkaline phosphatase. Whether or not this altered physiology is responsible for the increased sensitivity when grown in this medium is unknown.

The effect of copper ions alone on growth at high pH also was tested using this same β -glycerophosphate-buffered synthetic medium. The CuSO_4 was added in place of the citrate complex at a concentration of 33 ppm of copper. No inhibition of growth when compared to the control flask was seen in this case, which supports the claim that the 1:1 complex is the toxic agent.

The effect of the MCC on cell growth in deteriorated MWF is shown in Table 3. Some decline in cell numbers was evident with the higher concentrations, but this was not permanent. The initial increase in count in the control was due to the aeration during the experiment. At a concentration of 650 ppm (not shown in Table 3), which is considered impractical for actual use, a drop in count from $10^6/\text{ml}$ to less than $10^3/$

TABLE 3. *Cell numbers as a function of time in metal-working emulsion treated with monocopper (II) citrate*

Time (h)	Control	Concentration of MCC (ppm)		
		13	65	325
0	2.7×10^7	2.7×10^7	2.7×10^7	2.7×10^7
6	5.4×10^7	3.0×10^7	5.0×10^6	5.2×10^5
18	14.0×10^7	19.0×10^7	9.0×10^6	1.1×10^6
30	12.0×10^7	9.0×10^7	9.7×10^7	1.0×10^7
48	1.7×10^7	1.2×10^7	6.0×10^7	10.0×10^7

ml occurred in 6 h. However, after 48 h, the numbers increased to 4×10^5 cells/ml, further demonstrating the short-term nature of the inhibition.

The data in Table 3 also show that after 48 h, cell numbers were greater in the flasks treated with the higher concentrations of MCC than in the controls. This can be due either to a simple resurgence of growth following the MCC-induced lag, or possibly to the presence of citrate as a utilizable substrate contributed by the dissociated MCC itself.

CONCLUSIONS

The data show that MCC is not effective for the long-term control of bacterial growth. However, the fact that inhibition was possible with this compound is interesting from a theoretical point of view. As copper ion is most probably the actual toxic agent, the citrate complex has potentiated this metal toxicity since no inhibition was seen when using copper ion alone. The ligand may serve to facilitate delivery of the metal to its target.

The level at which heavy metals such as copper become toxic is unclear. Our data show that copper ion levels equivalent to those used in this study are not toxic (at pH 7.0) to this strain of *Pseudomonas* but do inhibit the production of pyocyanin pigment (Piet and Rossmoore, unpubl. results). Zimmerman (1966) has shown that ascorbic acid is able to potentiate the toxicity of low levels of Cu^{++} ion which are otherwise nontoxic. The presence of coordinating ligands thus may indeed influence metal toxicity.

With *Ps. aeruginosa*, the presence of porin channel proteins does not preclude the actual uptake of the charged citrate-metal complex by simple diffusion (Nikaido and Nakae 1979). However, the pH of the microenvironment near the cell surface may be lower than that of the bulk medium due to the presence of proton-attracting, negatively charged groups (James 1981). This would imply a dissociation into copper ions at the cell surface. These factors should be considered when defining the actual mode of action of MCC.

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