

# **COPPER As A Biocidal Tool**

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**Abstract**

Copper ions, either alone or in copper complexes, have been used for centuries to disinfect liquids, solids and human tissue. Today copper is used as a water purifier, algacide, fungicide, nematocide, molluscicide, and as an anti-bacterial and anti-fouling agent. Copper also displays potent anti-viral activity. Here we review (i) the biocidal properties of copper; (ii) the possible mechanisms by which copper is toxic to microorganisms; and (iii) the systems by which many microorganisms resist high concentration of heavy metals, with an emphasis on copper.

## **1. Copper as Biocide**

Metal ions, either alone or in complexes, have been used for centuries to disinfect fluids, solids and tissues [1,2]. The ancient Greeks of the pre-Christian era of Hypocrates (400 BC) were the first to discover the sanitizing power of copper thousands of years ago. They prescribed copper for pulmonary diseases and for purifying drinking water. The Celts produced whisky in copper vessels in Scotland around 800 AD and this practice has continued to the present day. Gangajal is stored in copper utensils in every Hindu household due to copper's anti-fouling and bacteriostatic properties. Copper strips were nailed to ship's hulls by the early Phoenicians to inhibit fouling, as cleaner vessels were faster and more maneuverable.

By the 18th century copper, had come into wide clinical use in the western world, being employed for the treatment of mental disorders and afflictions of the lungs. Early American pioneers moving west across the continent put silver and copper coins in large wooden water casks to provide them with safe drinking water for their long voyage. In the second World War, Japanese soldiers put pieces of copper in their water bottles to help prevent dysentery. Copper sulphate is highly prized by some inhabitants of Africa and Asia for healing sores and skin diseases. NASA first designed an ionization copper-silver sterilizing system for their Apollo flights.

Copper is considered safe to humans, as demonstrated by the widespread and prolonged use of copper intrauterine devices (IUDs) by women [3-5]. In contrast to the low sensitivity of human tissue (skin or other) to copper [6], microorganisms are extremely susceptible to copper. Several mechanisms to explain the toxicity of copper to microorganisms have been suggested (see Section 2). Bacteria, fungi and other microorganisms, but not viruses, have developed mechanisms to resist heavy metals in general and copper in particular (See Section 3).

### **1.1. Copper as a Bactericide.**

The bacteriostatic effect of copper was noted by Dr. Phyllis J. Kuhn [7], who was involved in the training of housekeeping and maintenance personnel in the Hamot Medical Center, Pennsylvania. To heighten their awareness of modes of infection, the students were given blood agar plates and instructions on their use, and they returned with bacterial cultures from such diverse sources as toilet bowl water (remarkably clean), salad from the employees' cafeteria (heavily colonized), and doorknobs. Brass (an alloy typically of 67% copper and 33% zinc) doorknob cultures showed sparse streptococcal and staphylococcal growth; stainless steel (about 88% iron and 12% chromium) doorknob cultures showed heavy growth of Gram-positive organisms and an array of Gram-negative organisms.

Based on this observation, she investigated bacterial growth on metals. Small strips of stainless steel, brass, aluminum, and copper, were inoculated with broths of *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus* group D, and *Pseudomonas* species. The broths contained approximately  $10^7$  bacteria/ml, a very heavy inoculum. The strips were then air-dried for 24 hours at room temperature, inoculated onto blood agar plates, and incubated for 24 hours at 37°C. The results were striking. The copper and brass strips showed little or no growth, while the aluminum and stainless steel strips produced a heavy growth of all microbes. The test was repeated at drying intervals of 15 minutes, 1, 5, 7, 20 and 24 hours. Brass disinfected itself in seven hours or less, depending on the inoculum size and the condition of the surface of the metal. Freshly scoured brass disinfected itself in one hour. Copper disinfected itself of some microbes within 15 minutes. Aluminum and stainless steel produced heavy growths of all isolates after eight days and growths of most isolates after three weeks.

In another experiment, stainless steel, aluminum, brass and copper strips were covered with seeded agar and incubated in culture for 24 hours. Replica plates from the stainless steel and

aluminum strips allowed the growth of bacteria, while replica plates from the brass and copper strips did not. Scanning electron micrographs pictures of the surfaces of the metals showed that *E. coli* was completely disrupted on the brass while remaining intact on the stainless steel.

More recently, the ability of various electroplated coatings (cobalt, zinc, copper, and cobalt-containing alloys of nickel, zinc, chromium, etc.) to inhibit the growth of pathogenic bacteria (Gram-positive bacteria, *Enterococcus faecalis* and methicillin-resistant *S. aureus*, and Gram-negative bacteria, *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*) was examined [8]. The amounts of H<sub>2</sub>O<sub>2</sub> produced and metal ions dissolved from the surfaces of the various electroplated coatings were measured and it was found that the inhibitory ability of the coatings corresponded to the amounts of H<sub>2</sub>O<sub>2</sub> produced. The bacterial survival rates on the surfaces of the coatings were almost zero when H<sub>2</sub>O<sub>2</sub> was produced in amounts greater than 10<sup>-6</sup> mmol/cm<sup>2</sup>. However, the dominant concentrations of metal ions dissolved from coatings were outside of the bacterial lethal range.

In another study it has been shown that although H<sub>2</sub>O<sub>2</sub> is toxic to bacteria in metal ion-depleted media, the H<sub>2</sub>O<sub>2</sub> dose level required for killing was sharply reduced if copper salts and reductants were added [9]. Similarly, it has been found that the antibacterial potency of several compounds is significantly higher when they are complexed with copper [10,11]. Likewise, a copper phosphate cement used as a restorative material for treatment of caries demonstrated the greatest antibacterial activity in vitro and in vivo among several restorative material tested [12,13]. Moreover, addition of activated copper significantly improved the antibacterial properties of calcium hydroxide used to kill bacteria in dentinal tubules [14].

The bacteriostatic effectiveness of copper when used in paints in rendering surfaces self-disinfecting has also been demonstrated [15]. Nearly all of the tested copper paints were capable of reducing the number of tested organisms (*S. aureus*, *E. coli*, *P. aeruginosa*, and *E. faecalis*) to negligible levels within 24 hours of exposure. This has led to the use of copper in paints,

including as an anti-fouling agent for the reduction of microbial biofilm formation in ships [16]. Fouling, the growth of barnacles, seaweed, tubeworms, and other organisms on boat bottoms, produces roughness that increases turbulent flow, acoustic noise, drag, and fuel consumption. In fact, an average increase of 10  $\mu\text{m}$  in hull roughness can result in a 1% increase in fuel consumption! Addition of copper to drinking glasses has been shown to reduce biofilm formation of *Streptococcus sanguis* reducing the risk of oral infections [17]. Recently, the potential use of copper as a bacterial inhibitor in various stages of food processing has been demonstrated [18]. The antibacterial activity of metallic copper surfaces was demonstrated against two of the more prevalent bacterial pathogens that cause foodborne diseases, *Salmonella enterica* and *Campylobacter jejuni*.

A platform technology was recently developed, which binds copper to textile fibres from which woven and non-woven fabrics can be produced [19]. The ability to introduce copper into textile fabrics may have significant ramifications. One example would be the possible reduction of healthcare-associated (nosocomial) infections in hospitals. Nosocomial infection ranks fourth among causes of death in the United States, behind heart disease, cancer and stroke. Nosocomial infections are estimated to add \$5 billion to US hospital and insurance costs each year [20]. Nosocomial infections can be bacterial, viral, fungal, or even parasitic. These infections are largely device-associated or surgically-related [21]. The main sources for contamination are the patient's skin flora, the flora on the hands of medical and nursing staff, and contaminated infusion fluids. However, it has been demonstrated that sheets in direct contact with a patient's skin and the patient's bacterial flora are an important source of infection [22,23]. Moreover, sheets were significantly more contaminated by patients carrying infection than by non-infected patients [22]. Therefore, use of self-sterilizing fabrics in pajamas, sheets, pillow covers, and robes in a hospital setting may reduce the spread of microorganisms in hospital wards, which could result in a reduction of nosocomial infections. Similarly, the use of gloves with anti-

bacterial and anti-viral properties by hospital personnel may also aid in reducing transmission of infectious microbes and viruses while providing increased protection to hospital personnel. The introduction of copper into latex, without changing the physical characteristics of the latex, enabled the manufacture of copper impregnated latex gloves [19].

An additional potential use of copper-impregnated fabrics is related to foot ulcerations, a common complication of type 1 and 2 diabetes, which afflicts approximately 130 million individuals around the world [24]. In many cases, these ulcerations can become highly infected due to cuts/bruises that do not heal, or heal at a very slowly pace. Infections that do not heal have been shown to cause the tissue to die (gangrene). In severe cases, toes and legs may have to be amputated in order to save the remaining healthy body parts of the patient. Use of socks containing copper-impregnated fibres by diabetics may significantly reduce the risk of foot infection by rendering the area aseptic.

## 1.2. **Copper as a Water Purifier.**

Some diseases can be acquired by contact with bacteria in water. Legionnaires' disease, for example, is acquired both by aerosols containing *Legionella pneumophila* and by microaspiration of water contaminated with *Legionella*. Thus, disinfection of water distribution systems in hospitals, where cases of Legionnaires' disease occur, has become commonplace with the knowledge that the water distribution is the source of the pathogen.

The bacteriostatic properties of copper have led to testing its capacity as a water purifier. Copper was found to be one of the most toxic metals to heterotrophic bacteria in aquatic environments. Albright and Wilson [25] found that sensitivity to heavy metals of microflora in water was (in order of decreasing sensitivity): Ag, Cu, Ni, Ba, Cr, Hg, Zn, Na, Cd. Sagripanti et al. [26] evaluated the efficacy of cupric chloride against 13 bacteria. Although cupric chloride

inactivated more than 5 logs of most bacteria within 30 minutes, incomplete inactivation was noted for *E. coli*, *P. aeruginosa*, *S. aureus*, and *Staphylococcus epidermis*.

In a study conducted by The Midwest Research Institute, USA, bacteria were introduced into 50 foot coils of different plumbing tube materials. Water with a suspension of *E. coli* was then pumped through the coils and changes in bacteria viability were periodically determined. While in different types of plumbing material, including glass, the level of bacteria remained the same, or in some cases even increased, in the copper loop only 1% of the *E. coli* bacteria remained viable after five hours (<http://www.mriresearch.org>). Based on a similar study, The Midwest Research Institute subsequently also reported that water distribution systems made of copper have a greater potential for suppressing growth and for decreasing persistence of *L. pneumophila* cells in potable water than do distribution systems constructed of plastic materials or galvanized steel [27].

A subsequent study conducted at the centre for Applied Microbial Research, Public Health Laboratories Service (PHLS) in England, compared the growth of *Legionella pneumophila* on copper and other plumbing materials by using a continuous culture model system. They found that the bacteria levels were reduced on copper surfaces compared with a glass control and other plumbing materials at all the temperatures tested (20°C, 40°C, 50°C and 60°C) and in the three different waters used (soft peaty water, moderately hard river derived water, and hard bore hole derived water) [28].

A controlled evaluation was also made of the efficacy of copper-silver ionization in eradicating *L. pneumophila* from a hospital water supply. Copper-silver ionization units were installed on the hot water recirculation line of a building with water fixtures positive for *Legionella*. Another building with the same water supply served as a control. *Legionella* species persisted within the system when copper and silver concentrations were < 0.3 and < 0.03 ppm, respectively. When copper and silver concentrations were > 0.4 and > 0.04 ppm, respectively,

there was a significant decrease in *Legionella* colonization, but the percentage of water fixtures positive for organisms remained unchanged in the control building. When the ionization unit was inactivated, water fixtures continued to be free of *Legionella* for 2 additional months [29]. Similar results were obtained in additional studies [30-33]. According to a report published in 1998, more than 30 hospitals in the USA are now using copper-silver ionization to control *Legionella* in their water distribution systems [34].

The efficacy of 1:10 silver/copper combinations for inactivation of *Hartmannella vermiformis* amoebas and the ciliated protozoan *Tetrahymena pyriformis* in vitro was also studied by a German group [35]. *Tetrahymena* and *Hartmannella* were inactivated for 2 log steps by 100 + 1000 µg/l Ag + Cu. The investigations clearly showed that levels within the limit of German drinking water regulations (10 + 100 µg/l Ag + Cu) could not inactivate these protozoas in vitro. In the case of *Naegleria fowleri*, the organism responsible for primary amoebic meningoencephalitis, it was demonstrated that a combination of silver and copper ions were ineffective at inactivating the amoebae at 80 and 800 µg/L, respectively. However addition of 1.0 mg/L free chlorine produced a synergistic effect, with superior inactivation relative to either chlorine or silver-copper used alone. A similar synergy was reported for *Staphylococcus sp.* and *P. aeruginosa* (Reviewed in [36]).

### **1.3. Copper as an Algicide, Fungicide, Molluscicide and Acaricide.**

Copper compounds have their most extensive employment in agriculture. The first recorded use of copper in agriculture was in 1761, when it was discovered that seed grains soaked in a weak solution of copper sulphate inhibited seed-borne fungi. Within a few decades, so general and effective had become the practice of treating seed grains with copper sulphate that today this seed-borne disease is no longer of any economic importance.

The greatest breakthrough for copper salts as fungicides undoubtedly came in the 1880's with the development of a lime-copper formulation by the French scientist Millardet. He showed that spraying of grapes and vines with a mixture of copper sulphate, lime and water engender them remarkably free of downy mildew. By 1885, his vintner's spray formulation was the fungicide of choice in the U.S. and was given the name of "Bordeaux mixture." Within a year or two of the discovery of Bordeaux mixture, Burgundy mixture, which also takes its name from the district of France in which it was first used, appeared on the scene. Burgundy mixture is prepared from copper sulphate and sodium carbonate (soda crystals) and is analogous to Bordeaux mixture.

Trials with Bordeaux and Burgundy mixtures against various fungus diseases of plants soon established that many plant diseases could be prevented with small amounts of copper applied at the right time and in the correct manner. From then onwards copper fungicides have been indispensable and many thousands of tons are used annually all over the world to prevent plant diseases (for a list of copper bactericides and fungicides see <http://www.copper.org/compounds>).

The discovery that many algae are highly susceptible to copper, led to the use of copper salts by water engineers to prevent the development of algae in potable water reservoirs. They are also employed to control green slime and similar algal scums in farm ponds, rice fields, irrigation and drainage canals, rivers, lakes and swimming pools.

As noted above, copper sulfate was one of the first chemicals used for algae control. However, copper sulfate reacts with hard water and forms copper carbonate, which is non-active and insoluble. Copper sulfate may be very toxic to fish. The toxicity of copper sulfate varies with water hardness and is greater in soft water. Copper sulfate solution is unstable in sunlight and warm temperatures. The sulfate ions tend to combine with hydrogen in an aqueous solution to form sulphuric acid, which is highly corrosive. The environmental hazards over copper build-up

in sediments, and the need for high dosages have resulted in the production of better compounds that provide the copper in a chelated form [37,38]. The chelated copper is non-reactive with other chemical constituents in the water. Even though there are more effective and safer products on the market, the use of copper sulfate for algae control is still very common, primarily because of its low cost and ease of application.

Anywhere there is significant humidity, roof algae is prevalent. Control of moss growth can be achieved by eliminating one source of its nutrients, algae infestation on the roofing granules. Copper is thus used as the active ingredient in products that prevent roof moss formation [39].

Copper sulfate is one of the most common therapeutics used to treat infected striped bass and redfish [40]. Both species of fish are affected by fungi (usually *Saprolegnia*) when the fish are injured or stressed. In addition, these fish and others are frequently infected with bacteria (*Vibrio* species and *Aeromonas hydrophila*), and protozoa (*Amyloodinium ocellatum*, *Trichodina*, *Ichthyophthirius*, *Cryptocaron*, etc.). The usefulness of two copper compounds (CuDequest-2041-hydroxide and Cu TETA) to control biological pollution of shellfish beds and microbial diseases in shellfish has also been demonstrated [41].

Copper sulphate has been used to inhibit timber and fabric decay, since it renders them unpalatable to insects and protects them from fungus attack. Copper sulphate has been in use since 1838 for preserving timber and is today the base for many proprietary wood preservatives.

Copper-8-quinolinolate and some of its derivatives have been shown to be fungicidal to *Aspergillus spp.* at concentrations above 0.4 µg/ml [42-45]. Since infection with this fungi is a major problem among immunocompromised patients (such as AIDS patients), this agent has been used to reduce environmental contamination of fungi in hospitals [46].

Use of copper by the wider population may also be beneficial for more benign conditions. About 15-20% of the population suffers from *tinea pedis* [47,48]. While there are many clinical

presentations of *tinea pedis*, the most common is between the toes and on the soles, heels and sides of the foot. Although this fungal infection is not usually dangerous, it can cause discomfort, may be resistant to treatment, and may spread to other parts of the body or other people. Affected feet can also become secondarily infected by bacteria. Recently it has been found that copper-impregnated socks may be useful in preventing and treating *tinea pedis* [19].

Another possible use of copper in fabrics is related to allergies and asthma. It is estimated that 15% of the general population suffer from one or more allergic disorders of which allergic rhinitis is the most common [49]. Allergic rhinitis affects an estimated 20 to 40 million people in the US alone. Similarly, nearly 15 million Americans have asthma, including almost 5 million children. Approximately 5,500 persons die each year from asthma [50]. Dust mites are considered to be an important source of allergen for perennial rhinitis and asthmatic attacks [51], and copper impregnated fabrics have been shown to destroy them [19]. Thus, elimination of house dust mites in mattresses, quilts, carpets and pillows, would be an important step in improving the quality of life of those suffering from dust-mite related allergies [19].

Copper sulfate has been found to be a potent molluscicide [52]. Control of snails may be an important strategy in fighting some human diseases, such as bilharziasis. This disease is caused by a trematode parasite, *Schistosoma mansoni*, which uses snails and humans as hosts. The International Copper Research Association screened 23 copper compounds, in addition to copper sulfate, for their effectiveness in killing snails in water of low- and high-alkalinity, with and without high levels of suspended solids. One compound, cupric chloride-bis-n-dodecylamine, was considerably superior to copper sulfate under all test conditions. A bivalent, organic complexed, copper nitrate was marginally superior to copper sulfate [52].

#### **1.4. Copper Antiviral Activity.**

In 1964, Yamamoto and colleagues [53] reported on the inactivation of bacteriophages by copper. Jordan and Nassar [54] in 1971 showed that copper (0.2 mg/l) present as copper carbonate or colloidal copper inactivated infectious bronchitis virus. Totsuka and Ohtaki [55] in 1974 showed that the effect of copper sulfate on poliovirus RNA is proportional to its concentration, and that most amino acids except cysteine had a protective effect as did  $\text{Fe}^{2+}$  and  $\text{Al}^{3+}$ . Similarly, Coleman and colleagues [56] in 1974 reported that herpes simplex virus (HSV) type I was quite sensitive to silver. Sagripanti and colleagues [57,58] found in 1992 that cupric and ferric ions were by themselves able to inactivate five enveloped or nonenveloped, single- or double-stranded DNA or RNA viruses (phi X174, T7, phi 6, Junin, and HSV). The metals were even more effective than glutaraldehyde in inactivating the viruses. The metal virucidal effect was enhanced by the addition of peroxide, particularly for  $\text{Cu}^{2+}$ . In every case, the viruses were more resistant to iron-peroxidase than copper-peroxidase on a metal concentration basis.

The inactivation of HSV by copper was enhanced by the following reducing agents at the indicated relative level: ascorbic acid  $\gg$  hydrogen peroxide  $>$  cysteine. Treatment of HSV-infected cells with combinations of  $\text{Cu}^{2+}$  and ascorbate completely inhibited virus plaque formation to below 0.006% of the infectious virus input. The logarithm of the surviving fraction of HSV mediated by 1 mg of  $\text{Cu}^{2+}$  per liter and 100 mg of reducing agent per liter followed a linear relationship with reaction time. The kinetic rate constant for each reducing agent was  $-0.87 \text{ min}^{-1}$  ( $r = 0.93$ ) for ascorbate,  $-0.10 \text{ min}^{-1}$  ( $r = 0.97$ ) for hydrogen peroxide, and  $-0.04 \text{ min}^{-1}$  ( $r = 0.97$ ) for cysteine. The protective effects of metal chelators and catalase, the lack of effect of superoxide dismutase, and the partial protection conferred by free-radical scavengers suggest that the mechanism of copper-mediated HSV inactivation is similar to that reported for copper-mediated DNA damage [59].

Sagripanti and Lightfoote [60] reported that Human Immunodeficiency Virus Type 1 (HIV-1) was inactivated by both cupric or ferric ions when the virus was free in solution and also

3 hr after cell infection. Fifty percent inactivation of cell-free HIV-1 was achieved with  $\text{Cu}^{2+}$  at a concentration between 0.16 and 1.6 mM, or by 1.8 to 18 mM  $\text{Fe}^{3+}$ . Thus, the dose to inactivate 50% of infectious HIV-1 ( $\text{IC}_{50}$ ) by  $\text{Cu}^{2+}$  or  $\text{Fe}^{3+}$  is higher than that reported for glutaraldehyde (0.1 mM), for sodium hypochlorite (1.3 mM), and for sodium hydroxide (11.5 mM). It is however significantly lower than that required for HIV-1 inactivation by ethanol (360 mM). Treatment of infected cells for 30 min at 20°C with 6 mM  $\text{Cu}^{2+}$  or  $\text{Fe}^{3+}$  completely inhibited the formation of syncytia and the synthesis of virus-specific p24 antigen in HIV-infected cells, while still preserving cell viability. We have recently reported the use of copper in free flow filters that deactivate HIV-1 and West Nile Virus. The copper filters reduced the infectious titers of both viruses by 5 to 6 log [19].

Wong *et al* [61] reported a  $10^6$ -fold reduction in bacteriophage R17 infectivity due to RNA degradation in the presence of both ascorbate and  $\text{Cu}^{2+}$ . A study published in 2001 reported on the inactivation of poliovirus and bacteriophage MS-2 in copper pipes containing tap water as a result of a synergistic effect between copper and free chlorine [62]. It was found that the log reduction/hour of the bacteriophage MS-2 in the presence of 400  $\mu\text{g/l}$  of leached copper was 0.385, in 20 mg/l free chlorine 7.605 and with both copper and chlorine, 10.906. This suggests that an oxidizing agent such as chlorine or hydrogen peroxide is necessary to break open the virus protein coat and allow the copper to bind to and denature the nucleic acid.

The International Copper Association, Ltd. investigated the effects of copper on the survival of waterborne viruses [63]. They found that poliovirus was completely inactivated by copper sulfate (20 mg/l) in the presence of hydrogen peroxide (10  $\mu\text{M}$ ), confirming observations of a synergistic effect of copper ions in the presence of oxidizing agents. The effect was reduced by the presence of a protective agent, L-histidine. Other proposed protective agents, disodium hydrogen orthophosphate, bovine serum albumin and catalase, were found to be relatively ineffective. Similarly, they found that copper reduced coxsackie virus types B2 & B4, echovirus

4 and simian rotavirus SA11 infectivity by over 98%. They concluded that there does not appear to be any significant difference between the capacity of copper to inhibit the different types of virus tested. The polio, coxsackie and echo viruses may be expected to behave similarly as they have a similar size and are common members of the enterovirus group. However, the rotaviruses are considerably larger (75 nm diameter as opposed to 28 nm) and belong to the Reovirus group, a completely different family of viruses. They thus suggest that whatever mechanism is removing or inactivating the viruses, it is not dependent on subtle properties associated with the surface of the viruses.

In another study, the efficacy of copper and silver ions, in combination with low levels of free chlorine, was evaluated for the disinfection of hepatitis A virus, human rotavirus, human adenovirus, and poliovirus in water [64]. There was little inactivation of Hepatitis A virus and human rotavirus under all conditions. Poliovirus showed more than a 4 log titer reduction in the presence of copper and silver combined with 0.5 mg of free chlorine per liter or in the presence of 1 mg of free chlorine per liter alone. Human adenovirus remained active longer than poliovirus with the same treatments, although it remained active significantly less than hepatitis A virus and human rotavirus. The addition of 700 µg of copper and 70 µg of silver per liter did not enhance the inactivation rates after exposure to 0.5 or 0.2 mg of free chlorine per liter, although on some occasions it produced a level of inactivation similar to that induced by a higher dose of free chlorine alone. This data indicates that copper and silver ions alone in water systems may not provide a reliable alternative to high levels of free chlorine for the disinfection of viral pathogens.

## **2. Mechanisms of Toxicity of Copper to Microorganisms.**

Metals at high concentration are toxic to microorganisms. Toxicity occurs through the displacement of essential metals from their native binding sites or through ligand interactions. In

general, nonessential metals bind with greater affinity to thiol-containing groups and oxygen sites than do essential metals. Toxicity results from alterations in the conformational structure of nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance. The redox properties that make some metals, such as copper, essential elements of biological systems, may also contribute to their inherent toxicity. For example, redox cycling between  $\text{Cu}^{2+}$  and  $\text{Cu}^{1+}$  can catalyze the production of highly reactive hydroxyl radicals, which can subsequently damage lipids, proteins, DNA and other biomolecules.

## **2.1. Copper Mediated Cell Membrane Damage.**

Copper's initial site of action is considered to be at the plasma membrane [65-67]. It has been shown that exposure of fungi and yeast to elevated copper concentrations can lead to a rapid decline in membrane integrity. This generally manifests itself as leakage of mobile cellular solutes (e.g.,  $\text{K}^+$ ) and cell death. For example, exposure of intact *Saccharomyces cerevisiae* to  $\text{Cu}^{2+}$  (100  $\mu\text{M}$   $\text{CuCl}_2$  in a buffer of low ionic strength) caused a loss of the permeability barrier of the plasma membrane within 2 min at 25°C. The release of amino acids was partial, and the composition of the released amino acids was different from those retained in the cells. Primarily glutamate was released, while arginine was retained in the cells. Cellular  $\text{K}^+$  was released rapidly after the addition of  $\text{CuCl}_2$ , but 30% of the total  $\text{K}^+$  was retained in the cells. These and other observations suggest that  $\text{Cu}^{2+}$  caused selective lesions of the permeability barrier of the plasma membrane but did not affect the permeability of the vacuolar membrane. These selective changes were not induced by other divalent cations tested [66].

Similar effects reported in higher organisms have now been largely attributed to the redox-active nature of copper and the ability of copper to catalyze the generation of free radicals and promote membrane lipid peroxidation [67-70]. For example,  $\text{Cu}^{2+}$  uniquely catalyzed peroxidation of rat erythrocyte membrane lipid in the presence of 10 mM  $\text{H}_2\text{O}_2$ , while several

other transition metal ions had no significant effect [70]. Thus, a copper-oxygen complex may be directly involved in the initiation of lipid peroxidation.

Extensive metal-induced disruption of membrane integrity inevitably leads to loss of cell viability. However, even relatively small alterations in the physical properties of biological membranes can elicit marked changes in the activities of many essential membrane-dependent functions, including transport protein activity [71], phagocytosis [72], and ion permeability [71]. The physical properties of a membrane are largely determined by its lipid composition, and one important factor is the degree of fatty acid unsaturation. Microbial membrane fatty acid composition is highly variable and is influenced by both environmental and intrinsic factors. For example, the unsaturated fatty acid content of microorganisms generally increase at low temperatures [71,73]. In addition, some variation can be attributed to inherent differences in fatty acid composition between microbial groups [74]. The relationship between plasma membrane fatty acid composition and copper toxicity was studied in *S. cerevisiae*, and it was found that copper-induced plasma membrane permeabilization and whole-cell toxicity increased markedly in cells enriched with polyunsaturated fatty acids [75]. In another study [9], the bactericidal potencies of copper towards several bacteria with different cell envelope structures (*Streptococcus lactis*, *E. coli* and *P. aeruginosa*) were found to be similar. However, the authors suggested that the  $\text{Cu}^{2+}$  ion-mediated killing appears to be related to the bacterial plasma membrane. Their conclusion was based upon the observation that the loss of metabolic functions localized to the membranes paralleled cellular death, while oxidation of susceptible biomolecules within the cytosol required considerably more extensive oxidative degradation of the cells.

Many antibacterial and antifungal compounds are more active as copper salts [43,45,76-84]. It has been suggested that some compounds, such as diethyldithiocarbamic acid (DDC), which form chelates with copper, and whose microbicidal effectiveness is enhanced greatly by

small amounts of copper, are cytotoxic by virtue of concentrating in the lipid bilayer and, perhaps, by forming amphipathic complexes which disrupt membrane integrity [84].

## **2.2. Copper Interaction With Nucleic Acids.**

The study of radiation effects on diverse biological processes has led to the finding that microwave radiation at nonthermal levels produces single- and double-strand breaks in purified DNA. Interestingly, however, it was found that this microwave-induced damage to DNA depends on the presence of small amounts of copper. Moreover, it was found that cuprous, but not cupric, ions were able to mimic the effects produced by microwaves on DNA [85]. This finding has led to more detailed investigations into the *in vitro* effects of  $\text{Cu}^{2+}$  on DNA [88]. Today it is clear that  $\text{Cu}^{2+}$  has a specific affinity for DNA and can bind and disorder helical structures by crosslinking within and between strands [86,87]. Copper reversibly denatures DNA in low ionic strength solutions competing with hydrogen bonding present within the DNA molecule [88]. Kinetic studies showed that the DNA double helix contains at least two kinds of binding sites for copper. One site is present every four nucleotides, has high affinity for copper. The other is an intercalating site for copper that is present in every base pair. This site is saturable, has a dissociation constant ( $K_d$ ) for  $\text{Cu}^{2+}$  of 41  $\mu\text{M}$ . In single-stranded DNA (ssDNA, such as found in many DNA viruses), an average copper binding site was found every three nucleotides with lower affinity than in double stranded DNA (dsDNA) [89]. The binding of copper to DNA shows an unexpected high specificity when studied in the presence of other metallic ions. The relative efficacy of several divalent cations to antagonize  $\text{Cu}^{2+}$  binding was:  $\text{Ni} = \text{Cd} = \text{Mg} \gg \text{Zn} = \text{Hg} > \text{Ca} > \text{Pb} \gg \text{Mn}$ , while  $\text{Cr}^{6+}$  enhanced  $\text{Cu}^{2+}$  binding to DNA [89].

Guanine-specific binding of  $\text{Cu}^{2+}$  in dsDNA was demonstrated following crystallization (1.2-Å resolution) of DNA soaked with cupric chloride [90,91]. Covalent  $\text{Cu}^{2+}$  bonded to guanine occurred at the N-7 position. This preferential  $\text{Cu}^{2+}$  binding to guanine bases in dsDNA

may explain the observed specificity of  $\text{Cu}^{2+}$ -induced oxidative DNA damage near guanine residues [92,93]. Hay and Morris [94] proposed that copper may stabilize the helix via a charge transfer complex formed when copper acting as an electron acceptor, intercalates between two adjacent G-C pairs which act as electron donors. Indeed copper has been shown to bind preferentially to G-C pairs [94].

DNA strand breakage is proportional to incubation time, temperature, and  $\text{Cu}^{2+}$  and  $\text{H}_2\text{O}_2$  concentrations [92]. DNA strand breakage is inhibited by metal chelators, catalase, and by high levels of free radical scavengers implying that  $\text{Cu}^{2+}$ ,  $\text{Cu}^{1+}$ ,  $\text{H}_2\text{O}_2$ , and OH radicals are involved in the reaction. This, together with the specificity of copper binding to DNA (see above), imply that nucleic acids degradation mediated by copper involves site-specific Fenton reactions [95-97]. Thus, subsequent to the specific binding of copper to nucleic acids, repeated cyclic redox reactions generate several OH radicals near the binding site causing multiple damage to the nucleic acids [67,98].  $\text{Cu}^{2+}$  may also generate radicals and co-ordinate with other toxic molecules such as adriamycin, hydroquinone, and reduced mitomycin C [99-102]. For example, addition of cupric acetate with adriamycin in the Ames salmonella mutagenicity test increases the mutagenicity of adriamycin by more than 700% [99]. This supports the contention that drug-metal ion-DNA associations might contribute to genotoxicity. Glutathione, a chelating agent, was shown to inhibit free radical formation by copper ions in the presence of hydrogen peroxide, ascorbate and DNA [103]. The protective effect of glutathione was attributed to its ability to stabilize copper in the  $\text{Cu}^{1+}$  oxidation state, preventing redox cycling and generation of free radicals.

It has been shown that the DNA damage, which is the main cause of cell death in *E. coli* cultures treated with  $\text{H}_2\text{O}_2$ , occurs through iron-mediated Fenton reactions [104,105]. However, under conditions of low iron availability, copper ions take part in the genotoxicity of  $\text{H}_2\text{O}_2$  in vivo in *E. coli* [106]. This phenomenon only occurs at high concentrations of  $\text{H}_2\text{O}_2$  (20 mM),

suggesting that copper ions only become available in the intracellular environment in the absence of iron and under severe oxidative stress [106].

DNA damage induced by many carcinogens is dependant on the presence of  $\text{Cu}^{2+}$  but not on the the presence of other metals. The main modifications in the DNA occur at guanine and thymine residues, resulting mainly in the formation of 8-hydroxy-2'-deoxyguanosine and piperidine-labile sites at thymine residues. There is a clear association between these modifications and DNA strand breakage [107].

It has been shown that in the cases of most carcinogens, copper-induced DNA damage occurs through the formation of  $\text{H}_2\text{O}_2$  by the carcinogens [108-113], and that the reactive species generated by the reaction of  $\text{H}_2\text{O}_2$  with  $\text{Cu}^{1+}$  participates in the DNA damage. In some cases the DNA damage is greatly enhanced by the reduction of the oxidized product by the addition of beta-nicotinamide adenine dinucleotide (NADH) [114-117]. These results suggest that an intermediate, derived from the reaction of  $\text{Cu}^{1+}$  with  $\text{H}_2\text{O}_2$ , participates in  $\text{Cu}^{2+}$ -dependent DNA damage, and NADH enhances the DNA damage via a redox cycle. A similar conclusion was reached by studying the mechanism of carcinogenesis caused by methylhydrazines [118]. Methylhydrazines in the presence of  $\text{Cu}^{2+}$  induce DNA cleavage at thymine residues only. The order of  $\text{Cu}^{2+}$ -mediated DNA damage was not correlated with the order of methyl free radical ( $\text{CH}_3$ ) generated during  $\text{Cu}^{2+}$ -catalyzed auto-oxidation. It was therefore suggested that the  $\text{Cu}^{1+}$ -peroxide complex rather than the  $\text{CH}_3$  plays a more important role in methylhydrazine plus  $\text{Cu}^{2+}$ -induced DNA damage. Mutations in DNA after exposure to copper ions were clustered and are predominantly single-base substitutions [119].

Interestingly, alpha-tocopherol (vitamin E), whose biological antioxidant activity is widely recognized, can act as a potent DNA-damaging agent in the presence of  $\text{Cu}^{2+}$  ions. It promotes the formation of copper-dependent reactive oxygen species (e.g. superoxide) from molecular oxygen, which results in DNA base oxidation and backbone cleavage. Electron spin resonance

spin-trapping investigations demonstrated that hydrogen peroxide interacts with  $\text{Cu}^{1+}$  to generate the reactive species responsible for DNA damage, which is either the hydroxyl radical or a species of similar reactivity [120].

An important host defense mechanism for dealing with invading bacteria involves the production of reactive oxygen species, such as superoxide, hydrogen peroxide and hypochlorous acid, by phagocytic cells. Several antibacterial antibiotics have been shown to participate with transition metal ions in chemical reactions leading to the formation of reactive oxygen species. This, together with the capacity of copper to induce DNA damage, was the rationale for adding copper salt to four structurally different antibacterial antibiotics (beta-lactam, tetracycline, bacitracin and rifamycin), leading to their enhanced DNA damaging activity and antibacterial potency [121].

Epinephrine, norepinephrine or dopamine in the presence of non-lethal concentrations of  $\text{Cu}^{2+}$  kill washed or growing *E. coli* cells. This effect is enhanced by anoxia and by sublethal concentrations of  $\text{H}_2\text{O}_2$ . The rate of killing is proportional to the rate of catecholamine oxidation. The copper epinephrine complex binds to *E. coli* cells, induces membrane damage and depletes the cellular ATP pool. The cells may be partially protected by superoxide dismutases (SOD) or catalase but not by OH radical scavengers. Addition of  $\text{H}_2\text{O}_2$  to cells, which were sensitized by preincubation with the epinephrine-copper complex, causes rapid killing and DNA degradation [122].

#### **2.4. Copper Mediated Protein Damage.**

Copper can alter proteins and inhibit their biological activities. For example, copper was the most potent metal inhibiting the protein tyrosine phosphatases VHR [123], a phosphatase that in conjunction with protein tyrosine kinases regulate cell growth and differentiation. Among the various metal ions ( $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cd}^{2+}$ ) examined for their inactivation effect on VHR,

$\text{Cu}^{2+}$  was found to be the most potent inactivator. The efficacy of  $\text{Cu}^{2+}$  as an VHR inactivator was about 200-fold greater than that of  $\text{H}_2\text{O}_2$ .  $\text{Cu}^{2+}$  also inactivated other protein tyrosine phosphatases including PTP1B and SHP-1. The  $\text{Cu}^{2+}$ -mediated inactivation was a consequence of the oxidation of a cysteine residue in the active site [123].

The reaction of a histidine-containing peptide (angiotensin I) with  $\text{Cu}^{2+}$ /ascorbate under physiological conditions has been studied chemically [124]. In the presence of a catalytic amount of  $\text{Cu}^{2+}$ , ascorbate mediated the oxidative damage to the peptide via selective loss of the histidine residue. It was shown that the reaction of  $\text{Cu}^{2+}$ /ascorbate occurs specifically at the C-2 position of the imidazole ring of the histidine residue within a peptide. In addition, the capacity of copper to intermediate free radical attack of proteins was investigated using proteins biosynthetically labeled with radioactive proline or histidine as targets [125]. It was found that protein-bound histidine was substantially converted into aspartate, and while much of the proline was modified during radical attack, it was not converted into glutamate. Thus, histidine and proline are important sites of protein attack mediated by copper radical formation. Such protein modification may result in cleavage. For example, a facile cleavage of peptide bonds of apolipoprotein B (apoB) by radical reaction was found when human LDL was subjected to oxidative damage using  $\text{Cu}^{2+}$  [126]. When human serum was treated with  $\text{Cu}^{2+}$ , a similar cleavage pattern of apoB was also observed [126]. Similar selective attack at particular residues due to oxidative damage using  $\text{Cu}^{2+}$  was shown with BSA, histones, cytochrome C, lysozyme and protamine [127].

It is suggested by a study on the biochemistry of copper [128], that the biocidal effects of copper are achieved mainly by the interaction of  $\text{Cu}^{2+}$  with SH- moieties present in the cell membrane, and within cells. This interaction results in the formation of thiol compounds and  $\text{Cu}^{1+}$  ions. The following redox reaction



glutathione

was suggested to occur when an excess of  $\text{Cu}^{2+}$  ions interact with living cells, leading to inactivation of 3 SH-groups per copper ion. This assumption was based on an equilibrium analysis of the system  $\text{Cu}^{1+}$  - glutathione and  $\text{Cu}^{1+}$  - pencicillamine system, in which they found that reduced glutathione, which is present in all cells and cell membranes in concentrations from 1 to 6 mM, decreased markedly in the presence of copper.

## **2.5. Mechanisms of copper antiviral, antifungal and anti-algae activities.**

All of the above copper mediated toxic mechanisms, elucidated mainly in studies with bacteria, are relevant to viruses as well. For example, viral inactivation via RNA damage, through the Fenton mechanism, was proposed by Carubelli and colleagues [129]. They found that incubation of the RNA phage Q beta at 37°C with a mixture of 100 mM ribose and 10  $\mu\text{M}$   $\text{CuSO}_4$  resulted in a complete loss of viable phage after 20 min. This cytotoxic effect required both ribose and cupric ions. There was a direct correlation between the decrease in the percentage of phage survival and: (a) the length of incubation, and (b) the concentrations of both ribose and  $\text{CuSO}_4$ . Addition of the strong chelator diethylenetriaminepentaacetic acid eliminated the cytotoxic effect. These results are consistent with an initial production of superoxide free radicals by transition metal catalyzed autoxidation of ribose and Amadori products, followed by dismutation of superoxide to hydrogen peroxide and generation of lethal hydroxyl radicals by the Fenton reaction. RNA isolated from phage incubated with ribose and  $\text{CuSO}_4$  retained its infectivity, suggesting that the cytotoxic effect may be mediated by a free radical attack on proteinaceous components of the phage through a site specific generation of hydroxyl radicals on protein-bound transition metal ions.

HIV-1 protease, an essential protein for the replication of the virus, was found to be inhibited by approximately stoichiometric concentrations of copper ions. Inactivation by  $\text{Cu}^{2+}$  is rapid and is not reversed by subsequent exposure to EDTA or dithiothreitol. The addition of

copper to the protease at pH 5.5 induces aggregation of the protein, providing a possible basis for the inhibitory action of copper. Direct inhibition by  $\text{Cu}^{2+}$  required the presence of cysteine residue(s) in the protease. Oxygen is not required for inactivation [130,131].

In contrast to the different mechanisms of resistance to copper and other heavy metals found in bacteria, fungi and other microorganisms (see Section 3 below), viruses do not possess resistance or repair mechanisms, making them highly susceptible to high concentrations of copper ions.

The mechanism of copper antifungal and anti-algae activity has not been well studied. It has been suggested that the copper ions form electrostatic bonds with negatively charged areas on the microorganism's cell walls. These electrostatic bonds create stresses which lead to distorted cell wall permeability, reducing the normal intake of life sustaining nutrients. Once inside an algae cell, copper may attack sulfur containing amino acid residues in the proteins used for photosynthesis. As a result, photosynthesis is blocked and lead to cell lysis and death [132].

### **3. Microbial Resistance to Copper**

#### **3.1. General**

Many microorganisms demonstrate resistance to metals in water, soil and industrial waste. Metal resistance systems are present in nearly all bacterial types and may have developed shortly after prokaryotic life started. Several factors determine the extent of metal resistance in a microorganism. These include the type and number of mechanisms for metal uptake, the role each metal plays in normal metabolism, and the presence of genes located on plasmids, chromosomes, or transposons that control metal resistance [133-140]. Microorganisms may be resistant to one metal and not to another. For example, clinical staphylococcal isolates that demonstrate reduced susceptibility to lead and potassium, are highly sensitive to silver and copper [141].

The mechanisms of tolerance include: exclusion by permeability barrier, intra- and extra-cellular sequestration by cell envelopes, active transport membrane efflux pumps, enzymatic detoxification, and reduction in the sensitivity of cellular targets to metal ions. Genes responsible for these processes may be encoded by the chromosome or by plasmids. Since some toxic metals are also essential micronutrients (i.e. copper, cobalt, zinc, nickel), bacteria must precisely adjust the uptake and efflux systems to maintain their adequate intracellular levels. In the case of metals with no biological function (i.e. cadmium, silver), transport systems must be oriented only to the extrusion of the toxic ions. In the last few years, several bacterial systems dedicated to the efflux of toxic metals were analyzed at the molecular level resulting in a detailed understanding of the mechanisms. Among these are the membrane pathways that extrude copper, cadmium, zinc, nickel, cobalt and silver cations. Two general mechanisms have been found: those involving P-type ATPases, and those using proton antiporter systems [135,140].

Natural resistance may take the form of mutations in cellular components that prevent interaction with metals or alterations in cell membrane composition. Microorganisms can possess one or a combination of several resistance mechanisms. There are six known resistance mechanisms to heavy metals in bacteria (see below). Each mechanism is discussed, with emphasis on resistance to copper, and examples are provided of microorganisms that display the characteristics of each resistance mechanism.

### **3.2. Metal Exclusion by Permeability Barrier.**

Alterations in the cell wall, membrane, or envelope of a microorganism are examples of metal exclusion by a permeability barrier. This mechanism is an attempt by the organism to protect metal-sensitive, essential cellular components. A prominent example is the exclusion of  $\text{Cu}^{2+}$  resulting from altered production of the membrane channel protein porin by *E. coli* B [142]. This is usually a result of a single mutation in the gene that decreases the permeability of the

membrane to metal ions. Another example is the nonspecific binding of metals by the outer membrane or envelope. This offers limited metal protection due to the possibility of saturation of binding sites [143,144]. Bacteria that naturally form an extracellular polysaccharide coating demonstrate the ability to bioabsorb metal ions and prevent them from interacting with vital cellular components [145]. The exopolysaccharide coating of these bacteria may provide sites for the attachment of metal cations. Several strains of bacteria demonstrate the ability to bind metals extracellularly. These include *Klebsiella aerogenes*, *Pseudomonas putida*, and *Arthrobacter viscosus*. A protective layer of exopolysaccharide, for example, improves the survivability of *Klebsiella aerogenes* strains in  $\text{Cd}^{2+}$  solutions. These strains demonstrate twice the  $\text{Cd}^{2+}$  levels as strains without the protective layer [145].

Although not fully proven, it is believed that some forms of copper resistance are based on periplasmic binding [146,147]. Periplasmic binding of  $\text{Cu}^{2+}$  is found in *Pseudomonas* species where resistance is coded for in a plasmid by an operon of four genes: *copA*, *copB*, *copC*, and *copD* [148-150]. *CopA* and *copB* confer partial resistance, while the addition of *copC* and *copD* provides for full  $\text{Cu}^{2+}$  resistance [146]. *copA* and *copC* proteins are located between the inner and outer membrane, *copB* is found in the outer membrane and *copD* in the inner membrane. *CopC* can exchange copper between two sites activated by a redox switch. *CopA* modulates the redox state of copper [149]. In addition, periplasmic proteins (e.g. PcoC) have been thought to bind copper ions and to increase the level of resistance to copper ions above that conferred by *cop* gene products [151-153].

### **3.3. Active Transport of the Metal Away From the Microorganism.**

Active transport or efflux systems represent the largest category of metal resistance systems. Microorganisms use active transport mechanisms to export toxic metals from their cytoplasm. These mechanisms can be chromosomal or plasmid-encoded. Non-essential metals

normally enter the cell through normal nutrient transport systems but are rapidly exported. These efflux systems can be non-ATPase or ATPase-linked and highly specific for the cation or anion they export.

Active transport of the essential metal ion  $\text{Cu}^{2+}$  away from the bacteria is achieved through an ATPase efflux mechanism. The *cop* operon has been found in the Gram-positive bacteria *Enterococcus hirae* and contains four genes: *copA*, *copB*, *copZ*, *copY*. The operon has nomenclature similar to that of the *cop* sequestration operon found in *Pseudomonas*. *CopA* is responsible for encoding a  $\text{Cu}^{2+}$  uptake ATPase and *copB* encodes a P-type efflux ATPase. The gene products of *copY* and *copZ* regulate the *cop* operon. CopY is believed to be a repressor protein that inactivates the operon in the absence of  $\text{Cu}^{2+}$ . When  $\text{Cu}^{2+}$  is present it may bind to copY, converting it to a DNA-binding repressor. Copper also binds to *copZ* at higher levels and together forms a *copY*-Cu complex that ceases repression. This allows *copA* and *copB* to be transcribed [154-157].

Recently, data has accumulated showing that *E. coli* encodes four proteins (CusC/F/B/A) that mediate resistance to copper and silver by cation efflux. All four proteins form a tetrapartite resistance system, which involves a novel periplasmic copper-binding protein CusF that directly transports  $\text{Cu}^{1+}$  from the periplasm across the outer membrane [158,159]. Data supporting a copper efflux system in *S. enterica serovar Typhimurium* has also been reported [160].

### **3.4. Intracellular Sequestration of Metals by Protein Binding.**

Intracellular sequestration is the accumulation of metals within the cytoplasm to prevent exposure to essential cellular components. Metals commonly sequestered are  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Zn}^{2+}$ . Two examples exist for this form of metal resistance: metallothionein production in *Synechococcus* sp. and cysteine-rich proteins in *Pseudomonas* species [142,157]. The metal resistance system in *Synechococcus* species consists of two genes; *smtA* and *smtB*. The gene

*smtA* encodes a metallothionein that binds the metals. This gene is induced by high levels of  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Cu}^{2+}$  and is repressed by the gene product of *smtB*. Cysteine residues in SmtA metallothionein may act as a sink for excess toxic cations. Another organism that demonstrates intracellular  $\text{Cd}^{2+}$  sequestration is a strain of *Pseudomonas putida* that was isolated from sewage. This organism produces three low-molecular-weight cysteine-rich proteins, which may be related to metallothioneins.

*Mycobacterium scrofulaceum* also demonstrates intracellular accumulation of  $\text{Cu}^{2+}$  by sequestering it in the form of a black copper sulfate precipitate [161].

### **3.5. Extracellular Sequestration.**

Metal resistance based on extracellular sequestration has been found in several species of yeast and fungi and has been hypothesized for bacteria [162]. One form of  $\text{Ni}^{2+}$  resistance in yeast may be based on this mechanism. *S. cerevisiae* may reduce absorption of  $\text{Ni}^{2+}$  by excreting large amounts of glutathione. Glutathione binds with great affinity to heavy metals. Yeast carrying the methylglyoxal resistance gene demonstrates the ability to form extracellular metal-glutathione complexes in metal-rich media [163]. Resistance results when the toxic metal is bound in a complex and cannot traverse the cell membrane. Yeast forms complexes during hydrogen sulfide production and *Citrobacter* uses phosphate. Other organisms such as yeast or *Citrobacter* species form insoluble complexes of cadmium phosphate to confer cadmium resistance [164]. A similar mechanism exists in  $\text{Cu}^{2+}$ -resistant fungi [140]. These fungi secrete oxalate to form a metal-oxalate complex.

Interestingly, it has been shown that *P. aeruginosa* biofilms were anywhere from 2 to 600 times more resistant to heavy metal stress than free-swimming cells. The exterior of the biofilm was preferentially killed after exposure to elevated concentrations of copper, while the majority of cells that remained alive were in the substratum. A possible explanation for this phenomenon

is that the extracellular polymeric substances that encase a biofilm may be responsible for protecting cells from heavy metal stress by binding the heavy metals and retarding their diffusion within the biofilm [165].

### **3.6. Enzymatic Detoxification of a Metal to a Less Toxic Form.**

There are several examples of enzymatic detoxification of metals to less toxic forms. Resistance to mercury may be achieved by enzymatic detoxification in both gram-positive and gram-negative bacteria. A set of genes encodes for the production of a periplasmic binding protein and membrane-associated transport proteins. The periplasmic binding protein collects  $\text{Hg}^{2+}$  from the surrounding environment and transport proteins take it to the cytoplasm where it is neutralized by redox chemistry [140]. Recently, a Gram-negative bacterium, a pseudomonad very similar to *Pseudomonas synxantha* (CRB5), was isolated from a chromium-contaminated site. It was found to reduce toxic hexavalent chromium [ $\text{Cr}^{6+}$ ] to an insoluble  $\text{Cr}^{3+}$  precipitate under aerobic and anaerobic conditions. CRB5 tolerated up to 520 mg of  $\text{Cr}^{6+}$  per liter and reduced chromate in the presence of copper and arsenate. Under anaerobic conditions it also reduced  $\text{Co}^{3+}$  and  $\text{U}^{6+}$ , partially internalizing each metal. Metal precipitates were also found on the surface of the outer membrane. Chromate reduction by CRB5 is mediated by a soluble enzyme that was largely contained in the cytoplasm but also found outside of the cells. Membrane-associated  $\text{Cr}^{6+}$  reduction under anaerobiosis may account for anaerobic reduction of chromate under nongrowth conditions with an organic electron donor present [166,167]. Metal ion-specific reducing enzyme systems function in the cell surface layer of microorganisms. These enzymes require NADH or NADPH as an electron donor and flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), as an electron carrier component. Electron transport may be operated by transplasma membrane redox systems. Metal ion reductases are also found in the cytoplasm [168].

### **3.7. Decrease in Metal Sensitivity of Cellular Targets.**

Some microorganisms adapt to the presence of toxic metals by altering the sensitivity of essential cellular components. Protection is achieved by mutations that decrease sensitivity but do not alter basic function or by increasing production of a particular cellular component to keep ahead of metal inactivation. DNA repair mechanisms also provide limited protection to plasmid and genomic DNA. The microorganism may also protect itself by producing metal-resistant components or alternate pathways in an effort to bypass sensitive components.

Adaptation has been found in *E. coli*. Upon exposure to  $\text{Cd}^{2+}$ , unadapted *E. coli* demonstrate considerable DNA damage; however, after subculture, the same organisms show resistance. The growth lag phase of the organism shortens the longer it is exposed to  $\text{Cd}^{2+}$ . It is postulated that the extended lag phase is initially due to a period of induction of DNA repair mechanisms.

Natural resistance can result from normal cellular functions that give the organism a base level of tolerance [142]. An example is glutathione, which may offer protection to metal ions like  $\text{Cu}^{1+,2+}$ ,  $\text{Ag}^{1+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Hg}^{2+}$  [169]. Glutathione may offer protection from  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$  by suppressing free radical formation [142].

There appear to be differences in the ability of gram-negative and gram-positive bacteria to tolerate certain metal ions. Gram-negative bacteria (*E. coli* and *Pseudomonas* species) are better able to carry on protein synthesis in the presence of  $\text{Cd}^{2+}$  than gram-positive bacteria. A species of *Pseudomonas* could tolerate 5 to 30 times more  $\text{Cd}^{2+}$  in growth media before protein synthesis was reduced 50% compared to gram-positive *S. aureus*, *Staphylococcus faecium*, and *Bacillus subtilis* [170]. On the other hand, gram-positive organisms are able to bind 28 to 30 times more  $\text{Cu}^{2+}$  when compared with *E. coli* [140].

Copper tolerance in fungi has also been ascribed to diverse mechanisms involving trapping of the metal by cell-wall components, altered uptake of copper, extracellular chelation or precipitation by secreted metabolites, and intracellular complexing by metallothioneins and phytochelatins. Only the metallothionein chelation mechanism has been studied in molecular detail [65].

#### **4. Summary**

In contrast to the low sensitivity of human tissue to copper [3-6], microorganisms are extremely susceptible to copper. Copper toxicity to microorganisms may occur through the displacement of essential metals from their native binding sites, from interference with oxidative phosphorylation and osmotic balance, and from alterations in the conformational structure of nucleic acids, membranes and proteins. In most microorganisms, but not in viruses, there is an integrated set of proteins that delivers copper to specific subcellular compartments and copper-containing proteins without releasing free copper ions. Although some organisms have mechanisms of resistance to excess copper, generally, exposure of most microorganisms to high concentrations of this trace element results in damage to cellular components. Viruses lack DNA repair mechanisms, permeability barriers, intra- and extra-cellular sequestration of metals by cell envelopes, active metal transport membrane efflux pumps, and enzymatic metal detoxification mechanisms, such as those found in bacteria and cells. The reduced capabilities of viruses may thus explain their high vulnerability to copper.

Copper is used today as a biocide [15,16]. However, based on the above, further applications of copper in improving health are possible. One example may be the reduction of nosocomial infections in hospitals by the use of self-sterilizing copper-fabrics in pajamas, sheets, pillow covers, and robes. Similarly, the use of copper containing gloves with anti-bacterial and anti-viral properties may also aid in reducing nosocomial infections while providing increased

protection to hospital personnel. As discussed in Sections 1.1 and 1.3, other possible uses of copper fabrics are related to the reduction of foot ulcerations, *tinea pedis* infections and dust-mite related allergies.

Another important potential application of copper-impregnated materials is related to the reduction of bacterial and viral transmission during transfusion of blood or blood related products. The safety of whole blood and its components is a continuing global problem [171,172]. A growing number of viral, bacterial, and protozoa pathogens have been identified in blood products and new pathogens are regularly identified as being present. The capacity of HIV-1 to be transported in whole blood by platelets and red blood cells has been demonstrated [173,174]. In areas of the world where screening tests are too expensive to be performed regularly, a cheap, rapid virus inactivation filter would be extremely helpful. Even in the US, hospitals can no longer afford to pay for expensive tests for each ‘pathogen du jour’ [175]. Accordingly, a filter that can inactivate a broad spectrum of viruses in blood products would be very valuable. Preliminary results showing the neutralization of HIV-1 and the infectivity of other viruses when passed through copper-containing syringes indicate the possibility of producing a generic anti-viral filter [19]. However, it must first be established that these filters do not damage filtered plasma and other blood components and that they do not harm individuals infused with these blood products. We are currently carrying out these studies.

Yet another application of such generic antiviral filters are related to the reduction of HIV-1 transmission through breast-feeding, which accounts for one third to one half of all HIV mother-infant transmissions [176]. Breast milk may be passed through a copper fiber-containing filter reducing HIV infectivity. If there is no degradation to the milk’s essential nutrients as a result of filtration, the filtered milk may be fed to infants thereby reducing the risk of HIV transmission.

In conclusion, there are many potential uses of copper in new applications that address medical concerns of the greatest importance. Implementation of even a few of these possible applications may have a major effect on our lives.

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