Virus Inactivation by Copper or Iron Ions Alone and in the Presence of Peroxide

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Cupric and ferric ions were able to inactivate five enveloped or nonenveloped, single- or double-stranded DNA or RNA viruses. The virucidal effect of these metals was enhanced by the addition of peroxide, particularly for copper(II). Under the conditions of our test, mixtures of copper(II) ions and peroxide were more efficient than glutaraldehyde in inactivating φX174, T7, φ6, Junin, and herpes simplex viruses. The substances described here should be able to inactivate most, if not all, viruses that have been found contaminating medical devices.

It has been recently reported that copper(II) and iron(III) inactivate microorganisms with an efficiency intermediate between that of glutaraldehyde and those of other substances recommended for liquid disinfection in the health-care industry (10). In that study, peroxide potentiated the microbicidal effect of copper(II) and iron(III), in a concentration-dependent fashion. The virucidal properties of these compounds have been demonstrated on one virus, Junin virus (JV) (10), an RNA-containing arenavirus pathogenic to humans (9).

Before copper(II) and iron(III) are utilized in formulations for liquid disinfection and sterilization of material potentially contaminated with the variety of viruses found in the clinical setting, the generality of the virucidal activity of metal-based formulations must be established.

Since testing every known human pathogenic virus for sensitivity to a given virucidal agent is not feasible, the use instead of an array of viruses representing different chemical compositions has been proposed (6). Four viruses, bacteriophages φX174, T7, and φ6 plus herpes simplex virus (HSV), have been chosen as surrogates for common human pathogenic viruses in the evaluation of the virucidal effects of different photodynamic treatments (6, 8). These viruses have been chosen because their different compositions include single- or double-stranded nucleic acid (DNA or RNA) and because some do and some do not have lipid envelopes. To establish the generality of the virucidal properties exhibited by copper(II)- and iron(III)-based formulations, the sensitivities of different surrogate viruses were investigated.

HSV type 1, MP strain, was grown and plaque assayed on CV-1 African green monkey kidney fibroblasts. Virus growth and assay and cell culturing conditions have been described previously (5). The growth of JV on Vero cells and the infectivity assay were performed as previously described (1). JV and HSV were tested individually at initial concentrations of about 10^6 and 2×10^6 PFU/ml, respectively, in Eagles's minimal essential medium (EMEM) containing 5% fetal calf serum (FCS).

The bacteriophages and their host cells were φX174 with *Escherichia coli* C, T7 (am28) with *E. coli* O11, and φ6 with *Pseudomonas syringae* subsp. *phaseolicola*. Virus growth and assay conditions have been described previously (6).

Because of exclusive host cell specificity, each virus in a mixture of all three bacteriophages could be independently assayed (7), at similar initial concentrations of approximately 3×10^7 PFU/ml. The viruses were diluted into the medium (EMEM plus 5% FCS) at 24°C immediately before each experiment. Cupric chloride (CuCl$_2$ · 2H$_2$O) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Ferric chloride (FeCl$_3$ · 6H$_2$O) and hydrogen peroxide (29.0 to 32.0% [wt/vol], analytical reagent grade with <0.0001% heavy metal content) were obtained from Mallinckrodt, Inc. (Paris, Ky.). Glutaraldehyde (8%) in sealed glass ampules was purchased from Ladd Research Industries (Burlington, Vt.). For stock solutions, metal salts, in amounts necessary to yield 1 g of either Cu(II) or Fe(III) per liter of solution, were dissolved in 1 ml of sterile distilled water and subsequently sterilized by passage through 0.22-μm-pore-size membrane filters (Millex GV; Millipore, Bedford, Mass.). All inactivating agents were serially diluted 1:10 in sterile distilled water at a neutral pH, and experiments were performed within 3 h thereafter. New stock solutions were prepared before each experiment.

Inactivation by metal alone. The virucidal activities of selected concentrations of copper [Cu(II)] or iron [Fe(III)] alone were determined for the different viruses. Virus was diluted 1:4 at 24°C in sterile conical 1.5-ml Eppendorf tubes containing phosphate-buffered saline (PBS), pH 7.4, to a final volume of 20 μl, then 8 μl of the inactivating agent was added at various concentrations. After 30 min of incubation at 24°C the reaction mixture was diluted to 1 ml with ice-cold EMEM–5% FCS and put on ice. The surviving virus in each sample was titrated immediately.

At least 99% inactivation of all five viruses was obtained at the 1,000-mg/liter level of either metal. The concentration needed to inactivate 90% of the virus in 30 min (D$_{10}$) is presented in Table 1. With copper(II), the D$_{10}$s were smaller for the RNA-containing viruses, φ6 and JV. The D$_{10}$ for HSV was intermediate between those values and the D$_{10}$s for the smaller DNA-containing viruses, φX174 and T7. In addition, the enveloped viruses (φ6, JV, and HSV) were more sensitive to copper(II) inactivation than the nonenveloped ones (φX174 and T7). These differences suggest that the presence of RNA or lipid may render the virus particle more sensitive to inactivation by copper(II). With iron(III), the smaller viruses were roughly equally sensitive, with the

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TABLE 1. Inactivation parameters of viruses exposed to copper, iron, peroxide, and glutaraldehyde

Virus	Genome type and mol wt $(10^6)^a$	Presence of envelope	D_{10} (mg/liter)		T_{10} (min)		
			Cu(II)	Fe(III)	Cu(II)-Px^b	Fe(III)-Px^c	Glutaraldehyde (10 mg/liter)^d
φX174	ssDNA (1.7)	No	10^2–10^3	10^2–10^3	7	>60	>60
T7	dsDNA (24)	No	10^2–10^3	10^2–10^3	<10	<10	59
HSV	dsDNA (96)	Yes	10^2	10^1–10^2	8	>60	53
φ6	dsRNA (9.3)	Yes	10^1–10^2	10^2	22	24	>60
JV	ssRNA (4.4)	Yes	10^1–10^2	10^2–10^3	33	>60	14

^{a} ss and ds, single stranded and double stranded, respectively.
^{b} Cu(II) (1 mg/liter) (as cupric chloride) plus H_2O_2 (Px) (100 mg/liter).
^{c} Fe(III) (30 mg/liter) (as ferric chloride) plus H_2O_2 (Px) (100 mg/liter).
^{d} Glutaraldehyde at 1 mg/liter showed little or no activity.

largest virus, HSV, being most sensitive (having a smaller D_{10}).

Inactivation by copper(II) plus peroxide. Copper(II) alone at a concentration of 1 mg/liter did not inactivate HSV or φX174 and produced only a low level of inactivation for the remaining viruses within 60 min. However, the addition to Cu(II) (1 mg/liter) of peroxide at 100 mg/liter, which also produces little virus inactivation by itself, resulted in an effective virucidal mixture. The kinetics of inactivation of the viruses by the mixture of copper(II) and peroxide was determined in 1.5-ml plastic tubes by adding 40 μl of virus (in EMEM–5% FCS) to 120 μl of sterile distilled water. The metal ion was added (in a total volume of 32 μl) at the appropriate concentration and was followed by hydrogen peroxide or water (32 μl). Immediately thereafter, a 28-μl aliquot from the mixture was withdrawn. This was considered the zero-time sample; the actual contact time of virus with metal was approximately 2 min before dilution in cold medium. At five additional preestablished times, 28-μl aliquots were withdrawn from each reaction tube. To stop any reaction, each aliquot was immediately diluted to 1 ml with ice-cold medium and kept in ice until the last time point was reached. Dilution and chilling of the samples in this fashion stopped the reactions as measured by virus survival under these conditions. Surviving virus was then titrated. Parallel untreated controls showed no loss of infectivity in this protocol. The data depicted in Fig. 1 clearly demonstrate that all viruses were sensitive to this copper-peroxide mixture, while most were not substantially inactivated by the individual compounds at those concentrations.

As a measure of resistance to inactivation by the copper-peroxide mixture, the time needed to inactivate 90% of the virus (T_{10}) (Table 1) was calculated for each virus by interpolation from data in Fig. 1. Addition of peroxide (100 mg/liter) to a low concentration of copper(II) (1 mg/liter) increased virus inactivation manyfold for each virus. Inactivation of JV was the slowest among the viruses tested, with a T_{10} of 33 min.

Whereas the DNA-containing viruses were less sensitive than the RNA-containing ones to copper(II) alone, the DNA-containing viruses were more sensitive to the copper-peroxide mixture. The DNA-containing viruses, φX174, T7, and HSV, were nearly equally sensitive (T_{10}s between 6 and 8 min), although their diameters differ by about 5-fold and the sizes of their genomes differ by over 50-fold. The reason for this apparent inconsistency between suspected target size and the observed response is unknown.

Inactivation by iron(III) plus peroxide. Inactivation of the viruses by a mixture of iron(III) (30 mg/liter) and peroxide (100 mg/liter) was analyzed by methodology similar to that

described for Cu(II) and peroxide, and the respective T_{10}s are shown in Table 1. The data indicate that iron(III) alone had some virucidal effect at that concentration but the addition of peroxide was not substantially synergistic for inactivating φX174 or HSV. Synergistic inactivation did occur with T7 and φ6, indicating that each virus is approximately equally sensitive to iron(III)-peroxide (30 mg/liter: 100 mg/liter) and to copper(II)-peroxide (1 mg/liter:100 mg/liter) (compare T_{10}s in Table 1). In every case, the viruses were more resistant to iron-peroxide than to copper-peroxide on a metal concentration basis. Unlike the case with copper(II), however, addition of peroxide (100 mg/liter) to iron(III) (30 mg/liter) substantially increased virus inactivation for only two viruses, φ6, which contains RNA and is
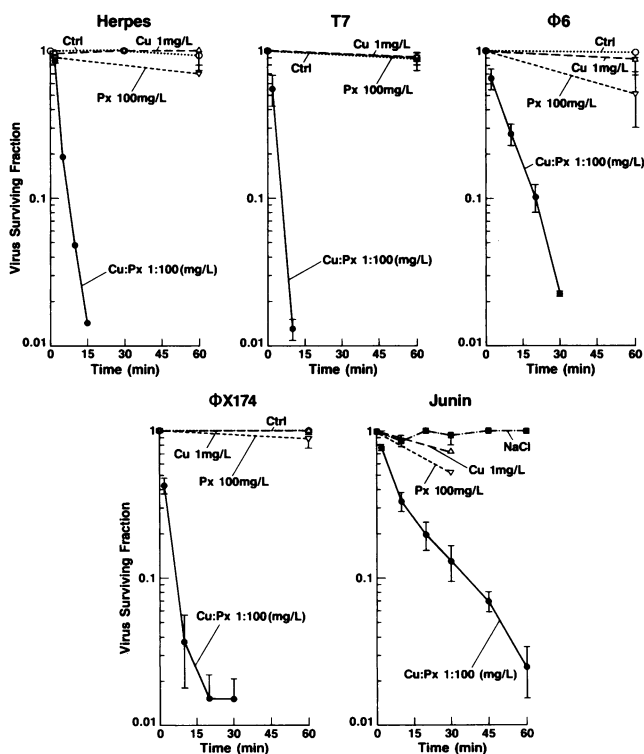

FIG. 1. Inactivation of different viruses at 24°C by Cu(II) (1 mg/liter) hydrogen peroxide (Px) (100 mg/liter) or the combination of both substances at those concentrations. The fraction of virus surviving treatment is plotted as a function of reaction time. The survival of untreated virus is included as a control (Ctrl).

enveloped, and T7, which contains DNA and is nonenveloped. The specific virus properties which govern sensitivity to iron(III) and to iron(III) plus peroxide remain unclear.

Inactivation by glutaraldehyde. Glutaraldehyde, a widely used liquid disinfectant (2–4), showed little or no virucidal activity at a concentration of 1 mg/liter (data not shown). Even at 10 mg/liter, glutaraldehyde inactivated four of five viruses with lower efficiency than the copper-peroxide mixture, as indicated by a comparison of the $T_{10}s$ (Table 1).

Significance. The results presented in this work demonstrate that five viruses of different biochemical and structural compositions may be inactivated by copper(II) or iron(III) alone. Peroxide synergistically increased the virucidal activity of copper(II) ions and to a lesser extent iron(III) ions. The synergism of peroxide with copper(II) extended to all viruses tested. For disinfection, a copper-peroxide mixture should therefore be preferable to an iron-peroxide mixture. Furthermore, comparative experiments (Table 1) demonstrated that a mixture of copper(II) (1 mg/liter) with peroxide (100 mg/liter) had 0.4 to 14 times the virucidal efficiency of glutaraldehyde at a concentration of 10 mg/liter for the viruses tested here. Thus, a formulation comprising 0.05% copper and 5% peroxide should have a virucidal efficacy comparable to that of glutaraldehyde at 2%, the concentration (used for 2 to 20 min) currently recommended for disinfection of a wide variety of medical devices (11, 12). The data presented here indicate that metal-based formulations, particularly copper-peroxide mixtures, may be useful virucidal agents.

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