Relationship between Residual Metal Ions in a Solution and the Inhibitory Capability of the Metal Ions for Pathogenic Bacterial Growth

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The inhibitory capability of various low concentrations of six kinds of metal ions [silver(I), copper(II), cobalt(II), nickel(II), zinc(II), and dichromate] for pathogenic bacterial (gram-positive bacteria *Staphylococcus aureus* and MRSA, gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*) growth was quantitatively determined exactly. Residual metal-ion concentrations in a phosphate buffer solution after being incubated with pathogenic bacteria were then measured by an atomic-absorption spectrophotometer. We found that the inhibitory capability of metal ions correlated with the residual metal concentrations. Based on the biochemical and chemical situation, the mechanisms of the inhibitory capability of the metal ions are discussed. In addition, the determined minimum inhibitory concentration (MIC) values of metal ions on tested bacteria are considered.

We have recently published the idea that electroplated coatings possess an inhibitory capability on the growth of pathogenic bacteria.[1,2] The effect of metal ions dissolved from the surface of a coating on their inhibition to bacterial growth as one of the mechanisms of these antibacterial activity coatings has been suggested. Therefore, it is necessary that relationships between the inhibitory capability of metal ions and the metal-ion concentrations, and the minimum inhibitory concentration (MIC) values of metal ions on bacteria be investigated.

Trace amounts of metal ions are primarily of interest with respect to their bactericidal properties in all microbiological fields.[3,4] The inhibitory capability of small quantities of metal ions on the growth of bacteria has been recognized since the nineteenth century.[5] The bactericidal capability of metal ions has been used to disinfect fluids, solids, and tissues.[6—9] Silver and silver(I) salts have been widely employed for centuries. Copper(II) has been known almost as long as silver(I). Copper and silver have usually been used in disinfecting water as containers. Numerous papers and review articles have been written on the bactericidal effects of copper(II) and silver(I).[10—12] Cobalt(II) complex compounds have been known for over a hundred years for the curare-like activity of some metal-amines in mammal's.[6] Zinc(II) and its organic or inorganic derivatives have also shown a wide spectrum of antibacterial and antifungal activities.[13] In recent years, the inhibitory capability of metal ions has been utilized worldwide for topical application.[14—16] Although they have been in use for many years, recently their anti-microbial activities have been systematically investigated.

On the other hand, trace amounts of some suitable metal ions are also used as nutrients to be adsorbed by microorganisms for forming new cellular walls and prompting cell growth.[17—19] The adsorbed metal ions are considered to be bound by the outer membrane.[20] Namely, as a mediate electrostatic phenomenon, the soluble metal ions can combine with fixed anionic carboxyl or phosphoryl groups in the structural polymer membrane of the bacteria. These cellular structures tenaciously bind metals and metal complexes, forming known natural compounds.[21,22]

We discuss here that the inhibitory capability of the metal ions for pathogenic bacterial growth is quantitatively determined exactly. Also, the mechanisms for inhibitory of the metal ions are discussed on the basis of the results of the residual metal concentrations in a solution.

Materials and Methods

Metal Ions. Cobalt(II) and nickel(II) ions were tested as cobalt(II) chloride and nickel(II) chloride, while zinc(II), copper(II), and silver(I) ions were tested as zinc(II) nitrate, copper(II) nitrate and silver(I) nitrate. The dichromate ion was tested as potassium dichromate. Solutions were freshly prepared in a sterile phosphate-buffer solution. Buffered saline contained potassium dihydrogenphosphate (0.2 mol dm^{-3}) and sodium hydroxide (0.2 mol dm^{-3}). The pH of the solution was adjusted to 7.4. All metal-ion solutions were diluted by a phosphate-buffer solution in parts per million (mmol dm^{-3}) or parts per thousand million (μmol dm^{-3}) concentrations for antibacterial experiments. The dilution series of silver(I) ion solutions was kept in the dark. The metal-ion solutions, after preparation, were immediately used for antibacterial experiments.

Bacterial Cultures. The gram-positive bacteria used in our

experiments were *Staphylococcus aureus* (*S. aureus*) IFO 12732 and methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA was isolated from Ehime University Hospital in Japan; MIC is resistant to a methicillin, 200 μg mL^{-1}. The gram-negative bacteria used were *Escherichia coli* (*E. coli*) IFO 3806 and *Pseudomonas aeruginosa* (*P. aeruginosa*) IFO 13275. Microorganisms they were cultured in a SCD (Soybean-Casein Digest broth) medium (Nihon Pharm. Co., Ltd.) at 37 °C for 18—24 h. A portion of the culture medium containing the bacteria in a phosphate-buffer solution was diluted to 10^4 CFU/tube bacteria for subjecting it to antibacterial experiments.

Antibacterial Experiments. As described above concerning the bacterial culture, 1 mL of an approximately 10^4 CFU/tube tested bacterial buffer solution was added to each 20 mL sterilized bottle, which each contained 1 mL of various concentrations of metal-ion solutions. After proper mixing, they were incubated at 25 °C for 24 h. Immediately after incubation, the incubated solutions were sufficiently mixed after 9 mL of a SCDLP (Soybean-Casein Digest Broth with Lecithin & Polysorbate 80) medium (Nihon Pharm. Co., Ltd.) was added to each sterilized bottle; 1 mL of each concentration of bacterial medium was added to 9 cm diameter Petri dishes followed by the addition of approximately 20 mL standard agar. After each was sufficiently mixed with the bacterial medium, the agar was solidified. The number of surviving bacteria and the bacterial survival rates were measured after incubation for 2 d at 37 °C.

Residual Metal Concentrations after Incubation with Pathogenic Bacteria. At 0.5 μg mL^{-1} (2.9, 3.9, 2.0, 2.7, 3.9, and 2.6 μmol dm^{-3}) the same initial concentration of 2 mL metal ions ($AgNO_3$, $CoCl_2$, $K_2Cr_2O_7$, $Cu(NO_3)_2$, $NiCl_2$, and $Zn(NO_3)_2$) was each added to separate 20 mL sterilized test tubes with screw caps, which contained 2 mL of an approximately 5×10^6 CFU/tube bacterial solution. After proper mixing they were incubated at 25 °C for 24 h. Various metal-ion controls that did not contain bacteria were made under the same conditions. Immediately after incubation, the incubated solutions were sufficiently precipitated by centrifugation at 9000 rpm at 5 °C for 30 min. The concentrations of residual metal ions in the solution were determined exactly with an atomic-absorption spectrophotometer. In order to eliminate the effect of various chemical reaction precipitations in the buffer solution, the residual metal ions in the control solutions were divided by the results achieved for solutions containing bacteria.

MIC Values. The MIC values of metal ions to four different kinds of pathogenic bacteria were measured. After the concentrations of metal ions were continuously diluted by half in each tube, each concentration of metal ions was added to 9 cm diameter Petri dishes containing 10 mL of Mueller Hinton Medium. The medium was solidified after being sufficiently mixed with each concentration of metal ion. Approximately 10^6 CFU/tube of the various bacteria solutions was poured onto the surface of the solidified medium by a Micro–Planter (Sakura Seisakusho Co., Ltd.). These Petri dishes were incubated at 37 °C for 20 h. The lowest metal-ion concentration in which the growth of the bacteria was completely inhibited was defined as the MIC value.[23]

Results and Discussion

Variation of Residual Metal Concentrations after Incubation with Pathogenic Bacteria. The residual metal concentrations in a phosphate buffer solution were subjected to measurements after being incubated with pathogenic bacteria at 25 °C for 24 h. The obtained experimental results are summarized in Table 1. The control concentrations of residual metals were not significantly different from the initial metal concentrations, except for the cobalt(II) ion. Silver(I) and copper(II) ions showed almost the lowest residual concentrations after being incubated with all of the tested pathogenic bacteria. The zinc(II) residual concentration was always higher than that of the silver(I) and copper(II) ions and somewhat lower than the other ions. The residual concentration of dichromate ion was close to the highest of all the tested metal ions.

Compared with the control concentrations, the decrease in each metal-ion concentration may be adsorbed or reacted with microorganisms either directly or in directly to be considered. A part of the metal ions that exist near the surface of bacteria are adsorbed into the outer layers of the bacteria and are diffused from the surface into the inside bacterial cell, the metal ions subsequently permeate through the membrane, until the metal-ion potential is equilibrated.[24—26] In addition, because the ability of the metal ions to react with a biological molecule may be different, the adsorption amounts of metal ions on microorganisms appear to be different, leading to a decrease in a different degree of residual metal ions in the solutions.

The higher are the charge and smaller ion radius that the metal ions have, the stronger are the complexes[27] with biological molecules that they can easily form, such as copper(II)

Table 1. Residual Metal Ion Concentrations in Phosphate Buffer Solution[a]

Ions	Compounds	Initial concentrations μmol dm^{-3}	Control concentrations μmol dm^{-3}	Residual metal ion concentrations (μmol dm^{-3}) against: Gram-negative bacteria *E. coli* IFO 3806	*P. aeruginosa* IFO 13275	Gram-positive bacteria *S. aureus* IFO 12732	MRSA (MIC to methicillin 200 μg mL^{-1})
Ag^+	$AgNO_3$	2.9	2.9±0.048	2.0±0.038	1.4±0.024	2.1±0.036	2.2±0.040
Co^{2+}	$CoCl_2$	3.9	3.0±0.052	2.9±0.058	2.5±0.052	2.8±0.054	2.9±0.056
$Cr_2O_7^{2-}$	$K_2Cr_2O_7$	2.0	2.0±0.036	1.9±0.042	1.8±0.038	1.9±0.038	1.9±0.036
Cu^{2+}	$Cu(NO_3)_2$	2.7	2.7±0.038	1.4±0.028	1.9±0.036	1.8±0.032	1.8±0.034
Ni^{2+}	$NiCl_2$	3.9	3.9±0.072	3.3±0.068	3.5±0.066	3.5±0.066	3.4±0.064
Zn^{2+}	$Zn(NO_3)_2$	2.6	2.6±0.054	2.2±0.046	2.1±0.044	1.9±0.038	2.0±0.038

a) After incubated with pathogenic bacteria for 24 h at 25 °C.

and silver(I) ions. Since they form stable compounds, the metal ions enable entry through a membrane or capsid. The silver(I) and copper(II) ions outside or inside of a bacterial cell can complex with electron donor groups. For example, the phosphoryl groups can be bonded to form a positive dipole on the phosphate and a cyclic phosphate and cleavage of these molecules at the phosphodiester bond.[28] The proton in thioketal groups is replaced and forms reversible thioketal or histidyl complexes[29] on the cell surface. The carbonyl group of a ketonic can be polarized to complex and assist in the transfer of electrons from a C–C bond.[30] Silver(I) and copper(II) ions can also facilitate hydrolysis or a nucleophilicity displacement either directly or indirectly.[31—33]

The silver(I) ion can bind bacterial deoxyribonucleic acid (DNA), thus increasing the rate of dimerization inside the bacteria. The intercalation of silver can lead to increased stability of the double helix.[34] Because the copper(II) ion can be reduced to the copper(I) ion, similar complexes have been suggested for silver(I) and copper(I) ions binding to DNA.[25] The copper(II) ion has a specific affinity for DNA, and can bind or disorder helical structures by cross linking within and between strands.[35] Regarding the binding of other divalent cations to DNA, the zinc(II) ion can only be explained by the formation of a chelate between nitrogen and a phosphoryl group as well as the copper(II) ion.[11] This may be one of the reasons why the zinc(II) ion had a lower residual concentration than did the other divalent ions (Table 1).

On the other hand, the silver(I) ion has a high positive standard reduction potential (+0.799 vs. NHE).[36] The biological molecules may also have sufficient reducing power for the spontaneous reduction of the silver(I) ion to its elemental form, thus enhancing the chemical reactions between the silver(I) ion and microorganisms.

On the basis of the date, a metal ion that is able to react with biological molecules of microorganisms can possibly be adsorbed by the microorganisms, resulting in a decrease in the residual metal ions in the solutions.

Effect of the Metal-Ion Concentrations on the Inhibitory Capability for the Growth of Bacteria. During the course of experimental studies, no significant difference was observed in the behavior of metal ions inhibiting the growth of MRSA and *S. aureus*. The latter was thus used in the antibacterial experiments described below. The experimental results are summarized in Fig. 1.

The silver(I) ion, which is well known as being one of the most significant inhibitory metal ions, can inhibit bacterial growth at extremely low concentrations. As shown in Fig. 1a), all of the tested bacteria were sensitive to any variation in the silver(I) ion concentration. When the concentration of the silver(I) ion was greater than 1.0 μmol dm^{-3}, the bacterial survival rates were close to zero. Also, the bacterial survival rates rapidly decreased with increasing silver(I) ion concentrations.

The copper(II) ion concentration of 27—270 μmol dm^{-3}, at which bacterial survival rates were close to zero, was 10—100 fold higher than that of the silver(I) ion. The curves of the copper(II) ion concentrations with respect to the bacterial survival rates are shown in Fig. 1b). The bacterial survival rates gradually decreased when the copper(II) ion concentrations began to increase from 2.7×10^{-4} μmol dm^{-3}. In the presence of similar copper(II) ion concentrations, the surviving rates of *S. aureus* were lower than that of *P. aeruginosa* and *E. coli*. The copper(II) ions may possess a more significant inhibition for gram-positive bacteria *S. aureus* than gram-negative bacteria *P. aeruginosa* and *E. coli*.

The relationship between the bacterial survival rates and the cobalt(II) ion concentrations is shown in Fig. 1c). In the presence of 390 μmol dm^{-3} cobalt(II) ion, the proliferation of pathogenic bacteria was completely inhibited. However, when the cobalt(II) ion concentrations were lower than 0.039 μmol dm^{-3}, the bacterial growth continued to grown and exceeded the buffer control.

The effect of the nickel(II) ion concentrations on the bacterial survival rates is shown in Fig. 1d). The bacterial survival rates appeared to decrease when the nickel(II) ion concentrations were greater than 0.039 μmol dm^{-3}. Even though the nickel(II) ion concentration, by which the bacterial growth was completely inhibited, was greater than 390 μmol dm^{-3} in the same concentration with the cobalt(II) ion. However, the decrease in the bacterial survival rates was slow with increasing nickel(II) ion concentration. It is noted that the low concentration of the nickel(II) ion appeared to have a somewhat less inhibitory capability than that of the silver(I), copper(II), and cobalt(II) ions.

As shown in Fig. 1e), the bacterial growth was inhibited in the ranges of low zinc(II) ion concentrations. Also, the bacterial survival rates appeared to rapidly decrease when the zinc(II) ion concentrations were greater than 0.26 μmol dm^{-3}. The bacterial survival rates were approximately close to zero when the zinc(II) ion concentrations were greater than 0.26 μmol dm^{-3}.

In the zinc-plated coating case, a significant inhibitory capability of the zinc plated coating was achieved.[1,2] It is postulated that zinc(II) ions dissolved from the surface of the zinc coating are an important factor.

The inhibitory capability of the dichromate ion on the growth of the tested bacteria was the weakest of all the metal ions used. Figure 1f) demonstrates that when the dichromate ion concentration was 0.51 mmol dm^{-3}, the proliferation of gram-negative bacteria *P. aeruginosa* and *E. coli* was completely inhibited. The complete inhibitory concentration of the dichromate ion was 200-fold greater than silver(I) ion. However, under the same dichromate ion-concentration conditions, for *S. aureus*, about 19% of the population continued to grow.

Since the dichromate can be reduced into the transient formation of trivalent-chromium by biological molecules of bacteria, the trivalent chromium are able to bond with both teichoic acid and peptidoglycan polymers, which are the basic chemical substances composing the bacterial cell wall.[18] A part of the adsorbed chromium ions was found immobilized in the polymer layer, thus preventing access to the cytoplasm, resulting in a decreased inhibitory capability of the dichromate ion.

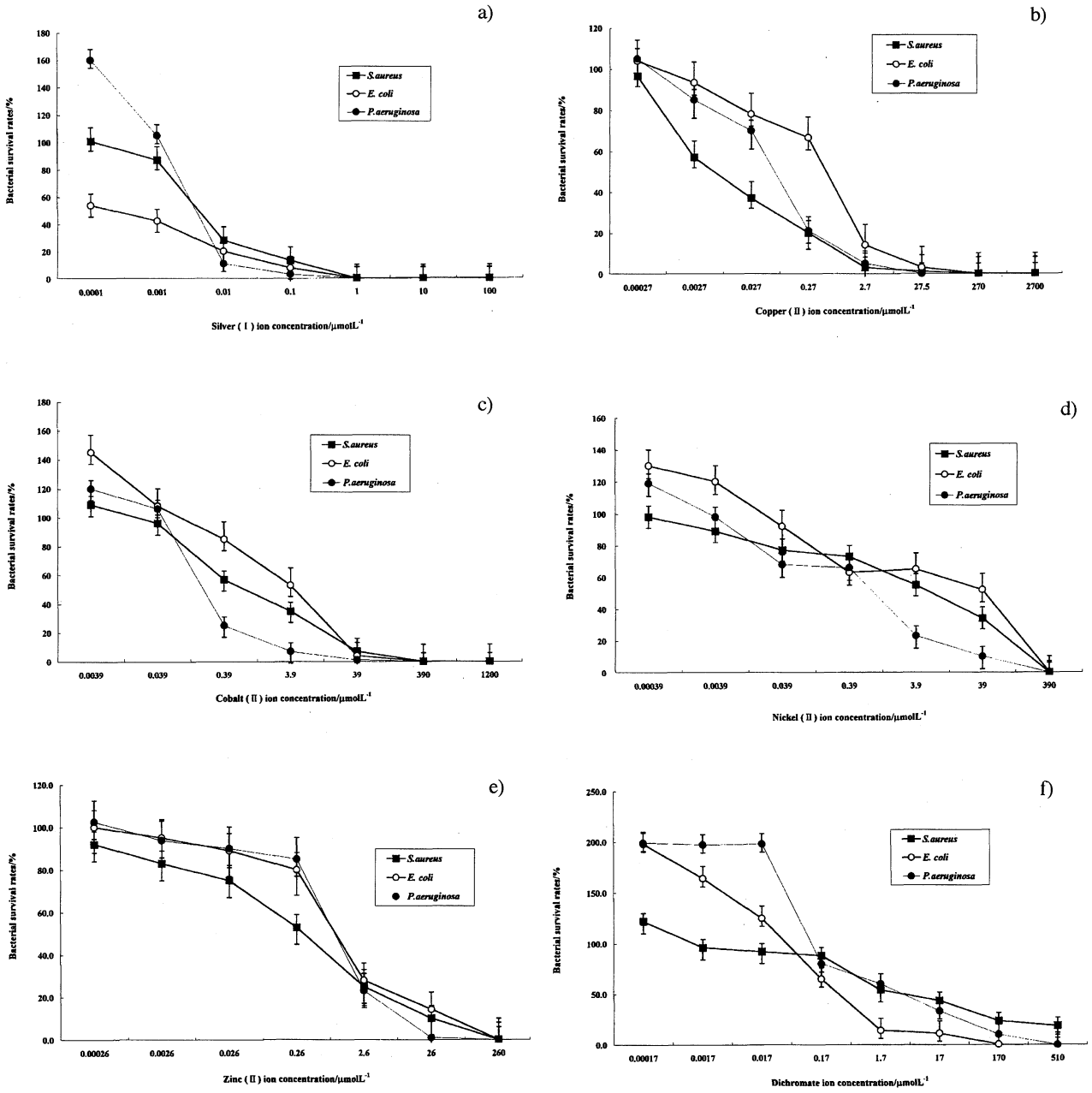

Fig. 1. Effect of metal ion concentrations on bacterial survival rates. Bacteria were incubated for 24 h at 25 °C in a phosphate buffer solution (pH 7.4).

In this study we also found that some tested metal ions, in low concentration ranges, almost did not inhibit, or had a slight inhibition on the normal proliferation of bacteria. In contrast to inhibiting, bacterial growth was promoted. As shown in Figs. 1c), 1d), and 1f), when the cobalt(II) and nickel(II) ion concentrations were lower than 0.039 μmol dm^{-3}, and the dichromate ion concentrations were lower than 0.17 μmol dm^{-3}, the bacterial growth continued to grow and exceeded the buffer control. The first reason is considered to be feasible, because the toxicity of these metal ions to bacteria is less than that of the silver(I) and copper(II) ions, and the low concentrations of the metal ions as nutrients may be easily accepted by the bacteria. The metal ions can form new cellular wall materials, such as the outer membrane and peptidoglycan layer,[16,17,19] thus promoting bacterial growth. The bacteria would absorb suitable trace elements from an external solution for satisfying their growth needs in order to compensate for the insufficiency of metal ions in the bacterial cellular structures. The second explanation is that the bioaccumulation of some metals is considered. The metal ions can be adsorbed by many bacteria into the outer layers of the cell. Also, the metal ions

Table 2. MIC Values of Metal Ions to Pathogenic Bacteria[a)]

Ions	Compounds	MIC ($\mu g\,mL^{-1}$) against: Gram-negative bacteria *E. coli* IFO 3806	Gram-negative bacteria *P. aeruginosa* IFO 13275	Gram-positive bacteria *S. aureus* IFO 12732	Gram-positive bacteria MRSA (MIC to methicillin 200 $\mu g\,mL^{-1}$)
Ag^{+}	$AgNO_3$	12.5	12.5	12.5	12.5
Co^{2+}	$CoCl_2$	25.0	12.5	12.5	6.25
Cr^{6+}	$K_2Cr_2O_7$	50.0	≥100	>100	>100
Cu^{2+}	$Cu(NO_3)_2$	25.0	12.5	12.5	12.5
Ni^{2+}	$NiCl_2$	25.0	12.5	12.5	12.5
Zn^{2+}	$Zn(NO_3)_2$	25.0	12.5	12.5	12.5

a) After incubation for 20 h at 37 °C.

are rapidly transferred a shorter diffusion distance and are appropriately accumulated at interfaces when they are not at the stimulatory concentration. The concentrations of accumulated metal ions are unaffected on the normal growth of bacteria. The metal ions that accumulated within cells in limited amounts affect the new synthesis of cellular macromolecules. The metal ions distribute between newly separated daughter cells, that are subsequently proliferated by accumulated metal-ion bacteria.

MIC Values of Various Metal Ions to Four Kinds of Pathogenic Bacteria. As described above, the MIC values of metal ions for various pathogenic bacteria were measured

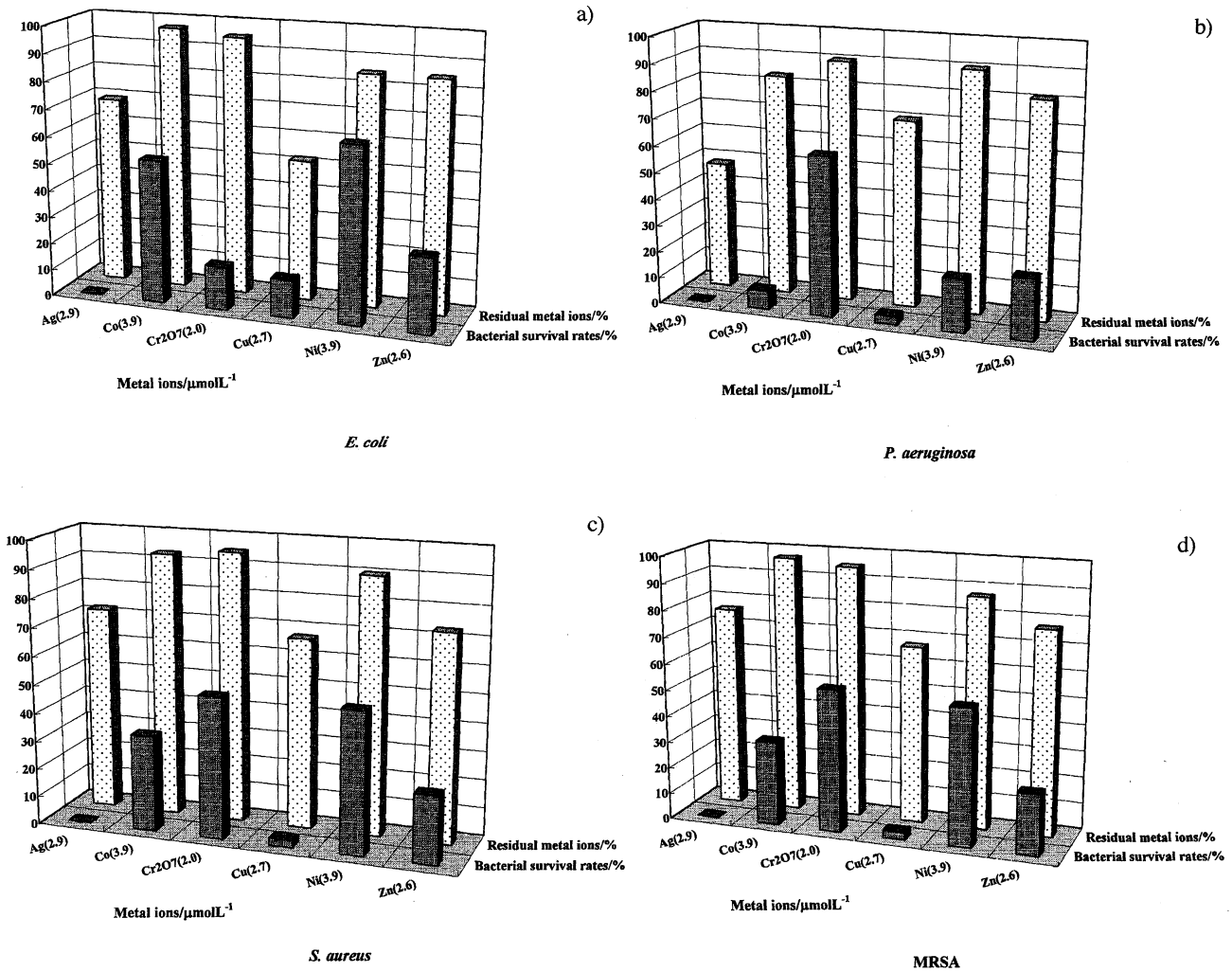

Fig. 2. Relationship between residual metal ions in a solution and inhibition of the metal ions to bacteria. Bacteria were incubated for 24 h at 25 °C in a phosphate buffer solution (pH 7.4).

on plates in Mueller Hinton Medium. The experimental results are summarized in Table 2. Because metal ions possessed a different inhibitory capability for the growth of diverse bacteria, to a certain extent, differences in the minimum concentrations of complete inhibition were apparent. For *E. coli*, the inhibitory capability of metal ions was in the order: $Ag^{+} > Cu^{2+} \geqq Co^{2+}$, $Ni^{2+}, Zn^{2+} > Cr_2O_7^{2-}$; The silver(I) ion appeared to have the most significant inhibition of all the metal ions. The MIC values of various metal ions to gram-positive bacteria, *S. aureus* and MRSA, were similar in magnitude to gram-negative bacteria, *P. aeruginosa*. However, the cobalt(II) ion, at only 6.25 $\mu g\,mL^{-1}$, was able to completely inhibit the growth of MRSA. The dichromate ion, at 100 $\mu g\,mL^{-1}$ or greater, was unable to achieve complete inhibition against *P. aeruginosa*, *S. aureus*, and MRSA. These experimental data were in good agreement with the measurement results of the bacterial survival rates of the dichromate ion [Fig. 1f)].

From the results given in Table 2, it was also found that the dichromate ion has a weaker inhibition for the growth of gram-positive bacteria than gram-negative bacteria. These differences may be due to the different biochemical and morphological characteristics of the gram-positive and gram-negative bacteria, which may be reflected in the distribution of metal ions in cellular fractions.

Relationship between Residual Metal Ion Concentrations and the Inhibition of Metal Ion on the Bacterial Growth. The inhibition of metal ions on the growth of bacteria and the variation in the residual metal-ion concentrations in the solutions has been discussed separately. We are interested in the relationship between both. The results of the residual metal percentages and the bacterial survival rates are shown in Fig. 2. It was demonstrated that the more significant inhibitory metal ions possessed lower residual percentages. However, the zinc(II) ion showed slight irregularities in the relationship between the inhibitory capability and residual percentages.

As shown in Fig. 2, the silver(I) and copper(II) ions appeared to have the most significant inhibition among all of the metal ions. Simultaneously, the lowest residual percentages of silver(I) and copper(II) ions after being incubated with bacteria were achieved. Especially, the survival rates of all the tested bacteria were close to zero, while the residual silver(I) ions decreased in a solution.

As described above concerning, the mechanisms for the adsorption silver(I) and copper(II) ions are due to chemical reactions, which occur with biological molecules inside a bacterial cell. The silver(I) or copper(II) ions went into the bacterial cells, where they could bind with dithioketal moieties of the cellular proteins and enzymes.[31,37—39] Accordingly, they replace natural metal ions existing in enzyme prosthetic groups,[40] resulting in a disruption of the enzyme structure and proper functions.[41,42] The silver(I) and copper(II) ions, which are able to bind and disrupt natural DNA, have also been reported.[17,43—46] Silver(I)–DNA complexes occur at bases, which causes denaturation[47] by displacing hydrogen bonds between adjacent nitrogen of purines and pyrimidines, thereby preventing replication.[48] Copper(II) reversibly denatures DNA, competing with hydrogen bonding within the macromolecule[49—51] and occurring without aggregation or disruption of the double helix. The effect of silver on the protein coat cannot also be ignored simultaneously. When proteins are compounded or altered by silver(I), they cannot perform normal functions. Copper(II), when chelating with a phosphoryl group, breaks hydrogen bonds within a double-stranded structure, opening the double helix. The stabilization or destabilization of molecules due to interactions with metal ions may affect their function and, subsequently, bacterial proliferation is significantly inhibited, decreasing residual metal ions in the solution at the same time.

On the other hand, as shown in Fig. 2b), regarding *P. aeruginosa*, the dichromate ion possessed the lowest inhibitory capability and appeared to have the highest residual percentage. The residual percentages of cobalt(II) and nickel(II) ions showed less decrease after being incubated with the tested bacteria and had higher bacterial survival rates than the other metal ions [Figs. 2a), 2c), and 2d)]. The possible reason is that the bacteria are only able to react with slight amounts of metal ions, forming complexes or chelate with several metal-binding agents to protect or decrease the metal-ion toxicity avoiding bacterial cell damage. Namely, the membrane proteins of bacteria show slight differences from the normal strains, suggesting a modification. As pointed out in the Fig. 2a) results, in the presence of the cobalt(II) ion, there was a greater than 20% survival rate of *E. coli* with only a slight decrease in the residual percentage. It has been reported that an impermeable barrier to cobalt(II) ion was formed on the specific membrane transport system for the cobalt(II) ion by a strain of *E. coli*.[52] Consequently, inheritable specific protection factors are produced for avoiding the particular toxicity of metal ions.

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