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Quantification, Qualification, and Microbial Killing Efficiencies of Antimicrobial Chlorine-Based Substances Produced by Iontophoresis

C. PATRICK DAVIS,[1]* MARK E. SHIRTLIFF,[1] NORMAN M. TRIEFF,[2] STEPHEN L. HOSKINS,[1] AND MICHAEL M. WARREN[3]

Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas 77555-1019[1]; Department of Preventive Medicine, Division of Environmental Toxicology, University of Texas Medical Branch, Galveston, Texas 77555-1010[2]; and Department of Surgery, Division of Urology, University of Texas Medical Branch, Galveston, Texas 77555-0540[3]

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The dependence of microbial killing on chloride ions present in solutions undergoing iontophoresis is addressed. A 400-μA current was applied to vials containing synthetic urine or saline, and the production of chlorine-based substances (CBSs) was detected by the *N,N*-diethyl-*p*-phenylene diamine colorimetric method. It was found that as the time of current application increased, the total concentration of CBSs also increased. The iontophoretic current converted (through oxidation) chloride ions present in the solutions into CBSs such as free chlorine, chlorine dioxide, chlorite, monochloramine, and dichloramine (the last two were produced by iontophoresis only when nitrogenous substances were present in the solution). Two of the CBSs (free Cl and ClO_2), when they were separately added back to microbial suspensions (approximately 3×10^5 CFU/ml) at the same concentrations at which they were detected in either 0.46% (wt/vol) NaCl solution or synthetic urine iontophoresed for 4 h at 400 μA, reduced or eliminated bacterial genera and a fungus. However, when free Cl and ClO_2 were jointly added back to microbial suspensions, bacterial and fungal killing was synergistic and more rapid and complete than when these chlorine-based biocides were added separately. Therefore, iontophoresis of solutions containing chloride ions produces chlorine-based biocides that are responsible for the antimicrobial effect of iontophoresis.

For many years urinary catheters have been identified as both the leading cause of nosocomial-induced urinary tract infections (UTIs) and the most common predisposing factor in fatal gram-negative sepsis in hospitals (13, 24). Therefore, reducing the threat of nosocomial UTIs from catheter usage has been an important goal for our laboratory. We have designed a catheter-power supply system that delivers an iontophoretic current of 400 μA to the urinary bladder through platinum electrodes (7). Previous research has demonstrated that this current will eliminate microbial populations only when it is passed through solutions containing chloride ions (11). We have also previously shown that platinum electrodes have the best durability and microbial killing efficiency and that iontophoresis for 4 h at 400 μA in synthetic urine generates a biocidal effect against selected fungi and gram-positive and gram-negative bacteria for more than 6 h after iontophoresis ceases (9, 10). Furthermore, bacterial killing efficiency was found to be directly related to increasing microamperage (up to 400 μA) and to chloride ion concentration, while it was found to be inversely related to the number of bacteria present in the solution undergoing iontophoresis (12). Preliminary work suggested that the reason for biocidal activity was the production of chlorine-containing oxidants. Therefore, the aim of the study described here was to both identify and quantify the various iontophoretically produced chlorine-based substances (CBSs) such as free chlorine (i.e., Cl_2, OCl^-, and HOCl), chlorine dioxide (ClO_2), chlorite (ClO_2^-), monochloramine (NH_2Cl), dichloramine ($NHCl_2$), and trichloramine (NCl_3). Another important goal was to determine which, if any, of the chlorine products were responsible for the antimicrobial effect produced by iontophoresis. Also, the relative efficiencies of CBS production by the iontophoretic system were determined.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555-1019. Phone: (409) 772-8498. Fax: (409) 772-5065.

MATERIALS AND METHODS

Constant current generator. The microamperage generator was constructed at the University of Texas Medical Branch (UTMB). This instrument provided either a constant current of 1 to 450 μA or a constant voltage of 0.01 to 12.5 V to 10 independently controlled channels (a wiring diagram is available upon request). Vented stoppered vials (cleaned and thoroughly rinsed with distilled deionized water) were connected to the generator by metal connectors. Platinum wires (0.2 mm in diameter) were attached to the base of the metal connectors so that approximately 2 cm of each wire was immersed in the 10-ml samples of 0.46% (wt/vol) NaCl solution or synthetic urine (the preparation method has been described previously [17]). A diagram of our iontophoretic system has been published previously (8).

Iodometric detection of oxidants. When aliquots of iontophoresed saline were titrated iodometrically with starch-iodide as an indicator and 0.01 N $Na_2S_2O_3$ (Spectrum Corp. Gardena, Calif.) as a titrant, an increasing titer was found with an increasing time of iontophoresis, demonstrating that an oxidant was produced iontophoretically (data not shown) (1).

Qualification and quantification of antimicrobial chlorine products. Because it was necessary that chloride ions be present for the iontophoretic production of oxidant, it was

concluded that the oxidants are most likely chlorine based (such as free Cl, ClO_2, and inorganic chloramines) (11). Further characterization was performed by a standard spectrophotometric technique (1). A standard graph was constructed by making multiple dilutions of 5.25% (wt/vol) household hypochlorite solution (i.e., Clorox) to final sample volumes of 100 ml. To each sample was added 10 ml of phosphate-buffered *N,N*-diethyl-*p*-phenylene diamine (DPD) indicator solution in a flask (29). After filling the spectrophotometer cell and measuring the A_{515} with a Bausch & Lomb model 340 spectrophotometer, the cell contents were returned to the flask and titrated with standard ferrous ammonium sulfate titrant to determine the free Cl concentration in the 100-ml sample as described in the DPD ferrous titrimetric and DPD colorimetric methods (1, 29). We were then able to construct a linear standard calibration curve of chlorine species concentration versus absorbance (data not shown). Samples (0.46% [wt/vol] NaCl solution or synthetic urine) were iontophoresed for 1, 4, 12, and 24 h. Immediately following iontophoresis, each 10-ml sample was quickly analyzed for the presence and concentration of the CBS by the DPD colorimetric method (1). The DPD-chlorine species complexes were measured by determining the absorbances and comparing these measured absorbances with those on the standard curve. Because the DPD method used different volumes of reagents that were added to the iontophoresed samples to detect the different types of CBSs in these samples, it was necessary to adjust the values extrapolated from the standard curve to compensate for the different dilutions. Dilutions of the household hypochlorite solution and the iontophoresed samples (when the total available chlorine concentration was greater than 4 mg/liter) were done with chlorine demand-free water (CDFW) instead of distilled deionized water. The use of CDFW as a diluting agent was preferred because impurities possibly present in distilled deionized water would have consumed some of the free and/or combined chlorine in both the diluted hypochlorite solutions and the iontophoresed samples, thus leading to an underestimation of the CBS concentrations detected in these solutions. The CDFW was prepared by adding 1.5 ml of 5.25% (wt/vol) household hypochlorite solution to 3 liters of best-quality distilled deionized water. The solution was allowed to sit for at least 48 h at room temperature to fulfill the chlorine demand of the possible water impurities in distilled deionized water, and the solution was then placed in direct sunlight for 8 h to remove the remainder of the free and/or combined chlorine (23). No chlorine-based substances were detected in the CDFW prepared in this way, as detected by the DPD ferrous titrimetric method (1). The CDFW was stored in the dark, the air inlet of the storage flask was attached to an H_2SO_4 trap, and the water was drawn out of the flask by gravity (1).

pH determination. The pHs of the saline and synthetic urine were measured with a Corning model 255 pH meter after 0, 1, 4, 12, and 24 h of iontophoresis at 400 μA. This procedure was performed in triplicate.

Percent relative efficiency. Once the types and amounts of the CBSs were determined in the iontophoresed samples, the relative efficiencies of production of these chlorine species were calculated. Relative efficiency was determined by dividing the number of electron equivalents used by the iontophoretic system in the production of CBSs by the number of electron equivalents (eq) produced by the iontophoretic system, multiplied by 100 (4, 16). Thus, $(eq_{utilized}/eq_{generated}) \times 100 =$ percent relative efficiency.

Microorganisms. The bacteria that were used included *Pseudomonas aeruginosa* (supplied by J. Reitmeyer at UTMB), *Escherichia coli*, and *Proteus mirabilis*. These bacteria were chosen because gram-negative bacteria are responsible for 80 to 90% of all UTIs, with *E. coli* causing more infections than all other genera combined (12, 24). *Staphylococcus saprophyticus* (obtained from the American Type Culture Collection through a commercial source) and *Candida albicans* (supplied by D. Niesel at UTMB) were used as representative members of gram-positive bacterial and fungal genera, respectively (10). Organisms were washed three times by centrifugation with sterile phosphate-buffered saline (PBS) solution (17). Initial microbe concentrations were approximated by using a Petroff-Hausser counting chamber and were diluted with sterile PBS to obtain the desired concentration of microorganisms (i.e., 10^9 microbes per ml). The concentrations of viable microbes were then determined by serially diluting 0.1-ml samples with sterile PBS and plating them onto brain heart infusion agar plates.

Free chlorine and chlorine dioxide preparation. In order to test which of the CBSs (or combination of CBSs) produced by iontophoresis was responsible for microbial killing in the system, free chlorine only, chlorine dioxide only, and a combination of free chlorine and chlorine dioxide were added to suspensions of selected bacteria and a fungus containing approximately 3×10^5 CFU/ml. The biocidal activities of the inorganic chloramines (i.e., NH_2Cl, $NHCl_2$, and NCl_3) were not studied because the killing efficiencies of the chloramines are reportedly only 4% of free chlorine's killing efficiency (34). The potential bactericidal action of chlorite was also not examined because other studies have demonstrated that chlorite has no effect on selected bacteria (18). Past studies revealed that 4 h of iontophoresis at 400 μA with platinum electrodes in synthetic urine was adequate for a 100% bacterial and fungal elimination (9, 10). Therefore, an amount of chlorine dioxide or free chlorine comparable to those detected after 4 h of iontophoresis was added back to the microbial suspensions of 0.46% saline or synthetic urine after correcting for the free Cl or ClO_2 removal by the various inorganic and possibly organic species in saline and synthetic urine (1). A 5.25% (wt/vol) household hypochlorite solution (i.e., Clorox) was used as a source of free chlorine (23, 34). Chlorine dioxide was generated from Purogene (Bio-cide, Inc. Norman, Okla.), a 2% chlorite solution. Chlorine dioxide can be released from solutions of chlorite by activation through acidification with HCl by the following mechanism (26):

$$5ClO_2^- + 4H^+ \leftrightarrow 4ClO_2 + 2H_2O + Cl^-$$

Since the concentration of chlorine dioxide produced from chlorite depends on the pH of the solution, activated Purogene was added to solutions of PBS matching the pHs of saline (i.e., pH 6.97) and synthetic urine (i.e., pH 5.90). By using the DPD colorimetric method, the chlorine dioxide concentrations were measured in each solution in order to quantify the proportion of the chlorine dioxide concentration at the pH of each solution. The exact volumes of activated Purogene to be added to the saline solution and the synthetic urine to equal the ClO_2 concentrations found after 4 h of iontophoresis were then calculated.

Model add-back system. Microbial inocula (0.1 ml) were added to sterile, sealed glass test tubes containing 9.9 ml of prewarmed (i.e., 37°C), sterile 0.46% saline solution or synthetic urine to give the desired concentration of approximately 3×10^5 CFU/ml. In order to obtain controls, 0.1-ml samples were acquired at this point and microbe counts were made by diluting the samples in sterile PBS and plating them onto brain heart infusion agar plates. A total of 0.1 ml of free Cl or ClO_2 was added to the microbial suspensions to produce the same

concentrations that were detected in the solutions after 4 h of iontophoresis at 400 μA. Samples of 0.1 ml were removed from the free chlorine- or chlorine dioxide-treated microbial suspensions at 1 min and 1, 4, 12, and 24 h after initial chlorine biocide inoculation. These samples, at the time of removal, were quickly (i.e., within 5 s of sample extraction) treated with 10% (wt/vol) sodium thiosulfate pentahydrate (J. T. Baker Chemical Co., Phillipsburg, N.J.) in order to neutralize any residual free Cl or ClO_2. No deleterious effects from the $NaS_2O_3 \cdot 5H_2O$ treatment were noted in the microbial genera represented in the study when they were compared with untreated controls (data not shown) (18). Samples were then serially diluted in sterile PBS, plated onto brain heart infusion agar, and incubated at 37°C for 18 h to determine the number of CFU remaining (18). The test tubes were all incubated at 37°C between the times of sample withdrawal. All glassware used in this add-back system was washed and thoroughly rinsed with CDFW.

Statistical analysis. Comparisons of the CBS concentrations detected by the DPD colorimetric method over the different iontophoretic durations were accomplished by the Student *t* test at a *P* value of <0.05. The differences in the pHs, relative efficiencies, and CFU of survivors over the different iontophoretic durations were also analyzed by this statistical test and with this *P* value.

RESULTS

DPD analysis of iontophoresed solutions. Figure 1A indicates that free Cl (the predominant species), ClO_2, and ClO_2^- were present after iontophoresis of 0.46% (wt/vol) NaCl for various times, while the chloramines (NH_2Cl, $NHCl_2$, and NCl_3) were absent. The predominant species found in iontophoresed synthetic urine (Fig. 1B) were chlorite and dichloramine, with lower levels of ClO_2 and free Cl detected; however, monochloramine (not shown on Fig. 1B) was detected only in trace amounts (0.002 mg/liter). The total chlorine substance concentrations in iontophoresed synthetic urine were approximately twice those detected in iontophoresed saline for all iontophoretic running times. An important similarity between Fig. 1A and B is that as the iontophoretic duration increased in synthetic urine and saline, not only did the total concentration of the CBS increase but the concentrations of each of the predominant chlorine species also increased (with the exception of ClO_2 concentrations in saline, which did not change over the different iontophoretic durations with any statistical significance [$P > 0.05$]). No chlorine-based substances were detected in the 0-h controls (i.e., when no current was applied) in either solution.

pH of iontophoresed solutions. Figure 2 reveals the pH measurements for samples of saline and synthetic urine that underwent iontophoresis for 0, 1, 4, 12, and 24 h at 400 μA. While the pH of the saline solution became more basic with increasing time of current application (i.e., the pH levels measured between the 0- and the 24-h iontophoretic durations rose an average of 3.6 units), synthetic urine samples became more acidic (i.e., the pH levels measured between the 0- and 24-h iontophoretic durations decreased an average of 0.20 units).

Percent relative efficiency. Maximum efficiency for the creation of the CBSs for the two solutions occurred at about 1 h after the onset of 400-μA iontophoresis and then steadily and significantly declined (Fig. 3) ($P < 0.001$). The efficiencies for NaCl solutions declined much more rapidly than those for synthetic urine solutions after the 1-h peak.

Add-back system. When free chlorine only, chlorine dioxide only, or free chlorine plus chlorine dioxide (at concentrations equal to those detected in 0.46% NaCl solution after 4 h of iontophoresis) were added back to microbes (approximately 3×10^5 CFU/ml) suspended in the NaCl solution, 100% elimination of each of the five organisms occurred at less than 1 min after chlorine biocide inoculation (data not shown). Figure 4A shows the effect of free chlorine on the selected microbes suspended in synthetic urine when the free chlorine existed at a concentration equal to that found in synthetic urine after 4 h

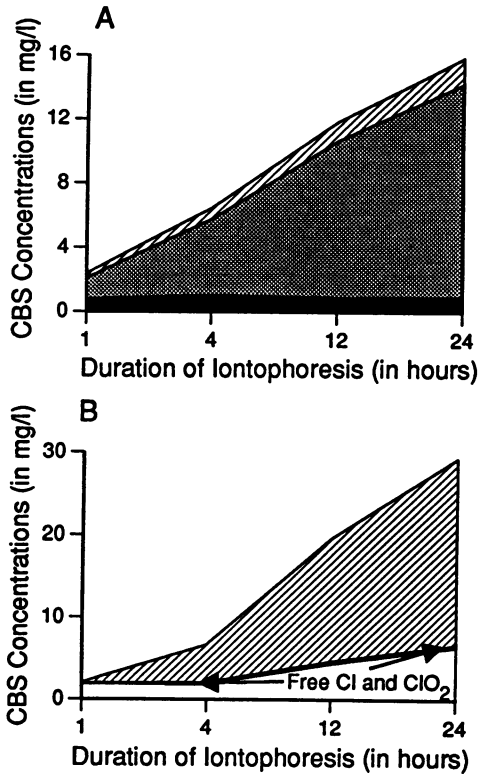

FIG. 1. Concentrations of CBSs detected by the DPD colorimetric method in samples of 0.46% NaCl solution (A) and synthetic urine (B) iontophorized at 400 μA for 1, 4, 12, and 24 h. CBSs include chlorite (▨), free chlorine (▤), chlorine dioxide (■), and dichloramine (□). Arrows point to the expanded dark line that represents the detected concentrations of free chlorine and chlorine dioxide.

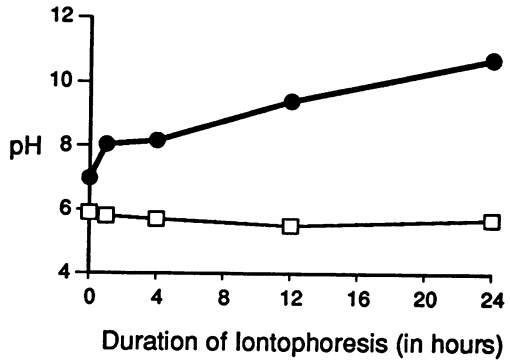

FIG. 2. pH values of samples of 0.46% NaCl solution (●) and synthetic urine (□) that were iontophorized (at 400 μA) for 0, 1, 4, 12, and 24 h.

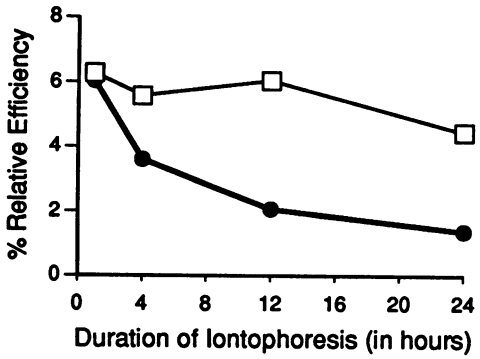

FIG. 3. Percent relative efficiencies for samples of 0.46% NaCl solution (●) and synthetic urine (□) that underwent iontophoresis at 400 μA for 1, 4, 12, and 24 h. Percent relative efficiency is the number of electrons used to form CBSs divided by the total number of electrons generated by the iontophoretic system and multiplied by 100.

of iontophoresis at 400 μA (as measured by the DPD colorimetric method). The populations of *S. saprophyticus* and *P. mirabilis* were eliminated within 12 h of initial free chlorine inoculation. *P. aeruginosa*, *C. albicans*, and *E. coli*, however, were reduced to a minimum within 4 h of free chlorine addition, but then the concentrations of all three organisms displayed net increases after the 4-h sampling period ($P < 0.01$), with *P. aeruginosa* showing the highest population increase. Figure 4B displays the effects of ClO_2 addition on microbial populations suspended in synthetic urine. *P. mirabilis* and *S. saprophyticus* were removed from the solution within 4 h of ClO_2 inoculation and all genera were eliminated within 24 h. Figure 4C shows the combined sterilizing effects of free chlorine and chlorine dioxide on microbial populations suspended in synthetic urine. All microbes were eliminated within 4 h of the addition of free Cl plus ClO_2. An average of 60% of the total organisms were eliminated within the first minute after free Cl or ClO_2 inoculation in synthetic urine (data not shown).

DISCUSSION

Our results have explained why chloride ions were necessary for the iontophoretic killing of microbes. The electrons provided by the current of the iontophoretic system converted the chloride ions present in the solution into the chlorine-containing biocides including free chlorine, chlorine dioxide, monochloramine, and dichloramine (the last two are produced by iontophoresis only when nitrogenous substances are present in the solution). We and other investigators have used the term iontophoresis (i.e., the use of current to produce ions of soluble salts) to explain the mechanism of bacterial killing (2). While microbial killing is linked to iontophoresis, electrosynthesis (i.e., chemical reactions effected by means of electricity) is occurring simultaneously to produce the chlorine-based biocides responsible for the antimicrobial effect (14). Two of these chlorine-based biocides, free Cl and ClO_2, were adequate for rapid bacterial and fungal killing in saline solutions and synthetic urine. Since the electrons from the iontophoretic system were responsible for the conversion of chloride ions into chlorine-based biocides, the increase in the length of time that the iontophoretic current was applied produced the measured increases in the CBS concentrations.

When the concentrations of chlorine-based biocides were measured by the DPD method, no chloramines were produced

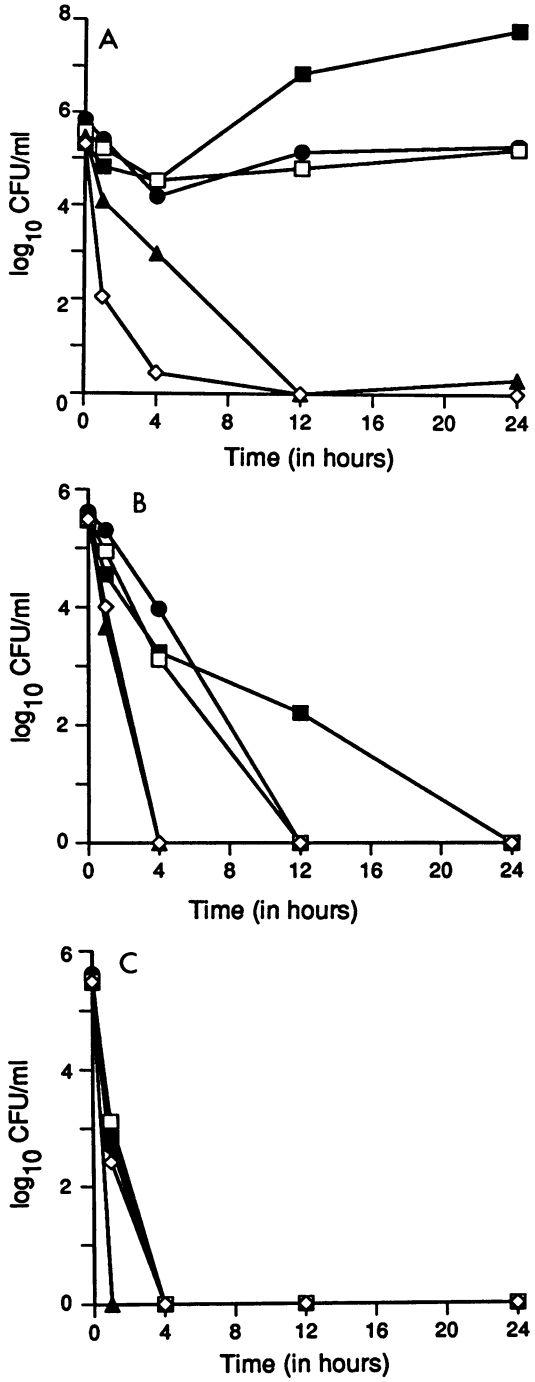

FIG. 4. Bacterial and fungal killing in synthetic urine at 1, 4, 12, and 24 h following inoculation of free chlorine alone (at an end concentration of 0.049 mg/l) (A), chlorine dioxide alone (at an end concentration of 0.115 mg/liter) (B), and free chlorine and chlorine dioxide combined (at end concentrations of 0.049 and 0.115 mg/liter, respectively) (C). These concentrations were equal to the free chlorine and chlorine dioxide concentrations detected in synthetic urine by the DPD colorimetric method after 4 h of iontophoresis at 400 μA. The organisms used in this study included *P. aeruginosa* (■), *E. coli* (●), *C. albicans* (□), *P. mirabilis* (▲), and *S. saprophyticus* (◇).

in iontophoresed saline solution because no ammonia or other nitrogen-containing substances were present to provide the necessary nitrogen atoms. The presence of chloramines did, however, occur in iontophoresed synthetic urine because the requirement for nitrogen atoms was fulfilled by the NH_3 that was formed from urea through the following reaction (5):

$$2e^- + H^+ + (NH_2)_2CO + H_2O \rightarrow 2NH_3 + COOH$$

Once the nitrogen-containing molecules were added to the system, as in the case of synthetic urine, the free chlorine produced by iontophoresis reacted with ammonia to produce chloramines in a stepwise and pH-dependent process (see below) (25, 32):

$$1/2Cl_2 + NH_3 \rightarrow NH_2Cl + H^+ + 1e^- \text{ (formation at pH} > 8)$$

$$1/2Cl_2 + NH_2Cl \rightarrow NHCl_2 + H^+ + 1e^- \text{ (formation at pH 4.5 to 5.5)}$$

$$1/2Cl_2 + NHCl_2 \rightarrow NCl_3 + H^+ + 1e^- \text{ (formation at pH} < 4.4)$$

We may assume that because of the very low concentrations of free chlorine in the iontophoresed synthetic urine samples (compared with those in the iontophoresed saline samples), nearly all of the free chlorine in synthetic urine quickly reacted with ammonia to create dichloramine. Also, minimal monochloramine and no trichloramine were produced because the pH of the iontophoresed synthetic urine (5.65 to 5.9) was closest to the optimal pH for dichloramine formation, and therefore, dichloramine production was highly favored (32).

Although the chlorite concentrations found in the iontophoresed saline rose with increasing time, the levels were very low when compared with the concentrations detected in synthetic urine. The levels of ClO_2 in iontophoresed saline, however, were elevated but demonstrated no statistically significant change with increased iontophoretic durations. Therefore, some of the large amount of highly volatile ClO_2 being iontophoretically produced in the saline may have been converted to chlorite, lost through evaporation, or removed from solution by reaction with other compounds. Nearly all of the ClO_2 in synthetic urine, however, appeared to be converted into chlorite. This augmented loss of ClO_2 in saline may have accounted for the reduced total CBS concentrations detected in iontophoresed saline compared with those in synthetic urine.

The saline became more basic with iontophoresis because OH^- was produced by the action of electric current on saline solutions by the following reaction (27):

$$2NaCl + 2H_2O \xrightarrow{\text{electric current}} 2NaOH + Cl_2 + H_2$$

The synthetic urine, nevertheless, became more acidic because H^+ ions were produced by chloramine formation (see above) and other reactions, such as the following (32):

$$4Cl_2 + NH_3 + 3H_2O \rightarrow 8Cl^- + NO_3^- + 9H^+$$

The H^+ apparently overcame the pH-increasing affect of the OH^- (produced by the action of the electric current on saline solution), making the iontophoresed synthetic urine more acidic. The pH of the synthetic urine, however, only slightly decreased because the phosphate buffer (i.e., $H_2PO_4^-$/HPO_4^{2-}) in synthetic urine maintained the pH at an approximately constant level.

When the efficiencies for the production of the chlorine-based species by iontophoresis were calculated, only a small percentage (range, 1.4 to 6.3%) of the total electrons generated by the iontophoretic system was used in the production of these chlorine substances in saline solution and synthetic urine (Fig. 3). The remainder of the electron energy may have gone into heating of the connecting wires and electrodes or the production of nonoxidant substances. Some electron energy may also have been lost through volatilization of unstable components (such as chlorine dioxide) or the production of an oxidant(s) that later reacted with other components of the solution, thereby making the oxidant(s) unavailable for detection by the DPD method. Because both solutions reached maximum efficiency at about 1 h and then steadily declined with longer iontophoretic durations, it could be assumed that at a constant current the CBS production was fastest in the first few hours of iontophoresis. The steady drop in measured efficiencies may have been due to competing reactions or changes in pH. The drop may have also been due to the removal of the finite number of chloride ions from the solutions by the iontophoretically produced reactions that formed both the CBSs and the other substances. As the concentration of chloride ion reactants decreased, the CBS formation became more unfavorable, causing the decline in the CBS production efficiency (27). Lastly, the steady decline in measured efficiencies after 1 h of iontophoresis may also have been an indication of the slow saturation of both the electrodes and the solutions undergoing iontophoresis with chlorine-based products, resulting in electrode overvoltage. Thus, production of the CBSs may have become less favorable with time. Also, reactions that may have both formed nonoxidants and caused more oxidants to react with other components of the solution (thereby making them unavailable for detection by the DPD method) were more likely to occur. This effect may have been less apparent in synthetic urine because the favored iontophoretic products (as demonstrated by their advantageous redox potentials), ClO_2 and free Cl, were removed from solution by forming chlorite and chloramine species, respectively. However, these ClO_2 and free Cl removal mechanisms were not active in saline because of the unavailability of nitrogen-containing substances and an unfavorable pH. This absence of removal mechanisms may have caused the more rapid drop in relative efficiency after 1 h of iontophoresis in saline compared with that in synthetic urine. Also, this rapid drop in the measured efficiencies of saline after 1 h of iontophoresis may have been caused by augmented evaporation of the CBS products. Evaporation of the CBSs may have played a larger role in efficiency reduction in saline compared with that in synthetic urine because saline preferentially produced free chlorine and chlorine dioxide, both of which exist as gases at room temperature (27). Therefore, gaseous CBS produced by saline iontophoresis may have escaped from the reaction system (i.e., out of the vents of the stoppered vials).

When the free chlorine alone, chlorine dioxide alone, and free chlorine plus chlorine dioxide were added at concentrations equal to those detected after 4 h of 0.46% NaCl solution iontophoresis at 400 μA to the microbes (approximately 3×10^5 CFU/ml) suspended in saline, all organisms were eliminated within 1 min after chlorine product inoculation. However, when the free chlorine or chlorine dioxide was added at the concentrations at which they were detected after 4 h of iontophoresis of synthetic urine to the microbial suspensions, the killing efficiencies were less than those of the chlorine species in saline. The reason for the very quick microbial killing quality in saline compared with that in synthetic urine was that the predominant species produced by iontophoresis in saline were free chlorine and chlorine dioxide, and these two species have been described as very potent chlorine-based

biocides (34). On the other hand, the predominant species produced by iontophoresis in synthetic urine were dichloramine and chlorite, with minimal concentrations of the strong biocides free chlorine and chlorine dioxide being produced. Because the dichloramine was a much weaker biocide and chlorite had no detectable biocidal effect, only free chlorine and chlorine dioxide were added to the microbial solutions (18, 34). Therefore, with the low concentrations of free Cl and ClO_2, one would expect the iontophoresed synthetic urine to have a lower biocidal capacity than that of iontophoresed saline. This reduced biocidal effectiveness was demonstrated by the inability of free Cl (at a concentration of 0.049 mg/liter) to eliminate all of the five test microbes suspended in synthetic urine. Three of the five microbes were able to survive the biocidal effects of free chlorine, and these microbes actually had net increases after overcoming the free chlorine. These net increases may have been due to either the consumption of the low levels of biocides or organism resistance. However, the development of resistance by the organisms seems less likely since previous experiments provided no good evidence that resistant organisms are generated by the products of iontophoresis (6). The ClO_2, however, was alone able to slowly eliminate all of the microbes because it existed at relatively higher concentrations (i.e., 0.115 mg/liter). Also, chlorine dioxide is a more potent oxidizing agent and, therefore, is a potentially stronger biocidal agent than free chlorine (20). While chlorine dioxide inoculation was sufficient for killing all of the test organisms, the combined effects of free chlorine and chlorine dioxide elicited a rapid (i.e., 100% microbial elimination within 4 h from free Cl plus ClO_2 inoculation) biocidal response to the microbial challenge of approximately 3×10^5 CFU/ml (Fig. 4C). According to a formula developed by Spector et al. (33), when free Cl and ClO_2 are combined, the biocidal action is synergistic. Because the plasma membrane is the major target site for many antibacterial and antifungal agents, one cause of the antimicrobial synergism may be that one of the CBSs may have affected the cell membrane and facilitated the entry of the other (22, 30). The synergistic biocidal action may also have been due to sequential blocking of one or more metabolic pathways (22).

The five genera of microbes possessed different survival rates and therefore different susceptibilities to chlorine-based biocides. *P. aeruginosa* had the highest level of resistance; this was followed by *C. albicans*, *E. coli*, and *P. mirabilis*, and lastly, *S. saprophyticus* was the microbe group with the least resistance. The reasons for the differences in the susceptibilities of the microbial genera to free chlorine and chlorine dioxide were likely due to the differences in the relative compositions of the cell walls. The outer cell envelopes of gram-positive bacteria (like *S. saprophyticus*) are mainly composed of peptidoglycan, with lesser amounts of lipids than those found in gram-negative bacteria (31). This small percentage of lipids in *S. saprophyticus* did not likely act as an adequate barrier to the CBSs, as it did in the lipid-rich cell walls of the gram-negative bacteria *P. aeruginosa*, *E. coli*, and *P. mirabilis* (31). Therefore, the CBSs were probably able to penetrate the outer and cytoplasmic membranes and reach their site of biocidal action in *S. saprophyticus* (30). *P. aeruginosa*, however, displayed the highest level of resistance to the biocidal effects of free chlorine and chlorine dioxide. The reason for this elevated level of resistance may be due to the very low number of pore complexes in the cell wall in contrast to the numbers in other microbes, thereby providing only a very low surface area of channels available for the diffusion of the hydrophilic molecules of biocidal chlorine (31). Since only a small portion of the biocidal chlorine may be able to pass through the cell wall and interact with the inner cell membrane, where the antimicrobial action likely occurs, the susceptibility of *P. aeruginosa* was greatly reduced (31). The fungus, *C. albicans*, also had an elevated level of resistance to the killing effects of free chlorine and chlorine dioxide, but little is known about the factors that limit the susceptibilities of fungi to biocides except that the plasma membrane is likely to be a major target site (30).

With our results we have answered the question of whether or not iontophoretically produced chlorine substances are factors responsible for the iontophoretic killing of microbes. Not only did we find that chlorine-based biocides are responsible for microbial killing but we have identified and quantified the concentrations of these substances. This is an important finding because the use of this type of technology is on the rise. For example, iontophoresis has been recently used on patients as a method of physical therapy, and some commercial localized drug delivery systems use iontophoretic technology (3, 19, 21). Many chlorinated compounds that are produced by the iontophoretic current have been shown to have toxic effects on humans, and the chlorination of solutions has recently been blamed by the Environmental Protection Agency for the creation of trihalomethanes, which are potent carcinogens (15, 28, 35). Therefore, studying the types and concentrations of the iontophoretically produced chlorinated compounds may allow us to discover and prevent any possible deleterious effects on patients undergoing treatments that use this technology. We have applied the iontophoretic system to a catheter design that may be able to reduce or eliminate morbidity and mortality because of catheter-induced nosocomial UTI. We are investigating the potential toxic effects of the chlorine-based biocides on human tissue and are conducting studies that test the in vivo effectiveness of our catheter design in sheep. Preliminary results suggest that a significant microbial population reduction occurs with no major deleterious effects on the sheep. We are also pursuing studies on the in vitro detection, quantification, and microbial killing efficiencies of the products of iontophoresed human urine. Early results suggest that chlorine biocide production in iontophoresed human urine produces the same or a similar antimicrobial property. Once these studies have been completed, we hope to begin human clinical studies to test the practicality of the iontophoretic catheter.

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