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Spectroscopic Characterization and the pH Dependence of Bactericidal Activity of the Aqueous Chlorine Solution

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Raman spectroscopic analysis has been used to identify the chemical species that exist in aqueous chlorine solution. The pH dependence of the Raman spectra obtained indicates that there is an equilibrium among hypochlorite ion, hypochlorous acid and chlorine. Bactericidal activities of the acidic electrolyzed water, which is generated by electrolysis of an aqueous NaCl solution, were evaluated in the pH range 2 – 9 against *Escherichia coli* K12 and *Bacillus subtilis* PCI219 by a semi-quantitative bioassay. The maximum activity was observed between pH 4 and 5 in both bacteria. The Raman and the ultraviolet spectroscopic data, along with chemical analysis data, were used to conclude that the bactericidal activity is quantitatively correlated to the concentration of hypochlorous acid in solution.

Keywords Acidic electrolyzed water, bactericidal activity, hypochlorous acid, Raman spectra, UV spectra

The history of the utilization of chlorine for disinfection dates back to the mid 18th century. Chlorinated lime and chlorine gas have since been used for conventional disinfection of municipal drinking water for the prevention of epidemic diseases such as cholera and typhoid.[1-3] Generation of chlorine by electrolysis of aqueous NaCl solution had come into use by the end of the 19th century. The advantage of the electrolysis is that transportation and storage of potentially hazardous chemicals are not needed.

In recent years, compact equipments designed for the electrolysis of aqueous NaCl solution have become available commercially. The acidic solution generated in the anode chamber of the equipment, which will hereafter be designated as acidic *electrolyzed water* (EW), has been widely used in medical applications such as for the prevention of the infection of methicillin resistant *Staphylococcus aureus* (MRSA). Acidic EW has also been used for various agricultural purposes, such as sterilization of vegetables. Significant attention is now being paid to acidic EW for disinfection of drinking water in military or refugee camps in remote areas.

The microbicidal activity of the aqueous solution of chlorine has not been fully understood in a quantitative way. The aim of the present study is to establish the chemical basis of the microbicidal activity of the aqueous solution of chlorine. An attempt was made successfully to establish the chemical basis of the microbicidal activity of the chlorine solution. Raman and ultraviolet spectral measurements were used along with conventional chemical analyses to follow the pH dependence of the chemical equilibrium of the solution. A semi-quantitative bioassay system using suspension culture of bacteria was designed for statistical treatment of the inhibitory effects to bacterial growth. The discussion of the microbicidal activity of acidic EW will be based on the results obtained using *E. coli* and *B. subtilis.*

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Experimental

Reagents

All chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan), except for *p*-hydroxybenzoic acid (Tokyo Chemical Industry, Tokyo, Japan) and Bis-tris (Aldrich Japan, Tokyo, Japan). Except sodium hypochlorite solution (practical grade, chlorine concentration, 1.5 – 1.8 M) and sodium chlorite (80% purity), all the chemicals were of reagent

grade. Milli Q-grade water (Millipore, Bedford, MA, USA) was used in all experiments.

Procedure

The concentration of hypochlorous acid was determined by iodometric titration[4] and/or the *o*-tolidine method.[5] In the latter method, a colorimetrical measurement was made at 437 nm employing a commercially available reagent set (Reagent Set for Water Analysis LR-11, Kyoritsu Chemical-Check Laboratory, Tokyo, Japan).

The concentration of chlorine determined here represents the sum of the concentrations of Cl_2, HClO and ClO^- and hereafter is referred to as the available chlorine concentration. The unit of concentration used is mM of HClO.

The chlorite and chlorate ions were quantitated by ion-chromatography after removing the chloride ions by passing acidic EW through a Ag-bonded resin cartridge.[6] Ninety milliliters of acidic EW was transferred into a 100 ml volumetric flask and 5 ml of NaOH (100 mM), 0.5 ml of ethylenediamine (400 mM) used as a masking agent for the hypochlorous acid and pure water were added to give a total volume of 100 ml. This solution was passed through a Ag-bonded resin cartridge (On-guard Ag, Dionex, Sunnyvale, USA) to remove chloride ions. An aliquot of the eluate was injected into an ion-chromatograph under similar conditions to those used for the chloride ion analysis, except that a mixture (pH 8.2) of *p*-hydroxybenzoic acid (2.2 mM) and 2-diethylaminoethanol (2.0 mM, Tokyo Chemical Industry) was used as an eluent at 35˚C.

The concentration of the perchlorate ion was also quantitated without any pretreatment by ion-chromatography.

The concentration of sodium ion was quantitated with an ion-chromatograph (LC-10A, Shimadzu) equipped with a cation-exchange column (Shim-pack IC-C3, Shimadzu) and a conductivity detector. The cations in acidic EW was eluted with oxalic acid (2.5 mM) at a flow rate of 1.0 ml min^{-1} at 40˚C.

An aqueous NaCl solution (20 mM, 1.4 l) was electrolyzed (7.3 V/850 mA) for 7 min at ambient temperature using a batch-type electrolyzer (Superwater mini, Janix, Atsugi, Japan) as previously described.[7] Freshly prepared acidic EW was used without delay in all experiments. The pH and oxidation-reduction potential (ORP) were measured with a pH meter (M-13, Horiba, Kyoto, Japan) using a glass-electrode (6366-10D, Horiba) and a platinum-electrode (6861-10c, Horiba), respectively. The acidity was determined by acid-base titration[4] with an end-point of pH 3.7 and was represented as the amount of the NaOH consumption. Dissolved oxygen (DO) value was measured with a DO meter (OM-14, Horiba) using a diaphragm-electrode (5420-10D, Horiba) at 23˚C.

Prior to ion-chromatography analysis, acidic EW was reacted with sodium sulfite, reducing the hypochlorite ion to the chloride ion. From the choride ion concentration determined by ion-chromatography, the initial concentration of the chloride ion was calculated by subtracting that of the chloride ion newly generated from hypochlorous acid. One milliliter of acidic EW was transferred into a 100-ml volumetric flask and 10 ml of sodium sulfite (10 mM) in carbonate buffer (100 mM, pH 10)[6] was added. The mixture was diluted with water, making a total volume of 100 ml. An aliquot of this solution was injected into an ion-chromatograph (LC-10A, Shimadzu, Kyoto, Japan) equipped with an anion-exchange column (Shim-pack IC-A3, Shimadzu) and a conductivity detector. Anions were eluted at a flow rate of 1.2 ml min^{-1} at 40˚C using an aqueous solution at pH 4.4 of *p*-hydroxybenzoic acid (8.0 mM) and Bis-tris (3.2 mM).

For Raman spectra, a commercially available sodium hypochlorite solution (2 ml) was mixed with a 500 mM aqueous solution (2 ml) of Na_2SO_4. The pH of this solution was adjusted to various pH values, ranging from 2.6 to 10.5, with a 100 mM HCl solution to make 0.15 M solution of hypochlorite (total volume 20 ml). The sample solution (2 ml) was sealed into an ampoule in order to avoid the evaporation of molecular chlorine. The Raman intensity was scaled using the S–O stretching band of SO_4^{2-} at 983 cm^{-1} as an internal standard.

The sample solution (500 ml) was mixed with 500 ml of a NaOH solution (5 mM) containing NaCl (20 mM) for UV spectra. An aliquot (45 ml) of this solution was adjusted to pH 4.5 to 9.8 with HCl (20 mM) to a total volume of 50 ml with NaCl (20 mM). The initial concentrations of the available chlorine of acidic EW used in these measurements were iodometrically quantitated. Based on the results of the analyses, the available chlorine in these acidic EW at various pH values were estimated to be 0.5 mM.

A commercially available sodium hypochlorite solution was diluted with a 20 mM aqueous solution of NaCl to make 1.1 mM solution of hypochlorite. The pH of this solution was adjusted to various pH values ranging from 4.1 to 9.9 as described above in the case of acidic EW.

The time course and pH-profile of the bactericidal activity of acidic EW were compared with those obtained using the authentic hypochlorous acid solution. Acidic EW preparations used in the experiments were at pH 2.7 with an available chlorine concentration of 1.2 mM and a NaCl concentration of 20 mM. Hypochlorous acid solutions at identical pH with the identical concentrations of available chlorine and NaCl were prepared using a commercially available sodium hypochlorite solution.

Escherichia coli K12 and *Bacillus subtilis* PCI219 were used to examine the bactericidal activity of acidic EW. In the case of *E. coli*, acidic EW solutions and hypochlorous acid solutions contained available chlorine at a concentration of 6 or 10 μM at pH values ranging from 2.7 to 9.0. The sample solutions were prepared by diluting acidic EW or a commercial sodium

hypochlorite solution using three kinds of 10 mM buffer solutions: phosphate buffers (pH 3.5, 6.0, 7.0 and 8.0), acetate buffers (pH 4.0 and 5.0) and carbonate buffer (pH 9.0). The buffer solutions were filtered (0.22 μm) and stored at 4˚C until use. During dilution, NaCl was also added until the final solutions contained 20 mM NaCl.

In the case of *B. subtilis* PCI219, higher concentrations of available chlorine of 0.5 or 0.8 mM were used. The same pH ranges were used as in the case of *E. coli*. The sample solutions were prepared by diluting acidic EW and sodium hypochlorite solutions using the same buffer solutions as employed above for *E. coli* with a higher concentration of 0.5 or 1.0 M. A 0.5 or 1.0 M stock buffer solution was added to acidic EW or sodium hypochlorite solution to make a final solution of 50 ml at a buffer concentration of 10 mM. The pH of the solution was adjusted to 3.5 – 9.0 using 0.1 or 1.0 M NaOH aqueous solution. During dilution, NaCl was added until the resulting solutions contained 20 mM NaCl.

Aqueous solutions of chlorous acid, chloric acid and perchloric acid (each 11 μM, pH 2.7, containing 20 mM NaCl) were also prepared for the evaluation of bactericidal effects. All the sample solutions were freshly prepared, kept in glass test tubes, encapped without any headspace and were used within 10 min for the bactericidal assays.

E. coli K12 cells were propagated in nutrient broth (Nutrient broth "Eiken", Eiken Chemical, Tokyo, Japan) for 18 – 20 h at 37˚C. The culture was centrifuged at 2600×*g* for 10 min at 4˚C and the resulting pellet was suspended in 0.85% NaCl solution. The suspension was centrifuged again and the pellet was resuspended in 0.85% NaCl solution. The cell number was adjusted to 1 – 5×10^9 ml^{-1}.

B. subtilis PCI219 cells were propagated in soy broth (Bacto Tryptic Soy Broth, Difco Laboratories, Detroit, MI, USA) containing 350 mg l^{-1} of $MnCl_2 \cdot 4H_2O$ on a rotary shaker at 180 rpm for 6 d at 27˚C to completely form spores. The culture was harvested by centrifugation at 2600×*g* for 10 min at 4˚C. The pellet was suspended in 0.85% NaCl solution and was vortexed. The cell was then heated for 30 min at 70˚C and stored at 4˚C until use. The number of *B. subtilis* PCI219 spores to form a colony was adjusted to 1 – 5×10^9 ml^{-1} after washing with 0.85% NaCl solution.

In order to follow the time course of bactericidal effects against *B. subtilis* PCI219, 1.35 ml of acidic EW or hypochlorous acid solution (1.2 mM, pH 2.7) with NaCl (20 mM) was added to 0.15 ml of cell suspension (1×10^9 ml^{-1}) in a 1.5 ml-sterilized microtube. The suspension was shaken quickly and left for 0 to 60 s at ambient temperature. Then 1 ml of suspension was added to a 4 ml solution of nutrient broth to quench the bactericidal effect with vigorous mixing. The suspension was incubated for 3 h at 37˚C in a water bath shaker at 170 strokes min^{-1}.

For the assay of the bactericidal effects against *E. coli* K12, 0.9 ml of diluted acidic EW or an aqueous hypochlorous acid containing NaCl (20 mM) was added with vigorous mixing to 0.1 ml of cell suspension (1×10^9 ml^{-1}) in a sterilized 15 ml centrifuge tube and this mixture was left at ambient temperature for 5 s. Then 4 ml of nutrient broth was added to the suspension with vigorous mixing to quench the effects of diluted acidic EW or hypochlorous acid and incubated for 2 h as described above. For the same assay using *B. subtilis* PCI219, 1.35 ml of diluted acidic EW or an aqueous hypochlorous acid containing NaCl (20 mM) was added to 0.15 ml of cell suspension (1×10^9 ml^{-1}) in a sterilized 1.5 ml microtube. The suspension was shaken vigorously and left for 1 min at ambient temperature. Then 1 ml of the suspension was treated and incubated for 3 h as described above. After incubation, absorbance of the cultures was measured at 600 nm using a Shimadzu UV-1600 spectrophotometer.

Evaluation of bactericidal effects of the chlorous, chloric and perchloric solutions (11 mM, pH 2.7) was performed in the same way.

Apparatus

Raman spectra were obtained at 25˚C using an excitation wavelength of 514.5 nm (cw, Ar-ion laser, laser power 600 mW, Spectra Physics) and the signal was dispersed using a single spectrograph (Instruments SA, HR 320; grating, 1800 line mm^{-1}) which was detected by a liquid nitrogen cooled CCD detector (Princeton Instruments, LN/CCD 1024 TKB).

Ultraviolet spectra were measured at 25˚C using capped 1 cm-quartz cells and a Shimadzu UV-1600 spectrophotometer.

Results and Discussion

Chemical characterization

The pH value of acidic EW used in the present experiment was 2.7. Based on the NaOH consumption recorded in the titration, the acidity of the acidic EW solution was calculated to be 2.3 mM. As shown in

Table 1 Chemical data of the acidic electrolyzed water

	Mean ± s.d.[a]	
pH	2.7±0.04	(*n*=10)
ORP(mV)	1170±5	(*n*=10)
Acidity(mM)	2.3±0.02	(*n*=10)
Dissolved oxygen(mV)	0.39±0.008	(*n*=7)
Cl^-(mM)	21.0±0.1	(*n*=7)
Available chlorine[b] (mM as HClO)	1.15±0.03	(*n*=10)
ClO_2^-(mM)	<0.0002	(*n*=5)
ClO_3^-(mM)	0.014±0.002	(*n*=5)
ClO_4^-(mM)	<0.0005	(*n*=3)
Na^+(mM)	18.8±0.1	(*n*=7)

a. The mean values and the standard deviation for *n* measurements.
b. The sum of Cl_2, HClO and ClO^-.

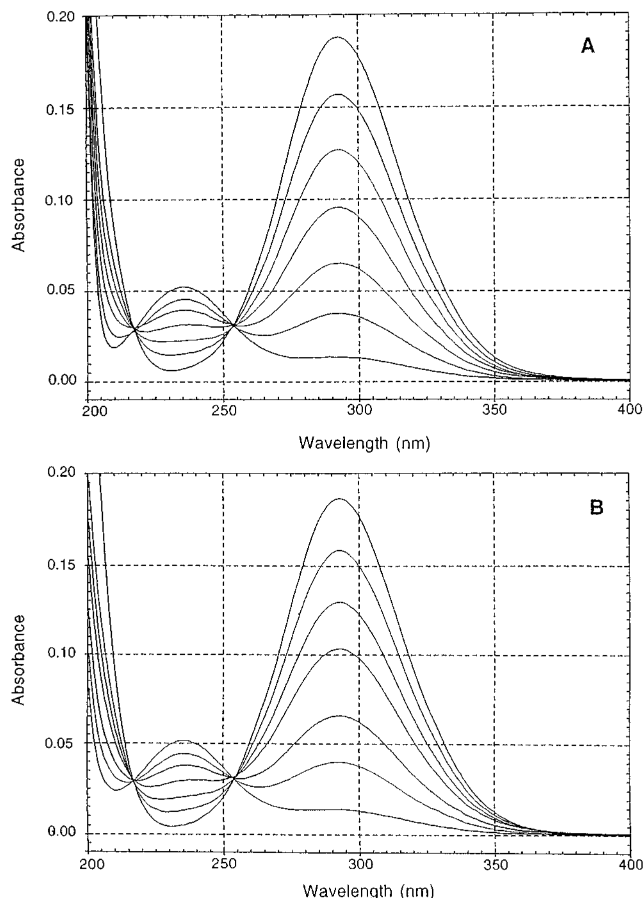

Fig. 1 UV spectra of diluted acidic EW (A) and sodium hypochlorite solution (B) at various pH. Observed in a 1-cm cell at 25˚C. The available chlorine concentration in all solutions is 0.51 mM. The peak at 292 nm decreases with decreasing pH with a concomitant increase of the intensity of the peak at 236 nm. The pH values are as follows: (A) 4.5, 6.8, 7.2, 7.5, 7.8, 8.2, 9.8; (B) 4.1, 6.8, 7.1, 7.5, 7.8, 8.1, 9.9.

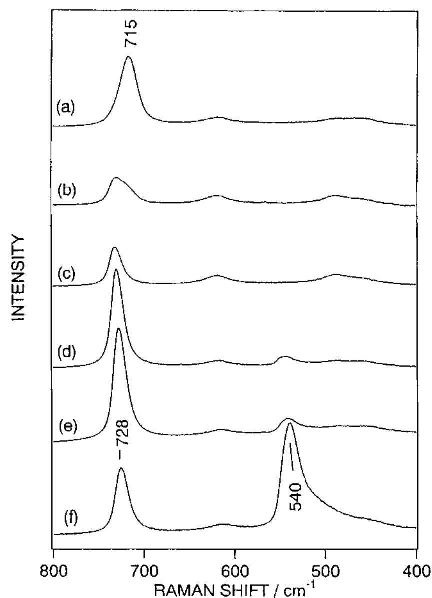

Fig. 2 Raman spectra of sodium hypochlorite solutions at pH (a) 8.7, (b) 7.9, (c) 7.5, (d) 5.9, (e) 3.9 and (f) 2.6 upon acidification by HCl. The spectra were scaled using the intensity of the band at 983 cm^{-1} of SO_4^{2-} (internal standard).

Table 1, the ORP value was 1170 mV, which was higher than that of 20 mM NaCl (*ca.* 400 mV) observed prior to electrolysis. Dissolved oxygen concentration was 0.39 mM. This value was higher than that of 20 mM NaCl air-saturated at 23˚C (*ca.* 0.27 mM).

The chloride and sodium ion concentrations were 21 and 19 mM, respectively. The chlorine concentration was 1.2 mM (as HClO), whereas that of the chlorate ion was 0.01 mM. The chlorite and perchlorate ions were not detectable.

The UV spectra of diluted acidic EW observed at various pH values showed two absorption maxima at 292 and 236 nm with two isosbestic points at 254 and 217 nm. See Fig. 1A. With a decrease in pH, the peak at 292 nm decreased in intensity, with a concomitant increase in the intensity of the peak at 236 nm.

A standard [HClO/ClO^-] solution was prepared using commercial sodium hypochlorite solution and was used to make similar UV spectral measurements. As Fig. 1B indicates, the acidic EW solution and the [HClO/ClO^-] solution gave the identical spectral features with the identical pH dependence and the identical isosbestic points. The values of molecular extinction coefficient are also the same for the two solutions. The results obtained here are quite consistent with those reported for hypochlorous acid by Adam and Gordon.[8] The pK_a value of hypochlorous acid reported by them is 7.4 at 25˚C.

For more detailed spectroscopic identification of chemical species that exist in EW, Raman spectra were observed. We primarily focus our attention on the bands due to HClO, ClO^-, and Cl_2 for the chemical identification of EW. In Fig. 2, which reproduces Raman spectra in the 800 – 400 cm^{-1} region, the Cl–O stretches of HClO and ClO^- and the Cl–Cl stretch of Cl_2 are observed at 728, 715 and 540 cm^{-1}, respectively. Spectral changes observed upon acidification may be summarized as follows: 1) The intensity of the band at 715 cm^{-1} became weaker as the pH was lowered and this band was shifted to 728 cm^{-1} at around pH 8. The observed spectral change reflects the protonation of ClO^-. 2) The 728-cm^{-1} band of HClO appeared at around pH 8; its intensity became stronger as the pH was lowered and showed the maximum at around pH 5. 3) The 540-cm^{-1} band of Cl_2 appeared below pH 6.5 and this intensity became stronger as the pH was lowered.

The population of HClO, ClO^- and Cl_2 at different pH values can be estimated using the signal intensities observed at 728, 715 and 540 cm^{-1}, respectively. The results observed in the range of pH 2.6 – 10.5 are shown in Fig. 3. The pH profiles clearly indicate that the major chlorine species are Cl_2 below pH 3, HClO in the pH range 4 – 7 and ClO^- above pH 8.5.

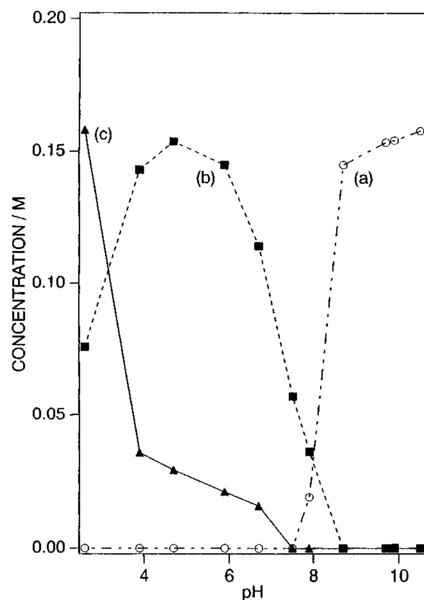

Fig. 3 The pH profiles of the concentrations of (a) ClO^-, (b) HClO and (c) Cl_2 in sodium hypochlorite solution upon acidification by HCl. The intensity of the Cl–O stretching band observed at 715 cm^{-1} (ClO^-) and at 728 cm^{-1} (HClO) and the Cl–Cl stretching band at 540 cm^{-1} (Cl_2) were used for estimating the concentration of each of the chemical species. The areas of each of the bands were calculated using a Lorentzian curve fitting technique. $I(ClO^-, 715\ cm^{-1})/I\ (HClO, 728\ cm^{-1})=0.7$ for equimolar aqueous solutions of ClO^- and HClO was obtained from the spectral changes observed upon deprotonation of HClO by adding solid sodium hydroxide to the Cl_2 aqueous solution (data not shown). It is difficult to estimate the concentration of Cl_2 exactly and therefore $I(Cl_2, 540\ cm^{-1})/I\ (HClO, 728\ cm^{-1}) \simeq 1.0$ for an equimolar aqueous solution of Cl_2 and HClO was a rough scaling factor estimated by the Raman spectrum of Cl_2 aqueous solution (data not shown).

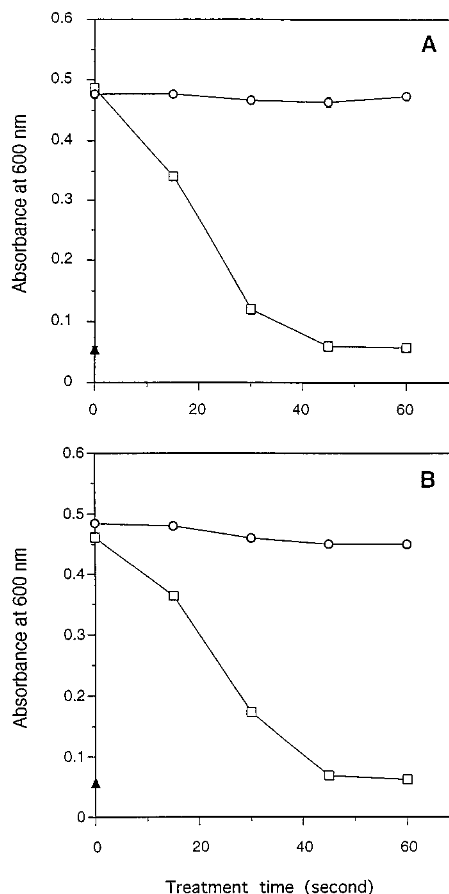

Fig. 4 Growth of *Bacillus subtilis* treated with acidic EW (A) and hypochlorous acid solutions (B) with varied treatment times. A cell suspension (*ca.* 1×10^8 ml^{-1} 0.85% NaCl aq.) of *B. subtilis* PCI219 was treated with the acidic EW containing 1.3 mM hypochlorous acid and with 1.3 mM hypochlorous acid solution for varied treatment times at ambient temperature. pH value of the acidic EW resulted from electrolysis for 7 min was 2.7, so that the pH of the hypochlorous acid solution was adjusted to 2.7 with 2.5 mM (final concentration) HCl in 20 mM NaCl. Initial absorbance value corresponds to about 2×10^7 ml^{-1} of *B. subtilis* cells in nutrient broth. The values represent means±S.E. of the results from duplicate experiments with four replicates. Symbols: A, □, acidic EW; B, □, 1.3 mM hypochlorous acid; ○, control; ▲, initial absorbance at 600 nm before incubation.

In the Raman spectra, the Cl–O stretching bands were observed at 933 and 800 cm^{-1}, which respectively correspond to ClO_3^- and ClO_2^- that are contained in the commercially available sodium hypochlorite solution used in the present experiment. We also observed weak bands at around 620 and 485 cm^{-1} due to ClO_3^- (data not shown).

On the basis of the chemical and spectroscopic data obtained, we conclude that the major component of the acidic EW is Cl_2/HClO in chemical equilibrium at given pH values.

Bactericidal activity

The bactericidal activity of acidic EW was quantitatively compared with that of hypochlorous acid by a semi-quantitative bioassay system which was established in this experiment using a suspension culture of bacteria. Since the bioassay system employed in the present experiment utilizes a linearity of the bacterial growth under constant conditions, the obtained data can quantitatively be dealt with by a statistical treatment.

In comparison with a conventional culture-plate method, the bioassay system used here is suited for the examination of many samples because the following procedures are not necessary: spreading cells on the culture plate, dilution of treated cells for the culture on the plate, overnight incubation at 37°C for the growth on the plate and counting the colonies to calculate growth inhibition.

The time course of the growth inhibition with acidic EW was followed using *E. coli* and *B. subtilis* cells. Figure 4A shows a typical result obtained for *B. subtilis*. In this experiment the turbidity of the culture medium was measured at 600 nm after 3-h incubation at 37°C. The initial absorbance value corresponded to a cell number of about 2×10^7 ml^{-1} suspended in nutrient

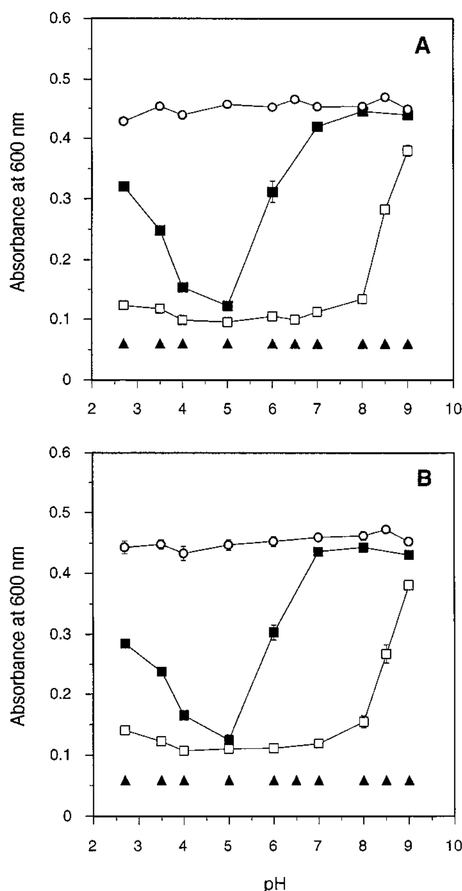

Fig. 5 Growth of *Escherichia* coli treated with diluted acidic EW (A) and hypochlorous acid solutions (B) with varied pH values. A cell suspension (0.1 ml; *ca.* 1×10^8 ml^{-1} 0.85% NaCl aq.) of *E. coli* K12 was treated with 0.9 ml of diluted acidic EW containing 10 and 6 μM hypochlorous acid (A) and 10 and 6 μM hypochlorous acid solutions prepared from reagent sodium hypochlorite (B) for 5 s at ambient temperature. pH values of the diluted acidic EW and hypochlorous acid solutions were adjusted with 10 mM phosphate buffer (pH 3.5, 6.0, 7.0 and 8.0), with 10 mM acetate buffer (pH 4.0 and 5.0) and with 10 mM carbonate buffer (pH 9.0). Then, the suspensions were added with 4 ml of nutrient broth to terminate the effect of the acidic EW and hypochlorous acid, and incubated in a water bath shaker at 37˚C. After incubation for 2 h, the absorbance at 600 nm was measured at 25˚C. Initial absorbance value corresponds to about 2×10^7 ml^{-1} of *E. coli* cells in nutrient broth. The values represent means±S.E. of the results from duplicate experiments with four replicates. Symbols: A, □, diluted acidic EW containing 10 μM HClO; ■, diluted acidic EW containing 6 μM HClO; B, □, 10 μM HClO; ■, 6 μM HClO; ○, control; ▲, initial absorbance at 600 nm before incubation.

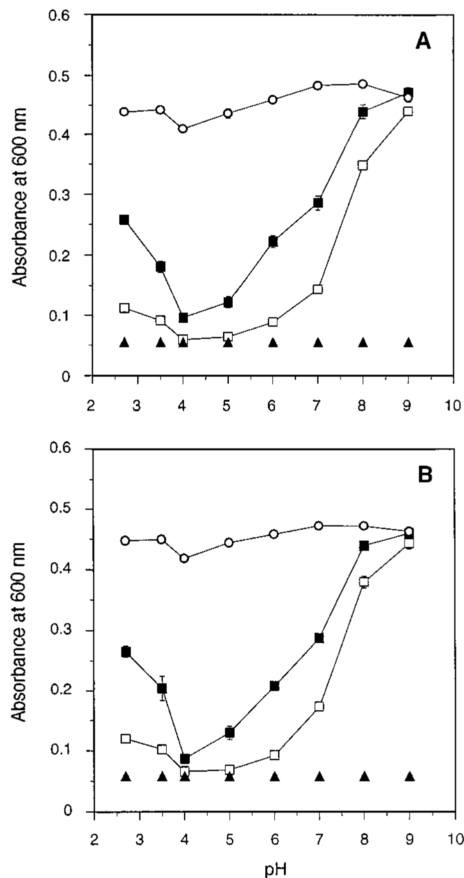

Fig. 6 Growth of *Bacillus subtilis* treated with diluted acidic EW (A) and hypochlorous acid solutions (B) with varied pH values. A cell suspension (0.15 ml; *ca.* 1×10^8 ml^{-1} 0.85% NaCl aq.) of *B. subtilis* PCI219 was treated with 1.35 ml of diluted acidic EW containing 0.8 and 0.5 mM hypochlorous acid (A) and 0.8 and 0.5 mM hypochlorous acid solutions prepared from reagent sodium hypochlorite (B) for 1 min at ambient temperature. pH values of the diluted acidic EW and hypochlorous acid solutions were adjusted in the same manner described in Fig. 5. Then 1 ml of the suspensions was added to 4 ml of nutrient broth to terminate the effect of the acidic EW and hypochlorous acid, and the mixture was incubated in a water bath shaker at 37˚C. After incubation for 3 h, the absorbance at 600 nm was measured at 25˚C. The initial absorbance value corresponds to about 2×10^7 ml^{-1} of *B. subtilis* cells in nutrient broth. The values represent means±S.E. of the results from duplicate experiments with four replicates. Symbols: A, □, diluted acidic EW containing 0.8 mM HClO; ■, diluted acidic EW containing 0.5 mM HClO; B, □, 0.8 mM HClO; ■, 0.5 mM HClO; ○, control; ▲, initial absorbance at 600 nm before incubation.

broth. The growth of *B. subtilis* cells was inhibited by a 45-s treatment with acidic EW.

A solution was prepared with the same amount of hypochlorous acid and NaCl as in the case of acidic EW used in the above experiments. The pH was adjusted at the identical pH as that of acidic EW and the time course of the growth inhibition was followed using *B. subtilis* cells. As Fig. 4B indicates, no significant difference was observed for the time course for the experiments performed using acidic EW and the hypochlorous acid solution.

Figure 5 compares the pH profiles of the growth of *E. coli* K12 obtained using acidic EW and hypochlorous solutions. In these two kinds of solutions the concentration of NaCl and the pH were the same as described above for the observation of the time course of the growth inhibition of *B. subtilis*. The initial absorbance

Table 2 Growth of *E. coli* cells after treatment with oxo acids of chlorine

	Absorbance at 600 nm	
Hypochlorous acid[a]	0.119±0.006[c]	(*n*=6)
Chlorous acid[a]	0.360±0.004[c]	(*n*=6)
Chloric acid[a]	0.424±0.006[c]	(*n*=6)
Perchloric acid[a]	0.426±0.004[c]	(*n*=6)
Control[b]	0.430±0.006[c]	(*n*=6)

a. Concentrations of the oxo acids were adjusted to 11 μM, pH 2.7.
b. Free from the oxo acids, pH 2.7.
c. The mean values and the standard deviation for *n* measurements.

value for the cell suspensions corresponded to about 2×10^7 cells ml^{-1} in nutrient broth. The *E. coli* cells were treated for 5 s; the concentrations of hypochlorous acid were 6 and 10 μM. Treatment of the *E. coli* cells with NaCl (20 mM) for 5 s was conducted as the control experiment.

Growth of *E. coli* was strongly inhibited in the pH region between pH 2.7 to 7. Above pH 7, growth of *E. coli* was observed to stay near the control level. The maximum growth inhibition was observed in the pH range 4 – 5. It should be noted that the pH profiles obtained using acidic EW and hypochlorous solutions are virtually identical. This result indicates that the bactericidal activity of acidic EW can quantitatively be explained by the concentration of hypochlorous acid that exists in the acidic EW.

Similar experiments were performed using *B. subtilis* PCI219 cells. Much higher concentrations of hypochlorous acid (0.6 or 0.8 mM) were used with about 2×10^7 cells ml^{-1}. The results obtained are quite similar to those for the *E. coli* system. See Fig. 6. Strong inhibition was observed between pH 2.7 to 6. Above pH 7, the growth of *B. subtilis* cells recovered to the control level. The maximum growth inhibition was observed in the pH range 4 – 5.

The results obtained using *B. subtilis* again established that the bactericidal activity of acidic EW was quantitatively correlated to the concentration of hypochlorous acid in solution.

We also examined the growth inhibitory effect of other members of oxo acids of chlorine: chlorous acid, chloric acid and perchloric acid. The results are summarized in Table 2. Chlorous acid possessed a small but significant bactericidal activity, whereas no bactericidal activity was detected for chloric acid and perchloric acid. In acidic EW, it was possible to detect chloric acid at a low concentration, in addition to hypochlorous acid. It was also possible that hydrogen peroxide was generated by the electrolysis of an aqueous NaCl solution. In the present experiments, no efforts were made to detect a minute amount of hydrogen peroxide in acidic EW. However, even if it was generated by electrolysis, it was quite unlikely for it to survive in solution for a significant amount of time because it must have been scavenged by free chlorine immediately.[9]

It was reported that hydroxy radicals were detected in the electrolyzed strong acid aqueous solution by electron spin resonance spectroscopy, and the generation of the hydroxy radicals was associated with sulfate ions contained in tap water. But hydroxy radicals were not observed in the electrolyzed water that was prepared with pure water and NaCl.[10] The high bactericidal activity of the acidic electrolyzed water that was prepared with pure water and NaCl was observed in the present experiments, as shown in Fig. 5A. The acidic electrolyzed water that was prepared with pure water and Na_2SO_4 showed little bactericidal activity (data not shown). These results suggest that the bactericidal activity of acidic electrolyzed water was not directly related to the hydroxy radicals.

On the basis of the results of chemical, spectroscopic and biological analyses, we conclude that 1) the microbicidal activity of EW can primarily be explained by the chemical equilibrium of Cl_2, HClO and ClO^-; 2) the major avtive component of acidic EW is Cl_2; 3) the microbicidal activity becomes maximum between pH 4 and 5, where the concentration of hypochlorous acid becomes maximum; and therefore 4) the microbicidal activity of acidic EW is quantitatively correlated to the concentration of hypochlorous acid that exists in solution. Whether hypochlorous acid is directly related to the bactericidal activity or one or more chemical species that are produced as secondary products from hypochlorous acid are actually responsible for the activity remains to be clarified.

The authors thank Professor Hiro-o Hamaguchi, Department of Chemistry, University of Tokyo, for the use of his Raman spectrometer and useful discussion.

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(Received March 20, 1998)
(Accepted May 12, 1998)