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Decontamination of Aflatoxin-Forming Fungus and Elimination of Aflatoxin Mutagenicity with Electrolyzed NaCl Anode Solution

TETSUYA SUZUKI,*,† TAKASHI NORO,† YUKIO KAWAMURA,‡ KENJI FUKUNAGA,§
MASUMI WATANABE,† MARI OHTA,† HISASHI SUGIUE,† YURI SATO,†
MASAHIRO KOHNO,‖ AND KUNIMOTO HOTTA⊥

Laboratory of Food Wholesomeness and Soundness, Division of Life Sciences, Graduate School of Fisheries Science, Hokkaido University, Minato, Hakodate 041-8611, Japan, Laboratory of Nutrition, Faculty of Agriculture, Kinki University, Nara, Japan, Laboratory of Public Health, Kansai Medical University, Moriguchi, Osaka, Japan, Kochi University of Technology, Tosayamada, Kochi, Japan, and National Institute of Infectious Diseases, Toyama, Shinjyuku, Tokyo

Electrolysis of a 0.1% (17.1 mM) solution of NaCl using separate anode and cathode compartments gives rise to solutions containing active chemical species. The strongly acidic “anode solution” (EW(+)) has high levels of dissolved oxygen and available chlorine in a form of hypochlorous acid (HOCl) with a strong potential for sterilization, which we have investigated here. Exposing *Aspergillus parasiticus* at an initial density of 10^3 spores in 10 μL to a 50-fold volume (500 μL) of EW(+) containing ca. 390 μmol HOCl for 15 min at room temperature resulted in a complete inhibition of fungal growth, whereas the cathode solution (EW(−)) had negligible inhibitory effects. Moreover, the mutagenicity of aflatoxin B_1 (AFB_1) for *Salmonella typhimurium* TA-98 and TA-100 strains was strongly reduced after AFB_1 exposure to the EW(+) but not with the EW(−). In high-performance liquid chromatography analysis, the peak corresponding to AFB_1 disappeared after treatment with the EW(+), indicating decomposition of the aflatoxin. In contrast, the routinely used disinfectant sodium hypochlorite, NaOCl, of the same available chlorine content as that of EW(+) but in a different chemical form, hypochlorite (OCl^-) ion, did not decompose AFB_1 at pH 11. However, NaOCl did decompose AFB_1 at pH 3, which indicated that the principle chemical formula to participate in the decomposition of AFB_1 is not the OCl^- ion but HOCl. Furthermore, because the decomposition of AFB_1 was suppressed by pretreating the EW(+) with the OH radical scavenger thiourea, the chemical species responsible for the AFB_1-decomposing property of the EW(+) should be at least due to the OH radical originated from HOCl. The OH in EW(+) was proved by electron spin resonance analysis.

KEYWORDS: ***Aspergillus parasiticus*; electrolyzed NaCl solution; inactivation of aflatoxin B_1; mutagenicity**

INTRODUCTION

Securing the safety of food and animal feed is one of the most important factors in the welfare of humans and livestock. Among the many types of microorganisms that cause food borne disease, toxic fungi and their products (mycotoxins) threaten the health of humans and livestock by contaminating food and feed materials (*1*−*4*). One of the most highly potent mycotoxins, aflatoxin, is produced by *Aspergillus flavus* and *Aspergillus parasiticus* and causes both acute liver damage and liver cancer (*5*−*9*).

Developing measures to control mycotoxin contamination is a high priority for the food and animal feed industries. The most reliable method to prevent mycotoxicosis is to avoid the use of contaminated materials, to disinfect fungi (*10*, *11*), and to inactivate mycotoxin. In the control against aflatoxins, many researchers have proposed and/or attempted measures to inactivate them (*12*, *13*) or to suppress their production by fungi (*14*), for example, through exposure to ammonia vapor at high temperature (*15*). Most of the proposed methods are not necessarily practical, however, because they not only decompose aflatoxin but also deplete the quality of the food and feed materials themselves; furthermore, most proposed methods are expensive and energy-consuming. Therefore, research into effective and energy-saving measures of decomtanimation has attracted much interest recently (*16*, *17*).

With the aim of finding a secure, effective, and energy-saving method to disinfect mycotoxin-producing fungi and to inactivate

* To whom correspondence should be addressed. Tel: +81-138-40-5564. Fax: +81-138-40-5563. E-mail: ted@fish.hokudai.ac.jp.
† Hokkaido University.
‡ Kinki University.
§ Kansai Medical University.
‖ Kochi University of Technology.
⊥ National Institute of Infectious Diseases.

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mycotoxin, we examined the effect of an electrolyzed dilute NaCl solution. This solution is generated by electrolyzing dilute NaCl solution, usually around 15−20 mM, with commercially available electrolysis devices at between 3 and 50 V of dc for several minutes in either a single-compartment chamber (*18*) or a two-compartment chamber separated by an ion exchangeable diaphragm (*19*). When the two-compartment chamber is used, a strongly acidic solution, or the "anode solution" (EW(+)), containing hypochlorous acid (HOCl) forms in the anode compartment (*20*). The chemical species that form in the cathode compartment that forms the "cathode solution" (EW(−)) have not been fully characterized yet, but this solution exhibits some antioxidative effects. Miyashita et al. (*21*) reported an antioxidative effect of EW(−) on highly unsaturated fat and oils such as linoenic acid ethyl ester, docosahexaenoic acid ethyl ester, trilinolein, and so on. Shirahata et al. (*22*), on the other hand, reported superoxide dismutase- and catalase-like activities of the EW(−). In an initial test, we found that the EW(+) could successfully disinfect a mycotoxicosis-causing fungus and inactivate its mycotoxins. Here, we report that the EW(+) sterilizes *A. paratisicus* and eliminates the mutagenicity of aflatoxin B_1 (AFB_1) for *Salmonella typhimurium* strains TA-98 and TA-100.

MATERIALS AND METHODS

Chemicals and Reagents. Aflatoxins B_1, B_2, G_1, and G_2 were purchased from Sigma-Aldrich (St. Louis, MO). The chemicals used were high-performance liquid chromatography (HPLC)-grade acetonitrile (Wako Pure Chemicals, Osaka, Japan), HPLC-grade methanol (Wako Pure Chemicals), trifluoroacetic acid (G. R. grade, Nakalai Tesque, Kyoto, Japan), chloroform (G. R. grade, Nakalai Tesque), ethyl acetate (Nakalai Tesque), sodium chloride (G. R. grade, Nakalai Tesque), mannitol (G. R. grade, Wako Pure Chemicals), and thiourea (G. R. grade, Wako Pure Chemicals). The water used for electrolysis and other procedures was purified with a Milli-Q system (Millipore, MA). Other chemicals used were guranteed reagent grade purchased from Nakalai Tesque.

Safety Precautions. Aflatoxins are extremely toxic, mutagenic, and carcinogenic compounds. As a safety precaution, all neat aflatoxin reagents were handled in a glovebox or thoroughly controlled safety cabinet in a P2 level facility. Degradation of aflatoxin solutions was performed by mixing with 10% KOH in ethanol and subsequent autoclaving in the tightly sealed vials. However, after the inactivation effect of the electrolyzed anodic NaCl solution (EW(+)) was confirmed, aflatoxin was mixed with enough volumes of excess EW(+) to destroy aflatoxins in a tightly sealed vial, kept for 30 min, and then subjected to autoclaving at 121 °C for 20 min. Contaminated glassware, vials, tubes, etc. were sealed in high-security disposals, autoclaved at 121 °C for 20 min, and thereafter incinerated.

Preparation of Electrolyzed NaCl Solutions. Electrolyzed NaCl solutions were prepared by electrolyzing 0.1% (17.1 mM) NaCl solution at 9−12 V of direct current (dc) for 10 min using a two-compartment type batch scale electrolysis apparatus (Super Oxseed Labo, Aoi Electronic Corp., Kannami, Shizuoka, Japan) divided by an ion exchangeable diaphragm. Electrolyzed NaCl solutions were prepared 5−10 min before their use in tests to evaluate their disinfectant and decomposition properties against AFB_1-forming fungus and its product aflatoxins.

The general definition of available chlorine includes free form chlorine such as HOCl, Cl_2, and hypochlorite ion (OCl^-) that accept an electron and bound form chlorine such as chloramines. However, considering the fact that the abundance of chemical formulas mentioned above is largely dependent upon pH and pK_a for HOCl = 7.4 (*23*−*25*), we mean the available chlorine for EW(+) and for sodium hypochlorite (NaOCl) kept at pH 3 molar concentration of HOCl, whereas those for NaOCl kept at pH 11 and for EW(−) mean OCl^-. The available chlorine concentration of both the EW(+) and the EW(−) was measured by iodometry and by electrotitration using an "available chlorine meter" (Central Kagaku, Tokyo, Japan). The oxidation/reduction potential (ORP) and pH were also measured. Physicochemical data obtained from five measurements are presented in **Table 1**, and a detailed analysis of the properties of disinfection and deactivation of aflatoxin-forming fungus, *Aspergillus parasiticus*, and its products by the electrolyzed NaCl solutions is given in the Results and Discussion.

Table 1. Physicochemical Parameters of Electrolyzed NaCl Solutions[a]

parameter	anode solution	cathode solution	ultrapure water[b]
	EW(+)	EW(−)	
pH	2.50 ± 0.06	11.65 ± 0.12	5.82 ± 0.04
available chlorine (ppm)	39.4 ± 0.89	0.51 ± 0.08	ND
[mM]	[0.75 ± 0.02]	[0.01 ± 0.002]	[ND]
dissolved oxygen (ppm)	14.4 ± 0.99	1.9 ± 0.11	5.0 ± 0.27
ORP (mv)	1,164 ± 33.62	−878 ± 8.43	264 ± 26.48

[a] Data were presented as the mean ± SD for 5 measurements. Available chlorine for EW(+) represents HOCl in parts per million and millimolar and that for EW(−) in OCl^- anion (ppm and mM). ND, not detected. [b] Data for ultrapure water were obtained from water freshly prepared from a Milli-Q filtration apparatus (Millipore Corp.). Electrolysis was carried out for 10 min at room temperature in a diaphragm type apparatus (Superoxseed Labo) using 17.1 mM (0.1%) NaCl in ultrapure water prepared with a Milli-Q filtration apparatus.

Disinfection of *A. parasiticus* with Electrolyzed NaCl Solutions. *A. parasiticus* IFO-30179 (equivalent to ATCC15517) was used to examine the antimicrobial activity of the electrolyzed solutions against mycotoxin-producing fungus. All experimental procedures were carried out in an isolated safety box for biohazard prevention. Freeze-dried fungus spores, purchased from the Institute of Fermentation Osaka (Dosho-machi, Kita ward, Osaka, Japan), were suspended in sterilized distilled water at a concentration of 10^6 spores/mL and then inoculated into a liquid medium specific for *A. flavus* and *A. parasiticus* (AFPA medium that consisted of 20 g of yeast extract, 10 g of peptone, 0.5 g of ammonium ferric citrate, 100 mg of chloramphenicol, and 2 mg of 2.6-dichloro-4-nitroaniline in 1 L of 1.5% agar). After 2−10 h growth of the fungus, an aliquot was taken to estimate the cell population with an adenosine 5′-triphosphate analyzer (Toa-Electronics Inc., Tokyo), and the culture was diluted with sterilized distilled water to make a stock cell suspension containing 10^5 colony-forming units (cfu)/mL. For the disinfectant experiment, 10 μL containing 10^3 cfu from the stock cell suspension was separately mixed with 0.01, 0.02, 0.1, 0.2, 0.5, and 1 mL of the electrolyzed NaCl solutions. The EW(+) of 0.01−1 mL contained ca. 8, 15, 80, 150, 390, and 800 μmol HOCl, respectively. The estimated concentration of available chlorine in the form of the OCl^- anion in the 1-, 2-, 10-, 20-, 50-, and 100-fold volumes of EW(−) are ca. 0.1, 0.2, 1.1, 2.2, 5.5, and 11 nmol, respectively. In the control test, cell suspensions were exposed to 17.1 mM (0.1%) NaCl. After each exposure for 15 min, sterilized distilled 10 mM mannitol as a terminator was added to the suspension to a volume of 2 mL. Subsequently, 1.0 mL of each sample solution was inoculated on to AFPA agar medium and incubated for 5 days at 25 °C. The disinfection effect was evaluated by the occurrence of fungal colonies on the agar plate. As the fungal growth could not be determined precisely by cfu, the growth was presented semiquantitatively.

Treatment of Aflatoxins with the Electrolyzed NaCl Solutions or NaOCl. AFB_1 and a mixture of aflatoxin B_1, B_2, G_1, and G_2 were dissolved in chloroform in use. Aliquots containing 40 ng of the aflatoxin mixture (10 ng of each aflatoxin) or 10 ng of AFB_1 were placed in vials, and chloroform was evaporated under N_2. Volumes of EW(+) or NaOCl solution (10−2000 μL representing 8−1600 μmol of the active chlorine species) HOCl (*20*) in EW(+), and NaOCl solution kept at pH 3 or OCl^- in NaOCl solution kept at pH 11 were added to tubes containing the aflatoxins so that the molar ratio of "active chlorine" to aflatoxin ranged from 1 to 200. Tubes containing aflatoxins and EW(+) were allowed to sit for 10 min. Similar volumes (10−2000 μl) of EW(−) were added to separate aflatoxin-containing tubes, but the concentrations of "active chlorine" in the form of OCl^- in these tubes were a one hundredth of those of EW(+) in each case. Samples were then examined for aflatoxin content as described below. Separate experiments with 10 ng of AFB_1 samples were conducted in a similar manner except that incubation times (0, 2.5, 5, 10, and 60 min) and temperatures (0, 20, 30, and 45 °C) were varied. At the end of the incubation periods, absolute ethanol was added to all tubes so that a final volume of 1 mL was achieved. Samples were prepared for and analyzed by HPLC as described below.

Effect of Radical Scavengers. Assuming that reactive chemical species, such as OH radicals and/or Cl radicals originating from HOCl, are involved in the disinfectant process, we examined the effect of pretreatment of the EW(+) before exposure to AFB_1 with mannitol that has been regarded as OH radical specific scavenger (*26*) and thiourea that has been regarded as a universal radical scavenger (*27, 28*). Mannitol was added to the EW(+) at ratios of 1 to 10 (molar ratio of mannitol to available chlorine as HOCl); similarly, thiourea was added to the EW(+) at ratios of 0.03 to 1 (molar ratio of thiourea to available chlorine as HOCl). Exposure of AFB_1 to the EW(+) containing mannitol or thiourea was performed at room temperature for 10 min**,** and then, the reaction products were extracted with $CHCl_3$ and separated by HPLC to examine changes in AFB_1. See the chromatographic analysis section below for HPLC conditions.

Chromatographic Analyses of Aflatoxins Treated with Electrolyzed NaCl Solutions. *(a) High-Performance Thin-Layer Chromatography (HPTLC).* AFB_1 and a mixture of equal amounts of AFB_1, AFB_2, AFG_1, and AFG_2 that were exposed to different molar ratios of EW(+), EW(−), NaOCl used at pH 11, and NaOCl at pH 3 in the separate experiments as explained in the previous section were subjected to HPTLC. After aflatoxin was exposed to increasing molar ratios of the electrolyzed NaCl solutions, 1.0 mL of ethyl alcohol was added to the mixture, and 0.5 mL of ethyl acetate was added and shaken vigorously to extract aflatoxin and its reaction products into the organic solvent phase. Extraction with ethyl acetate was repeated three times. Ethyl acetate was carefully evaporated off under an N_2 gas stream, and then, 100 μL of fresh ethyl acetate was added. Twenty microliters of the ethyl acetate extract of aflatoxin/electrolyzed solution reaction mixtures and 20 μL of either the AFB_1 solution or the aflatoxin mixture, with or without exposure to electrolyzed solution, were applied to HPTLC plates (10 cm × 10 cm, silica gel 60 precoated plate; Merck Darmstadt, Germany) and developed with a 9:1 (v/v) mixture of $CHCl_3$, acetone for 7 cm. Aflatoxin analogues were detected as a bluish-white spot under UV-A (365 nm) illumination.

(b) HPLC. (b-1) HPLC of Derivatized AFB_1. Following up the results of HPTLC, the change of AFB_1 was further examined quantitatively on HPLC. Because preliminary experiments with HPTLC revealed that exposing EW(+) containing 24−56 μmol HOCl to 1.3 nmol AFB_1 diminished the AFB_1 spot gradually, the decrease of AFB_1 was quantitatively determined by HPLC after derivatization to trifluoroacetic acid. For the sample without any treatment as a control, 400 ng (1.3 nmol) of AFB_1 in 10 μL of ethanol was put in the sealed vial, and then, ethanol was evaporated off under N_2, followed by trifluoroacetylation with 20 μL of trifluoroacetic acid anhydride (G.R grade, Wako Pure Chemicals, Inc.) in a tightly sealed glass vial by vigorous shaking and then incubated for 15 min at room temperature in the dark. Next, 180 μL of a 1:9 (v/v) mixture of acetone/water was added. The solution was further mixed vigorously, and then, 20 μL of the reactant was subjected to HPLC. For exposure to EW(+), 400 ng (1.3 nmol) of AFB_1 in 10 μL of ethanol was taken; then, ethanol was evaporated off under N_2; then, 10, 20, 30, 40, 50, 60, and 70 μL of EW(+) containing 8, 16, 24, 32, 40, 48, and 56 μmol of HOCl, respectively, were added to react with AFB_1 for 15 min. After 15 min of reaction, reacted AFB_1 solutions were evaporated off under vacuum and then trifluoroacetylated by the same procedure as described for the AFB_1 without EW(+) treatment. HPLC was run on a Hitachi high-performance liquid chromatograph equipped with Intelligent Pump (Hitachi L-6200), a Hewlett-Packard HP1046A programmable fluorescence detector and integrator (Hitachi D-2500). Reacted samples were separated on a reversed-phase octadecyl silane column (Zorbax ODS, Du Pont Co.) (4.6 × 250 mm) and eluted with a 35:65 (v/v) mixture of acetonitrile and water at a flow rate of 1.0 mL/min at room temperature. AFB_1 was detected by fluorometry with excitation at 365 nm and emission at 412 nm. Twenty microliters was injected onto the column. Chromatographic analysis was run for three times.

To examine how the elimination of AFB_1 with EW(+) was affected by reaction temperature, free radical scavengers (mannitol or thiourea) AFB_1 were quantified on the HPLC without derivatization to save analysis time.

(b-2) Effect of Reaction Temperature with EW(+) and AFB_1 on the Elimination of AFB_1. To examine the effect of temperature on the deactivation of AFB_1 by EW(+), 25 μg (80 nmol) of AFB_1 was exposed to 0.17 μmol of HOCl in 0.1 mL of EW(+) for 5 and 60 min at 0 °C. The EW(+) was evaporated off under vacuo and then dissolved in 1 mL of ethyl acetate; 10 μL was injected onto the column. The decrease of AFB_1 (80 nmol) and the appearance of secondary product by the reaction with EW(+) (0.17 μmol) at 0, 20, 30, and 45 °C for 0−60 min were also monitored by HPLC.

(b-3) Effect of Pretreament of EW(+) with Radical Scavengers on the Elimination of AFB_1. To confirm the participation of free radical (OH radical) in EW(+) on the elimination of AFB_1 , 0−200 μL of 30 mM mannitol was added to 600 μL of EW(+) containing 0.38 μmol HOCl by the mixing molar ratio of 0-, 1-, 2-, 5-, and 10-fold for 10 min prior to exposure to AFB_1 (128 nmol).

To examine the effect of pretreatment with thiourea on the elimination effect of EW(+), 1 mM thiourea was added to 600 μL of EW(+) containing 0.38 μmol HOCl by the mixing ratios of thiourea/HOCl in EW(+) 0-, 0.03-, 0.1-, and 1-fold. Kept standing for 10 min at 0 °C, the thiourea-treated EW(+) was exposed to 128 nmol of AFB_1 for 10 min at 30 °C.

After 10 min of reaction with radical scavenger-treated EW(+) at 30 °C, the reactant was dissolved in 40 μL of chloroform, and then, 10 μL was injected onto an HPLC column to determine the remaining AFB_1.

AFB_1 solutions reacted with/without EW(+) and pretreatment of EW(+) with/without radical scavenger were analyzed on an HPLC equipped with a Hitachi L-6200 Intelligent Pump, fluorescence detector (Hitachi F-1050), Hitachi D-2500 Chromato-Integrator, and reversed-phase column ODS-80 (4.6 mm × 150 mm) (TOSOH TSK-Gel) without any chemical derivatization. Gradient elution was used at a flow rate of 1.0 mL/min with 30% acetonitrile in water from 0 to 40 min, followed by a linear increase in acetonitrile from 30 to 100% between 40 and 70 min. Eluants were monitored fluorometrically using excitation wavelength at 365 nm and emission wavelength at 450 nm.

For the radical scavenger experiments, elution peaks were monitored at 365 nm, but all other analytical conditions were the same as described above. The chromatographic run was repeated three times in each analysis.

Mutagenicity Test for *Salmonella typhimurium* TA-98 and TA-100. The mutagenicity of AFB_1 was evaluated by a conventional Ames test using *S. typhimurium* strains TA-98 and TA-100 (*29*). Cultures of *S. typhimurium* TA-98, containing a histidine frame-shift mutant, and TA-100, containing a histidine missense mutation (*30*), were purchased from the Institute of Fermentation, Osaka. After 200 ng of AFB_1 (0.64 nmol) was mixed with either 100 μL of EW(+) containing a 0.76 nmol equivalent of HOCl as the available chlorine and the mixture was kept standing for 10 min, a 20 μL aliquot was added to 500 μL of S9 mixture or phosphate buffer (pH 7.5 for S9 mixture), followed by 100 μL of *S. typhimurium* TA-98 or TA-100 strains containing 10^7 cfu/mL. For the control, 100 μL of dimethyl sulfoxide was added in place of the electrolyzed solution. The mixture was incubated for 20 min at 37 °C with shaking, and then, 2 mL of soft agar was added before inoculation on to Vogel−Bonner E agar medium (*31*) for 48 h at 37 °C. Mutagenicity was assessed by the numbers of colonies of revertant occurring in the presence of the S9 mixture. Other experimental conditions were the same as for the mutagenicity assay of AFB_1-2,3-dichloride by Swenson et al. (*32*). The mutagenicity test was repeated five times, and data were presented as the mean ± standard deviation (SD) for $n = 5$.

Identification of Radical Species by Electron Spin Resonance (ESR). The anode solution was analyzed by ESR spectroscopy to determine whether hydroxyl radicals ($OH^\bullet$) might be involved in its disinfection properties. The anode solution (200 μL) was mixed with

Table 2. Effect of Electrolysis Time on Physicochemical Properties of the Anode Solution [EW(+)][a]

electrolysis time (min)	available chlorine (μM)	pH	ORP (mV)
0	ND	5.50 ± 0.10	376 ± 29
1	ND	3.53 ± 0.08	983 ± 25
3	88 ± 22.2	2.94 ± 0.07	1065 ± 5
6	371 ± 17.4	2.66 ± 0.12	1109 ± 9
10	624 ± 14.5	2.56 ± 0.05	1120 ± 3
15	976 ± 25.4	2.50 ± 0.05	1157 ± 2
20	1617 ± 5.6	2.45 ± 0.07	1163 ± 1
30	2356 ± 26.1	2.37 ± 0.04	1165 ± 1

[a] A 17.1 mM (0.1%) NaCl solution was electrolyzed at 9–11 V dc at room temperature (25 °C) using a diaphragm type device (Superoxseed Labo). Data are the means ± SD for $n = 4$.

10 μL of an 890 mM solution of the spin-trapping agent DMPO (5,5-dimethyl-1-pyrroline-1-oxide) to make a spin adduct and then analyzed by ESR spectrometry using a JES-RE3X/ESR data system ESPRIT330 (JEOL) and previously described experimental conditions (*33*).

Namely, ESR spectra were recorded with a JEOL JES-RE3X that was controlled by a computer system JEOL ES-ESPRIT 300. Measurement conditions were as follows: magnetic field, 335.5 ± 5 mT; resonance frequency, 9.41 GHz; modulation frequency and modulation width, 100 kHz and 0.063 mT; microwave power, 4 mW; response time, 0.1 s; amplitude, × 300; sweep time, 2 min. The spin trap reagent, DMPO (8.9M), was purchased from Dojin Laboratories (Kumamoto, Japan).

ESR measurements were done under the experimental conditions. An 890 mM DMPO water solution, 10 μL, anode solution, 200 μL, and $FeSO_4$ solution, 50 μL, and a volume of 130 μL were mixed and were transferred to a flat quartz ESR cuvette. The cuvette was placed in an ESR spectrometer, and recordings were made at room temperature. The ESR parameter, hyperfine-coupling constant (hfcc) obtained from ESR spectra of spin adducts, was finally determined by the computer simulation by using hfcc assigned to a spin adduct (DMPO−OH) generated by the reaction of DMPO and hydroxyl radical (HO) in anode solution.

RESULTS AND DISCUSSION

Physicochemical Properties of Electrolyzed NaCl Solutions. The physicochemical parameters of the EW(+), EW(−), and ultrapure water used for electrolysis varied considerably (**Table 1**). The EW(+) was strongly acidic, had high levels of dissolved oxygen and available chlorine in the form of HOCl, and had a high ORP. By contrast, the EW(−) was strongly alkaline, had a low level of dissolved oxygen, and had an extremely low ORP (−878 mV). Available chlorine was almost negligible in the EW(−). In other words, the EW(+) is highly oxidative, whereas the EW(−) is highly reductive; in addition, the parameters of both of these solutions were distinctly different from those of the ultrapure water. The data presented in **Table 1** were obtained from the electrolysis of 0.1% (or 17.1 mM) NaCl for 10 min; however, the values of these parameters, in particular the available chlorine that functions as an oxidant, were dependent on the length of electrolysis time (**Table 2**).

Disinfection of *A. parasiticus* with Electrolyzed NaCl Solution. To disinfect 10^3 cfu of *A. parasiticus* completely, at least a 50-fold volume of the EW(+), providing 20−30 ppm of available chlorine, or equivalent to 380−580 μmol of HOCl, was required (**Figure 1**). In other words, at least 0.4−0.6 mM of HOCl in the EW(+) must be secured in the suspension to disinfect 10^3 cfu of *A. parasiticus* completely. By contrast, the EW(−) did not disinfect *A. parasiticus* (**Table 3**). These data were obtained from a model experiment.

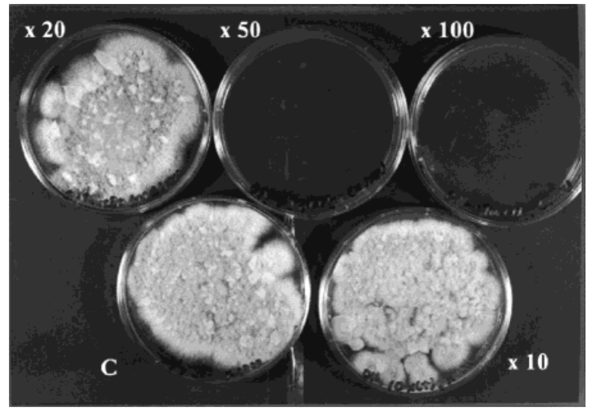

Figure 1. Disinfection of *A. parasiticus* by electrolyzed NaCl anode solution (EW(+)) *A. parasiticus* at a concentration of 10^3 cfu in 0.01 mL was immersed in increasing volumes of EW(+) for 12 h at room temperature. The solution was then inoculated on to AFPA agar medium and incubated for 5 days at 30 °C. C (lower left Petri dish): untreated control; ×10 (lower right Petri dish): 10-fold volume of EW(+), containing ca. 80 μmol HOCl; ×20 (upper left Petri dish), 20-fold volume of EW(+) containing 160 μmol HOCl; ×50 (upper middle Petri dish), 50-fold volume of EW(+) containing 390 μmol HOCl; ×100 (upper right Petri dish), 100-fold volume of EW(+) containing 800 μmol HOCl. Note white fluffy mycelia present on the control, ×10, and ×20 Petri dishes.

Table 3. Fungicidal Effect of Electrolyzed NaCl Solutions on *A. parasiticus*[a]

added solution	volume ratio of electrolyzed solution: control	×1	×2	×10	×20	×50	×100
anode solution	+++	+++	+++	+++	+++	±	–
cathode solution	+++	+++	+++	+++	+++	+++	+++

[a] Ten microliters of the cell suspension (10^3 cfu) was added to freshly prepared anode or cathode solution at different mixing ratios (0–100 times the volume ratio). The estimated concentrations of HOCl in the 10-, 20-, 50-, and 100-fold volumes of EW(+) are ca. 8, 15, 80, 150, 390, and 800 μmol, respectively. The estimated concentration of available chlorine in the form of OCl^- anion in the 10-, 20-, 50-, and 100-fold volumes of EW(–) are ca. 0.1, 0.2, 1.1, 2.2, 5.5, and 11 nmol, respectively. In the control test, cell suspensions were exposed to 0.1% NaCl. After each exposure, cells were then inoculated on to AFPA agar plates and incubated at 25 °C for five days. As the fungal growth could not be determined precisely by cfu, the growth is presented semiquantitatively: +++, full growth on plate; ±, few colonies; –, no growth. Experiment was repeated in triplicate.

To examine the practical capability of EW(+) to disinfect microorganisms including fungi attaching on the surface of food materials, we compared its effect with black pepper corn, turmeric finger, and coriander seeds. At least 50 mL of EW(+) 0.57 mM HOCl, satisfying concentration, to kill microbes mentioned above worked to clean up attaching microbes on the surface of 1 g of spices when EW(+) was used in combination with EW(−) (*34*) (Suzuki et al., unpublished data). Notably, the attached microorganisms, including fungi and spores observed by scanning microscopy, were not killed after a single 15 min exposure to a 50-fold volume of the EW(+) containing 30 μmol HOCl. However, a 15 min exposure to a 50-fold volume of the EW(−), followed by a 15 min exposure to a 50-fold volume of the EW(+), did effectively kill the microbes. Thus, although the EW(+) seems to be an effective disinfectant in model situations, for practical applications, a combined use of the EW(−) and the EW(+) might be needed. Detailed information will be reported later in an appropriate journal (Suzuki et al., in preparation).

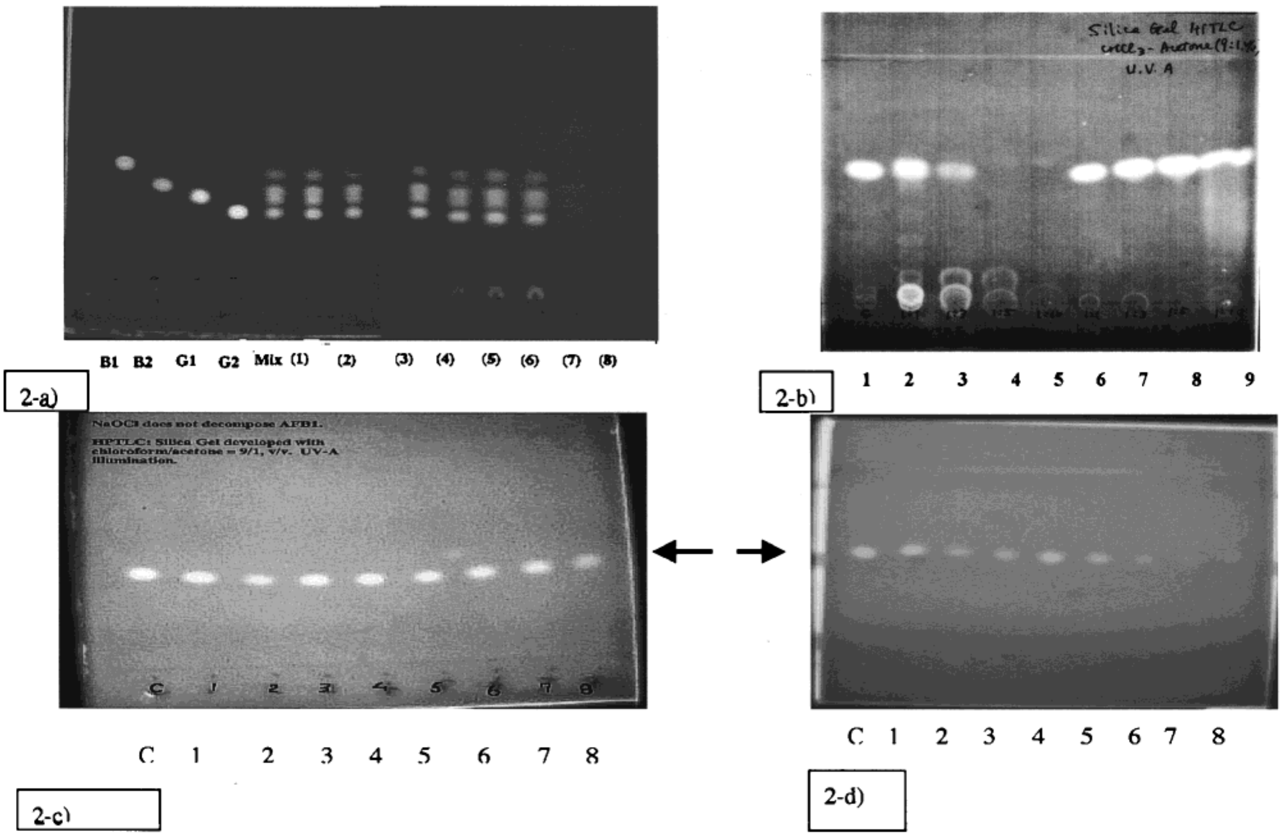

Figure 2. HPTLC analysis of aflatoxin exposed to electrolyzed NaCl solutions and NaOCl. After the aflatoxin was exposed to increasing molar ratios of the electrolyzed NaCl solutions, solutions were prepared for HPTLC as described in Materials and Methods. (a) HPTLC of individual aflatoxins B_1, B_2, G_1, and G_2 (10 ng of each) and a mixture of all four aflatoxins with or without exposure to EW(+). B_1, B_2, G_1, G_2, and mix indicate aflatoxins without treatment; numbers indicate the molar mixing ratio of the aflatoxin mixture to the EW(+). Lane 1, 1:0.7 $\times$ 10^5; 2, 1:2.8 $\times$ 10^5; 3, 1:4.2 $\times$ 10^5; 4, 1:5.6 $\times$ 10^5; 5, 1:7.0 $\times$ 10^5; 6, 1:14 $\times$ 10^5; 7, 1:28 $\times$ 10^5; 8, 1:42 $\times$ 10^5. Note that no fluorescent spot is seen with an approximately 28 $\times$ 10^5-fold molar addition of HOCl in the EW(+) over the 40 ng (ca. 0.12 nmol) of sum of 10 ng each of 4 aflatoxin analogues mixture. (b) AFB_1 after exposure to EW(+) or EW(–). Ten nanograms (0.03 nmol) of AFB_1 was mixed with EW(+) by the molar ratio of 1:0.7 $\times$ 10^5 to 1:7 $\times$ 10^5 in molar equivalent terms of AFB_1 to HOCl in EW(+). The EW(–) does not contain significant concentration of HOCl; however, 1–10-fold volumes were added to the AFB_1 solution. Lane 1, control AFB_1 (10 ng); Lanes 2–5, AFB_1 exposed to EW(+) of 0.7 $\times$ 10^5-fold, 2.1 $\times$ 10^5-fold, 3.5 $\times$ 10^5-fold, and 7.0 $\times$ 10^5-fold molar equivalents of HOCl. Lanes 6–9, AFB_1 exposed to 1-, 3-, 5-, and 10-fold volumes of EW(–). (c) AFB_1 after exposure to NaOCl at pH 11.5. Ten nanograms (0.03 nmol) of AFB_1 was mixed with NaOCl solutions containing between molar ratio of AFB_1 to EW(–) by 1:0.7 $\times$ 10^5 and 8 (1:5.6 $\times$ 10^5) in terms of molar equivalents of available chlorine as OCl^- anion at pH 11.5. Lane C, control AFB_1; lanes presented by numerals 1–8 mean mixing ratio with NaOCl: 1, 0.7 $\times$ 10^5; 2, 1.4 $\times$ 10^5; 3, 2.1 $\times$ 10^5; 4, 2.8 $\times$ 10^5; 5, 3.5 $\times$ 10^5; 6, 4.2 $\times$ 10^5; 7, 4.9 $\times$ 10^5; 8, 5.6 $\times$ 10^5. Note that AFB_1 does not disappear even at 5.6 $\times$ 10^5-fold mixing ratio. The arrow indicates AFB_1. (d) AFB_1 after exposure to NaOCl at pH 3. Ten nanograms (0.03 nmol) of AFB_1 was mixed with NaOCl solutions by the molar ratios of AFB_1 to HOCl between 1:0.7 $\times$ 10^5 and 1:5.6 $\times$ 10^5. Molar equivalents of available chlorine of NaOCl can be regarded as HOCl after pH adjustment at 3.0. Lane C, control AFB_1; lanes represented by numerals 1–8 mean mixing ratio with NaOCl the same as panel c except for pH 3. The arrow indicates AFB_1. The AFB_1 spot disappeared after exposure to 4.9 $\times$ 10^5 times the molar amount of NaOCl at pH 3. Note that the mixing ratio and experimental conditions were exactly the same as panel c except for pH value. Note that AFB_1 disappears at 4.9 $\times$ 10^5-fold molar mixing ratio (lane 7). Compare the chromatogram in panel c.

HPTLC Assessment of the Degradation of Aflatoxin. The fluorescent spots of AFB_1 and the B_1, B_2, G_1, and G_2 mixture of aflatoxin on HPTLC plates disappeared after exposure to seven times the molar amount of available chlorine in the form of HOCl to each aflatoxin (**Figure 2a**). The EW(+) completely abolished the AFB_1 spot; however, the strongly alkaline EW(−) had no effect on AFB_1 (**Figure 2b**). Theoretically, reactive chlorine that can be expressed as available chlorine including HOCl, OCl^-, or dissolved Cl_2 cannot be formed in the cathode compartment. The existence of low level available chlorine in the EW(−) should be due to leakage of EW(+) in the anode compartment into the cathode solution through the ion exhangeable membrane of the device.

Exposure of AFB_1 to eight times the molar amount of NaOCl at pH 11.5 did not abolish the AFB_1 spot (**Figure 2c**); however, AFB_1 disappeared if NaOCl was adjusted to pH 3.0 (**Figure 2d**). These results indicate that NaOCl exists in the form of HOCl at pH 3 and OCl^- at pH 11. Namely, the chemical species involved in destroying aflatoxin is not the ClO^- ion but is HOCl, which can give rise to a reactive OH radical and probably a Cl radical.

HPLC Assessment of the Degradation of Aflatoxin. Figure 3a shows that the trifluoroacetylated AFB_1 peak on HPLC disappeared after exposure to the EW(+). The peak height of trifluoroacetylated AFB_1, which eluted at 5.1 min, decreased with increasing molar ratios (on the basis of available chlorine as HOCl) of the EW(+) and disappeared completely after exposure to 43 $\times$ 10^3 times the molar amount of HOCl in EW(+) (**Figure 3b**). Although the peaks eluting at 3.1 and 8.3 min have not been assigned as yet, the peak at 8.3 min is likely to be a reaction product of AFB_1 formed after exposure to the EW(+).

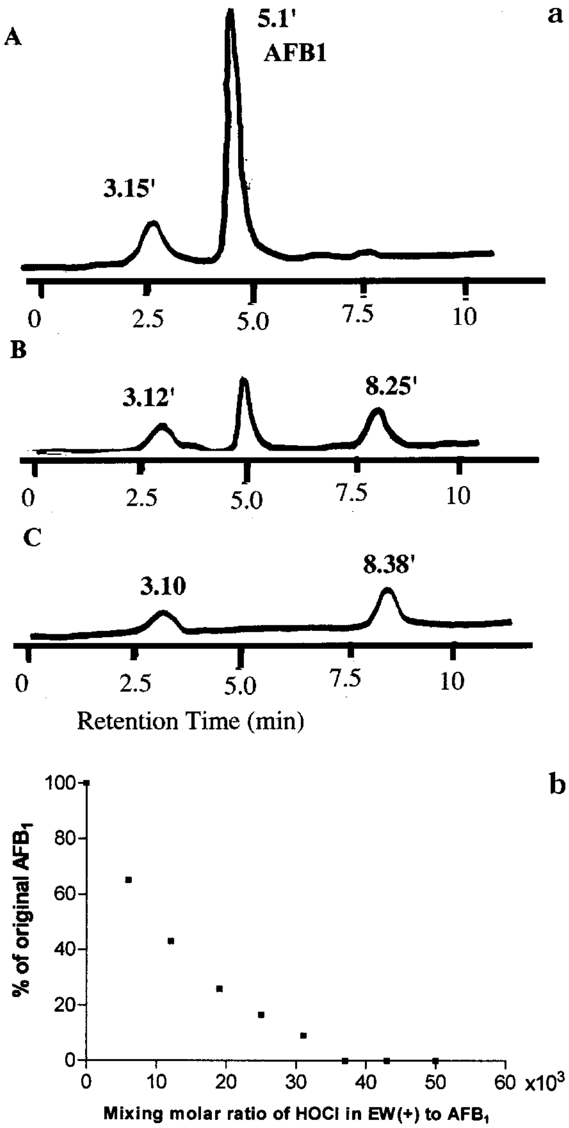

Figure 3. Reduction in AFB_1 peak height on HPLC after exposure to the anode solution. (a) Reduction in AFB_1 peak height with exposure to increasing volumes of EW(+) containing from 0 to 4.9×10^5 times the molar amount of HOCl. (A) AFB_1 without EW(+) exposure. The peak at 5.1 min corresponds to trifluoroacetylated AFB_1. The peak eluted at 3.15 min was not identified. (B) AFB_1 exposed to 0.19×10^5-fold molar excess of HOCl in EW(+). Eluted peaks at 3.12 and 8.25 min were not identified. (C) AFB_1 exposed to 0.44×10^5-fold molar excess of HOCl in EW(+). Other experimental conditions are in the Materials and Methods. (b) Decrease of AFB_1 content with increasing contact molar ratio of HOCl in EW(+). AFB_1 (400 ng corresponding to 1.3 nmol) was contacted to EW(+) varying its HOCl as described in the Materials and Methods. The content of the remaining AFB_1 was determined by HPLC and plotted. Ordinate, relative amount of remaining AFB_1 (percent); abscissa, mixing molar ratio of HOCl in EW(+) to AFB_1. Each plot represents the mean of three measurements.

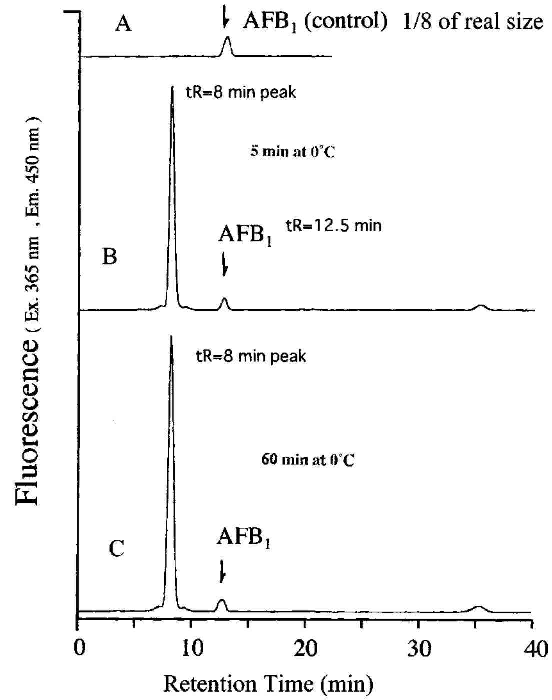

Figure 4. Effect of EW(+) exposure time on AFB_1 and its reaction products at 0 °C. AFB_1 (80 nmol) was exposed to EW(+) (8.5 μmol HOCl as available chlorine) at pH 2.3, for either 5 or 60 min at 0 °C. AFB_1 was analyzed by HPLC without derivatization as described in the Materials and Methods. Chromatogram on top (represented by A), AFB_1 without exposure (the AFB_1 peak on the chromatogram is 1/8 scale of the real size); chromatogram in the middle (represented by B), exposure of 6.8 μmol HOCl in EW(+) to 80 nmol AFB_1 for 5 min at 0 °C; chromatogram at the bottom, exposure of 6.8 μmol HOCl in EW(+) to 80 nmol AFB_1 for 60 min exposure at 0 °C. Note that the decrease in the AFB_1 peak eluting at 12.5 min was accompanied by the appearance of a major peak at t_R = 8 min (8-OH-9-Cl-AFB_1) and a minor peak at t_R = 35 min. The minor peak at t_R = 35 min was identified as an artifact. Note that the chromatograms shown were obtained without derivatization of the reaction products and so differ from those shown in **Figure 3**.

Identification of the reaction product was carried out on liquid chromatography mass spectroscopy (LC-MS) in another experiment without derivatization. LC-MS analysis of AFB_1 and its reaction products with EW(+) revealed the formation of 8-OH-9-Cl-AFB_1, 5,9-dichloro-8-OH-AFB_1, and 5,8,9-trichloro-AFB_1. Detailed information on the identification of the reaction products will be reported later (Suzuki et al., in preparation).

Effect of Reaction Time, Temperature, and Available Chlorine Concentration. The effect of exposure time and temperature on the annihilation of AFB_1 by EW(+) was assessed by HPLC (**Figure 4**). Free AFB_1 eluted as a single peak at 12.5 min, but this peak was replaced with a major peak at 8 min that was identified as 8-OH-9-Cl-AFB_1 and a minor peak at 35 min that was identified as 5,9-dichloro-8-acetoxy-AFB_1 (an artifact product) after AFB_1 was exposed to EW(+) at 0 °C for either 5 or 60 min (**Figure 4A−C**). The relation of the exposure temperature to both the disappearance of AFB_1 and the appearance of a reaction product, 8-OH-9-Cl-AFB_1 (t_R = 8 min), is shown in **Figure 5**. As the amount of AFB_1 decreased, the amount of reaction product increased. Low temperature slightly slowed the disappearance of AFB_1, but even after 5 min of exposure even at 0 °C, almost 40% of AFB_1 had disappeared. Considering the fact that the peak eluting at 8 min is 8-OH-9-Cl-AFB_1, the significant disappearance of AFB_1 with an increase of the OH, Cl adduct of AFB_1 at 0 °C suggests that this reaction is initiated by free radicals. Exposing AFB_1 to an equimolar amount of HOCl in the EW(+) for 5 min decreased the amount of AFB_1 by 40%.

Effect of Radical Scavengers on AFB_1 Degradation. Figure 6 shows the effect of the addition of mannitol and thiourea to the EW(+) before exposure to AFB_1. As the molar ratio of mannitol to available chlorine in the EW(+) increased, the percentage of the decomposed AFB_1 decreased. Similarly, when AFB_1 was exposed to EW(+) containing one-tenth of the molar

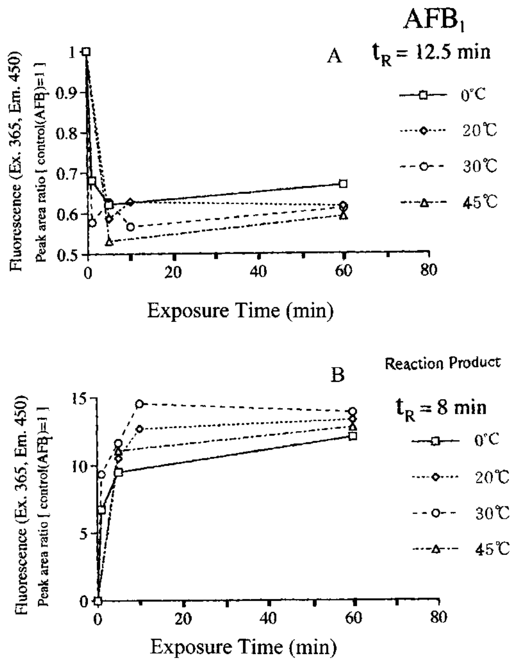

Figure 5. Effect of temperature and exposure time on the AFB_1 and the appearance of its reaction product 8-OH-9-Cl-AFB_1 eluting at 8 min. The ordinate indicates the fluorescence intensities at 450 nm; the abscissa indicates the exposure time in minutes. (A) Decrease in AFB_1 with increasing exposure time at various temperatures. (B) Increase in appearance of the reaction product, 8-OH-9-Cl-AFB_1 ($t_R = 8$ min), with increasing exposure time at various temperatures. Experimental conditions are the same as in **Figure 4**. Each plot represents the mean of three measurements.

amount of thiourea to HOCl in EW(+), only 25% of AFB_1 was destroyed, and with an equal molar ratio of thiourea to HOCl, AFB_1 was not destroyed at all. The known radical scavenging effect of mannitol and especially thiourea indicates distinctly that the disappearance of AFB_1 is due to a free radical-mediated reaction. The reason mannitol did not protect AFB_1 completely from the EW(+) may be due to an insufficient concentration of mannitol. For mannitol to exhibit an OH radical-scavenging effect, a higher concentration to scavenge OH radicals satisfactorily is required because 10 times as much molar amount of mannitol to HOCl protected only 16% of AFB_1 (**Figure 6a**).

Mutagenicity of Electrolyzed Solution Treated AFB_1. Three nanomoles of AFB_1 showed a high mutagenicity for both *S. typhimurium* TA-98 and TA-100 strains (**Table 4**), but this mutagenicity disappeared after a 10 min exposure to EW(+). **Table 4** shows the reduction in AFB_1 mutagenicity caused by increasing molar amounts of HOCl in the EW(+). Mutagenicity was reduced markedly after exposure to ca. 20-fold molar amount of HOCl in the EW(+) and showed less than 200% of relative mutagenicity indicating mutagenicity negative by exposure to ca. 60-fold molar amount or equivalent to 173 nmol of HOCl in both TA-98 and TA-100 strains.

ESR Analysis of Chemical Species in Anode Solution. To identify radical species, the EW(+) was subjected to ESR using the spin-trapping agent DMPO. At first, a weak spectrum indicating the formation of the OH radical was obtained (data not shown). Subsequently, to enhance the spectrum, we added 10 mL of 1 mM $FeSO_4$ and 10 mL of DMPO to the EW(+). In the presence of Fe^{2+} ion, an ESR spectrum typical of the reaction of DMPO with hydroxyl radicals (Fenton reaction) (*35*), as shown in **Figure 7**, was obtained, suggesting that the EW(+) also contains H_2O_2. Thus, these results indicate the presence of

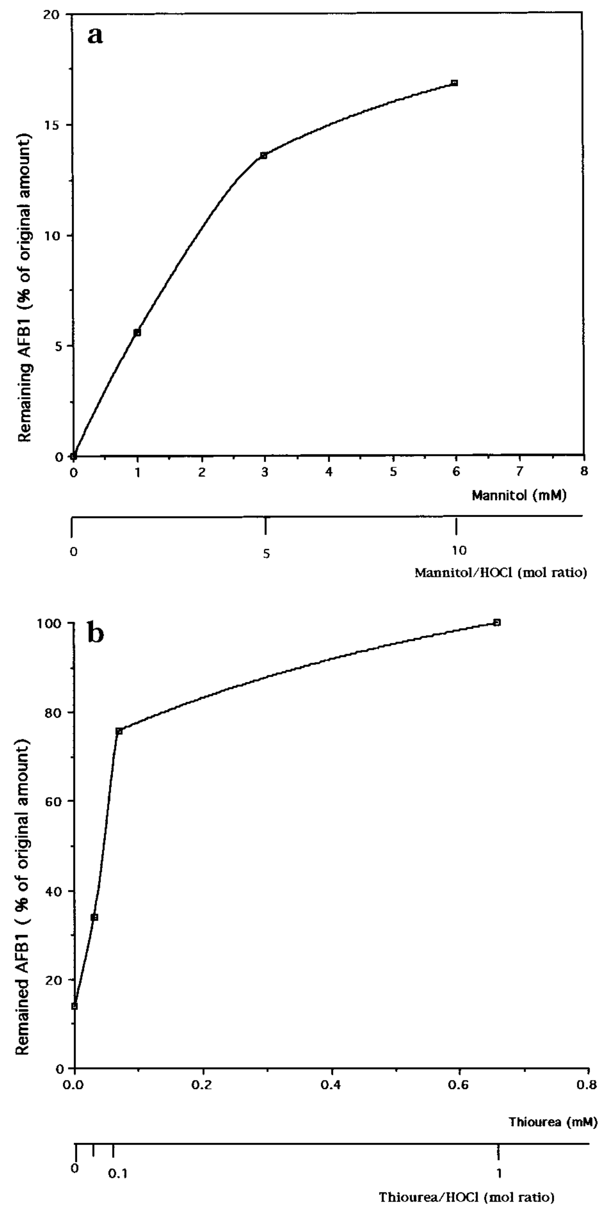

Figure 6. Effect of radical scavengers on the degradation of AFB_1 by EW(+). Either mannitol or thiourea was added to the EW(+) at increasing molar ratios as HOCl (0.640 mM) before exposure to AFB_1 (0.128 mM). Exposure of radical scavenger-treated EW(+) was done for 10 min at 30 °C. The ordinate shows the relative amount of remaining AFB_1, calculated as a percentage of the sum of the total peak area of control AFB_1 monitored at 365 nm; the abscissa indicates molar ratios of radical scavenger to HOCl in EW(+). (a) Effect of mannitol addition to EW(+) on the of AFB_1. Mannitol was added to EW(+) at 2–10 times the molar amount of HOCl in EW(+) prior to exposure of EW(+) to AFB_1. (b) Effect of thiourea addition to EW(+) on the degradation of AFB_1. Thiourea was added to EW(+) at 0.1–1.0 times the molar amount of HOCl in EW(+) prior to exposing EW(+) to AFB_1.

OH radicals and H_2O_2 in the EW(+). Furthermore, we confirmed the presence of HOCl at the acidic pH range in the EW(+) by comparing ultraviolet spectra between 200 and 350 nm; i.e., we obtained similar spectra identical to those of standard HOCl and NaOCl and EW(+) under different pH values as reported by Nakagawara et al. (*30*) with EW(+) and NaOCl under different pH conditions.

In summary, we have shown that electrolyzed NaCl anode solution (EW(+)) sterilizes *A. paraciticus* and eliminates the

Table 4. Effect of Anode Solution on the Mutagenicity of AFB_1[a]

sample	relative mutagenicity for *Salmonella typhimurium* (%) TA-98	TA-100
AFB_1 (3 nmol)	4039 ± 250	1010 ± 36
AFB_1 + anode solution		
3 + 58 nmol	514 ± 12	224 ± 8
3 + 173 nmol	NG	27 ± 1
3 + 288 nmol	NG	9 ± 1
3 + 575 nmol	NG	NG

[a] The mutagenicity test was carried out on His⁻ *S. typhimurium* TA-98 and TA-100 according to Maron and Ames (1983). Three nanomoles of AFB_1 was exposed to increasing volumes of the EW(+) with 0.77 mM (40 ppm) HOCl as available chlorine for 10 min at room temperature. This solution was then preincubated with an S-9 mixture for 20 min and incubated with *S. typhimurium* for 48 hours at 37 °C. Data are represented as the percentage of relative mutagenicity, which was calculated by [(cfu of the sample – cfu of the blank)/cfu of the blank] × 100. NG, negative growth (i.e., equal number of colony formation or less than the blank that did not include AFB_1). Different volumes of the EW(+) are expressed as nanomoles of HOCl. Data represent (mean ± SD) for five separate experiments.

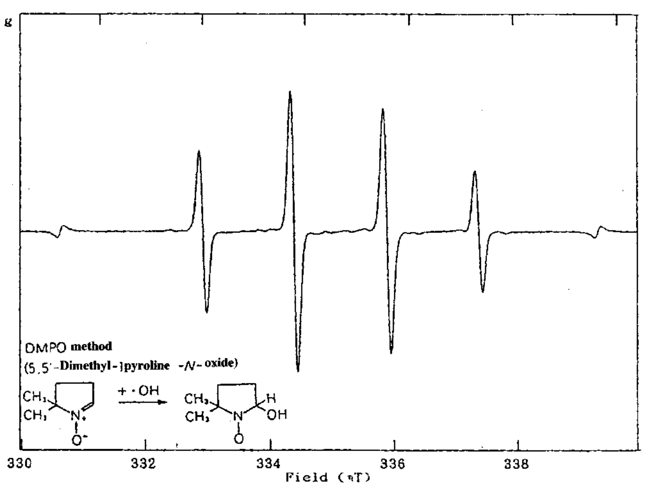

Figure 7. DMPO spin trapped ESR signal of the EW(+). ESR spectrum of DMPO incubated with EW(+). The ordinate represents the intensity of signal; the abscissa indicates the intensity of the magnetic field. The typical 1:2:2:1 "fingerprint", demonstrating electron adduction, shows that OH radicals are present in the EW(+).

mutagenicity of aflatoxin AFB_1. Aflatoxin breaks down after exposure to EW(+), as determined by HPLC analysis; however, the chemical formula of the reaction products was not described in detail here. We have succeeded in identifying the major reaction product to be 8-OH-9-Cl-AFB_1 by LC-MS and nuclear magnetic resonance techniques. In the following paper, we describe the chemical formulas of the products formed after the exposure of ABF_1 to EW(+).

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