Formation of Chemical Species and Their Effects on Microorganisms Using a Pulsed High-Voltage Discharge in Water

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Abstract— **The primary mechanism for sterilization of microorganisms by high-voltage pulses has been considered to be an electrical breakdown of the cell membrane. However, it is expected that many kinds of chemically active species would be generated by an electrical discharge in a needle-plate or rod-rod electrode system. Therefore it is necessary to identify the chemical species produced by the discharge and to investigate lethal effects of the active species on microorganisms. Using a nozzle-plate electrode configuration, the authors previously reported that magenta colored streamers propagated from the nozzle tip during a pulsed discharge in water with various conductivities. The authors also investigated the generation of ozone from oxygen bubbled through the discharge. In the present study, the formation of active species in water (without O_2 flow) and their effects on yeast cells were investigated using needle-plate electrodes. In the presence of the streamer discharge, H and OH radicals were detected by means of emission spectroscopic analysis of the discharge light. Hydrogen peroxide (H_2O_2) was also detected by absorption spectrophotometry using a reaction of peroxidase and catalase. The effect of the electrical conductivity of the water on the formation of the active species was investigated. Maximum ·OH and H_2O_2 concentrations were obtained at a water conductivity of about 10^{-5} S/cm. The H_2O_2 formation mechanism was considered to be a recombination reaction of ·OH. The lethal effects on beer yeast of ·OH and H_2O_2 generated by the pulsed electrical discharge in water were also investigated. It was found that ·OH had almost no effect in reducing the survivors. However, the H_2O_2 did kill the yeast cells: the logarithm of the survival ratio decreased linearly with increasing H_2O_2 concentration.**

I. Introduction

THE LETHAL effects of high-voltage pulses on microorganisms have been investigated by one of the authors [1], [2] and also by others [3]–[5] using a plate-plate electrode configuration (no discharge). Irreversible breakdown of the cell membrane by the electric field occurs due to electrical compression of the membrane or the Born energy effect [6], [7]. The former is a compression of the electrically charged membrane as a result of the strong applied electric field. In the latter, membrane breakdown occurs when ions in the water are injected into the membrane by the electric field. However, there are phenomena reported elsewhere which cannot be explained by electric field effects only. For example, one type of virus was killed by a high-voltage pulse and its RNA was cut into small fragments [3]. The diameter of a virus is usually only about 30 nm, so the voltage difference across the membrane due to the applied field is very small. Therefore it is most likely that some other mechanism may exist which kills viruses and cuts RNA molecules.

There are various types of high voltage pulsed discharges in water [8]–[10]. If the geometry is nonuniform (needle-plate or rod-rod configuration), a streamer or spark discharge tends to occur [11], [12], and many chemically active species can be produced by the discharge [13]. In this case, we have to consider not only physical effects (breakup of the cell membrane by electric field or shock wave) but also chemical effects (radical reactions, etc.) which can kill microorganisms. Many reports about the effects of radicals or chemical species on microorganisms can be found in the fields of radiation or medical chemistry. Recently, due to the large amount of cancer research, the action and the role of O_2^- (superoxide), H_2O_2 (hydrogen peroxide), ·OH (hydroxyl radical), and $^1O_2$ (singlet oxygen) in the human body are gradually being elucidated. Superoxide reacts with biological molecules: for example, hyperoxidation of lipids, degeneration of proteins, inactivation of enzymes, and damage to DNA [14]. The damage to DNA is very important because of the death of cells, mutation, and carcinogenesis [15].

In a study of chemical effects, Sale and Hamilton [4] conducted an experiment using agar to fix the cell suspension. They concluded that lethal effects occur not only in the region close to the electrode surface (where electrolytic reactions occur) but also in the region at the center (far from the electrode surface). Gilliland [16] obtained a fairly effective sterilization by using a spark discharge (shock wave) in water. The sterilization effectiveness was changed by the kind of metals used for the electrode: a copper electrode was better than iron or aluminum. This was because of the metal ion effect after elusion of the metal. Hayamizu [17] showed that ultraviolet light emitted by a spark discharge in water kills microorganisms [18], [19]. However, very little work could be found on the lethal effects of radicals produced by an electrical discharge in water. In the present study, the formation of active chemical species by a pulsed discharge in water was

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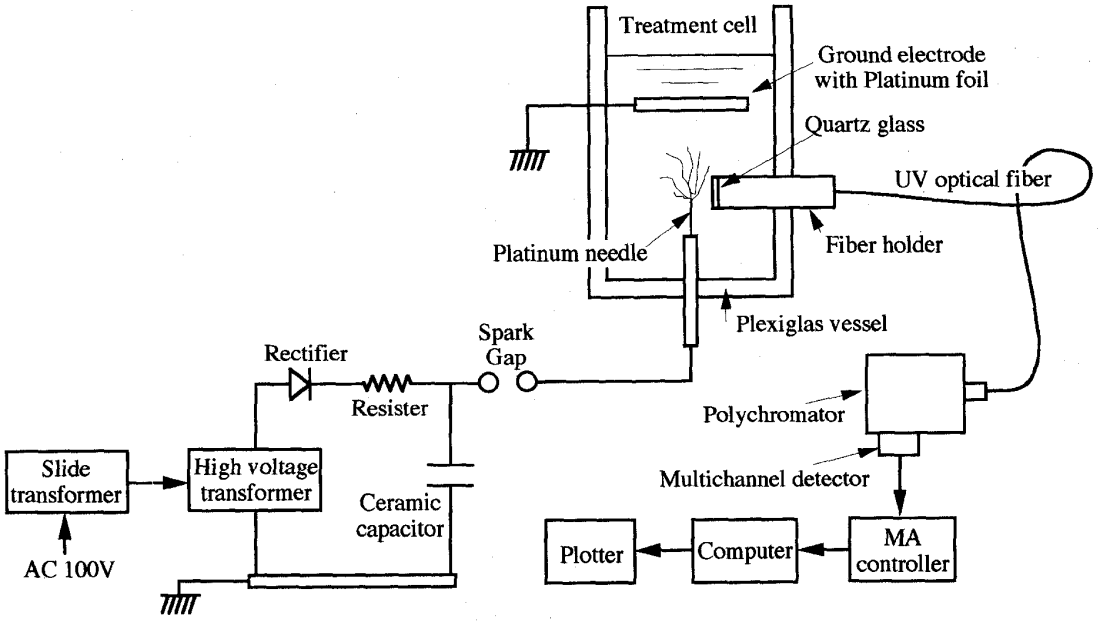

Fig. 1. Treatment cell and multichannel analyzer system for emission spectroscopic analysis.

first investigated without microorganisms. The results of this study were then used to facilitate an investigation of the lethal effects of the chemical species produced by the discharge.

II. Experimental Apparatus and Method

The experimental apparatus was similar to that used in our previous study [13]. However, in this work two treatment cells were used: a batch-mode cell and a recirculation-mode cell. The batch water sample was treated in a rectangular pulse treatment cell. The 5 × 5 × 10 cm treatment cell (see Fig. 1) had a volume of 250 ml. It contained platinum point-to-plane electrodes: the tip of the sharp 0.2-mm diameter needle electrode was located 5 cm from the ground plate. A quartz fiber was inserted into the cell to transmit ultraviolet and visible light emitted by the streamer discharge to a spectrophotometer. (The light was emitted as the streamers propagated from the needle toward the ground plate.) The pulsed power source used to supply high voltage pulses to the needle electrode was identical to that used in the previous work [13], but in this study two types of spark gaps were used: 1) for obtaining emission spectra of the single discharge light, a triggered spark gap (EG&G Inc., Type GP-22B) was used with a trigger module (EG&G Inc., Model TM-11A); 2) for measuring stable, long-lived chemical species generated by multiple discharges, a rotating spark gap was used (operating at a 50-Hz pulse repetition frequency). The pulse waveforms were recorded using an Iwatsu HV-P30 high voltage probe connected to an Iwatsu SS-5806 high-speed oscilloscope. Pulse shapes and rise times were the same as those in the previous work [13]. The conductivity of the water samples was varied between 1.33 and 1300 μS/cm and was measured using a TOA Electronics CM-5B conductivity meter. An Iwaki M13-D meter was used to determine the pH of the water samples.

In order to detect and identify radicals (·H, ·OH, etc.) formed in the discharge, it is necessary to measure them *in situ* because they react very quickly (nanoseconds). Therefore discharge emission spectra were used to identify the radicals and determine their relative concentration. The water samples were not circulated for these measurements because the pulse discharge was only operated for short time periods. To differentiate radicals from other chemical species, various alcohols (methanol, ethanol, and propanol) were added in some cases to the water sample before treatment in order to scavenge any radicals produced. Emission spectra (between 200 and 750 nm wavelength) of the discharge light were obtained using an Unisoku USP-500 MA (multichannel analyzer). The MA apparatus is shown in Fig. 1. The discharge light travels through the quartz optical fiber to a polychromator which disperses the various wavelengths of light to a diode array detector. The detector simultaneously measures the light intensity at each wavelength (for a single discharge). The detector data are processed and recorded by a microcomputer which generates a plot of light intensity versus wavelength.

Stable, long-lived chemical species generated by the discharge can be measured after pulsing (treatment). The recirculation-mode cell was used for these measurements because long treatment times (5 to 30 min) were required. As can be seen in Fig. 2, the cylindrical treatment cell had an inside diameter of 3 cm and a volume of 40 ml. It contained platinum point-to-plane electrodes: the tip of the sharp 0.2-mm diameter needle electrode was located 5 cm from the 3-cm diameter ground plate. During the pulsing a peristaltic pump was used to recirculate the water sample through both the cell and a cooling vessel. This was necessary in order to control the water sample temperature because long-term, continuous 50-Hz pulsing heated the water. The water temperature (measured

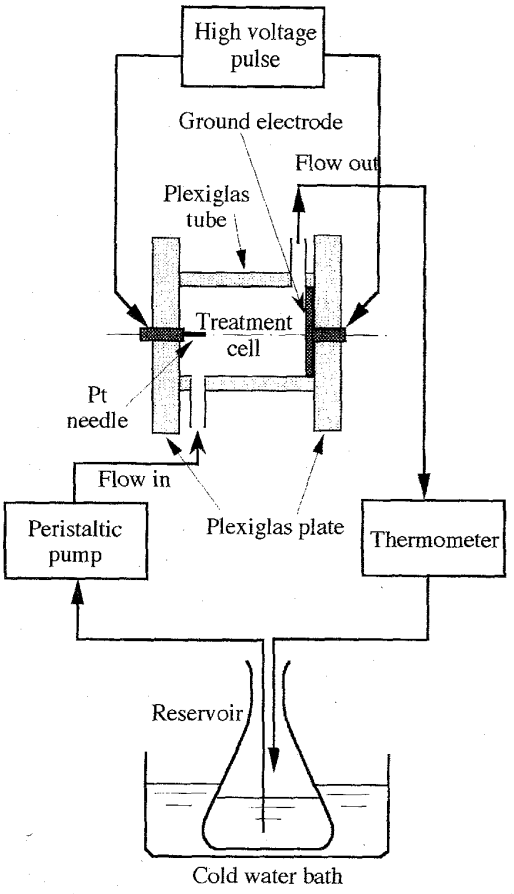

Fig. 2. Treatment cell and water sample recirculation system.

with a thermometer) had an equilibrium value of 25 to 30°C. The total volume of water in the flow system was 60 ml, the water flow rate was 100 ml/min, and the time required for total recirculation of the water sample was 36 s.

In these studies of stable chemical species, the total amount of oxidizing agent (O_3, H_2O_2, etc.) was measured by modifying a biochemical test originally designed to measure glucose concentrations by using enzymes (Glucose CII-Test Wako Method) [20], [21]. Glucose normally contains both the α-D and β-D types, but this test only works with the β-D type. Therefore, the mutarotase is used to convert the α-D to β-D-glucose. This allows all the glucose to be oxidized into H_2O_2. Then the H_2O_2 reacts with the pigment and the peroxidase yielding the red-colored quinone. The degree of color change is proportional to the concentration of quinone which is directly related to the amount of H_2O_2 present. In other words, the treated water sample is mixed with the biochemical indicator and the change in optical absorbance of the mixture (at 505 nm) indicates the total concentration of oxidizing species.

In order to distinguish H_2O_2 from O_3 or other oxidizing agents, it was necessary to make two measurements: 1) the total concentration of oxidizing agents, 2) total oxidation without H_2O_2 (a catalase is used to selectively remove the H_2O_2). The concentration of H_2O_2 is obtained by subtracting the result of the second measurement from that of the first. This method determined that the only oxidizing agent present in significant amounts was H_2O_2. Therefore it was only necessary to measure the total oxidizing agent (which was just H_2O_2). The absorbance of the mixture was measured by pouring it into a sample cell and inserting the cell into a Shimazu UV-1200 spectro-photometer (see our previous paper [13] for a discussion of absorbance).

Suspensions of beer yeast cells (*Saccharomyces cerevisiae* IFO 0259) were prepared by preculturing the cells for three days at 30°C in a yeast-malt (YM) medium. The YM medium was composed of the following: 5 g/l bactopeptone, 3 g/l yeast extract, 3 g/l malt extract, 10 g/l glucose, and 1000 ml distilled water. The cells (now increased in number to about 10^7 cells/ml were then suspended in sterile water. The cell suspension was then treated, i.e., subjected to the pulse discharge or mixed with treated water. The yeast cell survival ratio was determined using the following procedure: 1) the cell solution was diluted with sterile water; 2) this solution was used to inoculate a culture dish containing malt agar (45 g/l malt agar medium, 100 mg/l chloramphenicol, and 1000 ml distilled water); 3) the culture was allowed to incubate for three days at 30°C; 4) the number of colonies was counted and the survival ratio calculated (by dividing the final number of colonies by the number of cells in an untreated sample).

III. Results and Discussion

A. Radical ($\cdot H, \cdot OH$) Identification Using Emission Spectra (In Situ)

The emission spectrum between 200 and 750 nm obtained using the MA for the pulsed discharge in distilled water is shown in Fig. 3. The high-voltage pulses generated a white spot at the needle tip and magenta colored streamers which propagated from the needle electrode. The pulse voltage was +14 kV and the water conductivity was 4.3 μS/cm. The two peaks (H_α: 656.3 and H_β: 486.1) in the long-wavelength end of the spectrum are part of the Balmer series of emission lines from gaseous atomic hydrogen (H). There was also a continuous emission region in the spectrum between 250 and 550 nm. The largest peaks were in the short-wavelength (ultraviolet) section of the spectrum, and are most likely molecular emissions from the OH radical. The strong peak corresponds to the ·OH (0,0) $A^2\Sigma^+$ to $X^2\Pi$ transition, and the weaker peak to the ·OH (1,0) $A^2\Sigma^+$ to $X^2\Pi$ transition [22], [23]. Since the platinum metal in the needle has a known atomic emission line at 306.5 nm, it was necessary to determine if the 310-nm peak was due to ·OH, platinum, or both. This was accomplished by obtaining a spectrum after adding 500 mM of 2-propanol to the water. In this case the peak at 310 nm was gone. Since the propanol reacts very rapidly with ·OH (and thereby scavenges it), this result suggests that the 310-nm peak was due to ·OH and not platinum.

When 1 mM KCl was added to the water to increase its conductivity to 100 μS/cm, the streamer discharge became stronger and more steady, and the white spot disappeared.

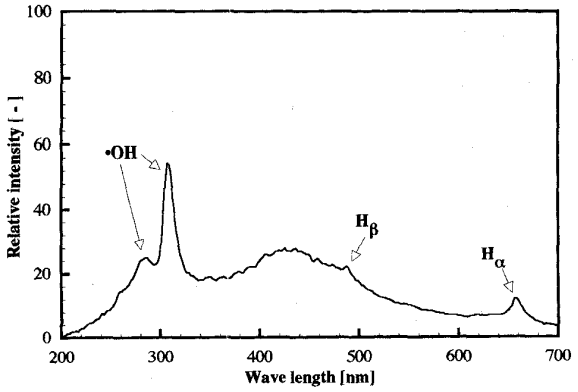

Fig. 3. Emission spectra from pulsed streamer discharge in distilled water (applied voltage: 14 kV).

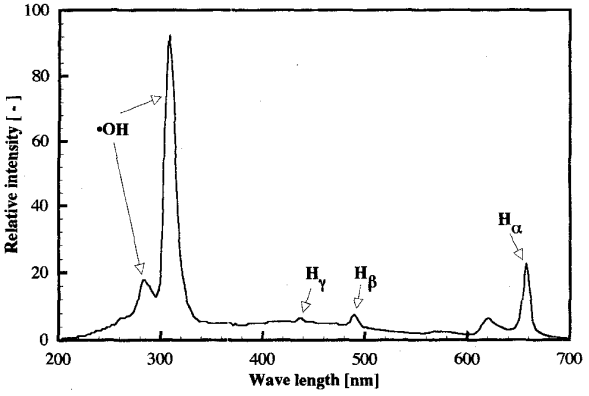

Fig. 4. Emission spectra from pulsed streamer discharge in 1 mM KCl aqueous solution (applied voltage: 14 kV).

The emission spectrum obtained under these conditions is shown in Fig. 4. The same emission bands were observed as in pure distilled water, but the peaks were much stronger and the continuous emission region was weaker. In addition, the next peak in the Balmer series (H_γ: 434.0) could barely be discerned (the intensity of the Balmer lines decreases serially).

These emission spectra indicate that H and OH radicals are being produced by the streamer discharge. Most likely, water molecules are being dissociated in the high electric field regions near the needle electrode and streamer tips (see our previous paper [13] for a discussion of streamer propagation theories). The hydrogen Balmer lines and ·OH peaks are stronger in the more conductive water because the magenta streamers are more intense. The continuous region is weaker because there is not a white-spot discharge in the conductive water.

B. Hydrogen Peroxide Measurement

Distilled water was exposed to +19 kV pulse discharges (treated) and then analyzed for the presence of long-lived oxidizing species. Water samples with and without catalase were treated for various exposure times and then analyzed using the previously described Glucose CII Test Wako method. Fig. 5 contains a plot of the concentration of total oxidizing agent versus the accumulated pulse energy. When the catalase (selective H_2O_2 scavenger) was present (1.67 mg/l, no oxidizing species were detected. Without the catalase, an oxidizing agent was detected. This indicated that the oxidizing agent was H_2O_2, and therefore the ordinate was labeled H_2O_2 concentration. As can be seen in the figure, the H_2O_2 concentration increased approximately linearly with increasing treatment time (total pulse energy applied).

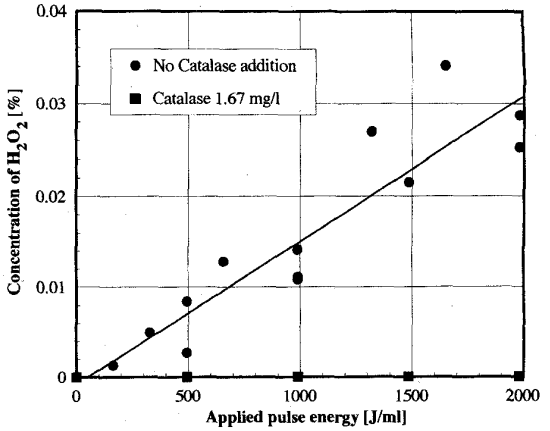

Fig. 5. Variation of H_2O_2 concentration due to pulse treatment in distilled water with and without addition of Catalase (pulse voltage: 19 kV).

The effect of water conductivity on the production of chemical species was investigated because conductivity has a large effect on streamer production and propagation [13]. Since the concentrations of the H and OH radicals are directly related to the intensities of their corresponding emission bands, these values were measured as a function of the initial conductivity of the water sample (see Fig. 6). The ordinate contains the relative intensities: measured intensity divided by that in distilled water. The H_2O_2 concentration after treatment was also measured and plotted, and is shown on the right-hand ordinate in the figure. The intensity of the H_α line (H concentration) decreased with increasing conductivity. However, the ·OH intensity and also the H_2O_2 concentration both went through a maximum at a conductivity around 10^{-5} to 10^{-4} S/cm. The correlation in the ·OH and H_2O_2 concentrations versus conductivity suggests that H_2O_2 formation may be related to ·OH production.

Radiation chemistry research has shown that when OH radicals are produced by irradiating homogeneous aqueous solutions [24], the OH radicals can recombine and form H_2O_2 through the following reaction pathway [25]:

$$\cdot OH + \cdot OH \rightarrow H_2O_2.$$

The rate constant (k) for this reaction is: $k = 5 \times 10^9$ 1/Ms. It is likely that a similar reaction pathway exists for the pulsed streamer discharge in water.

In order to verify the existence of this pathway, it is necessary to rule out the other reaction mechanism in which H_2O_2 is formed in alkaline solutions by electrolytic reaction of dissolved O_2 on the negative electrode [26]–[28]. This mechanism requires a dc current, but repetitive application of wide pulses could result in a small dc current flowing between

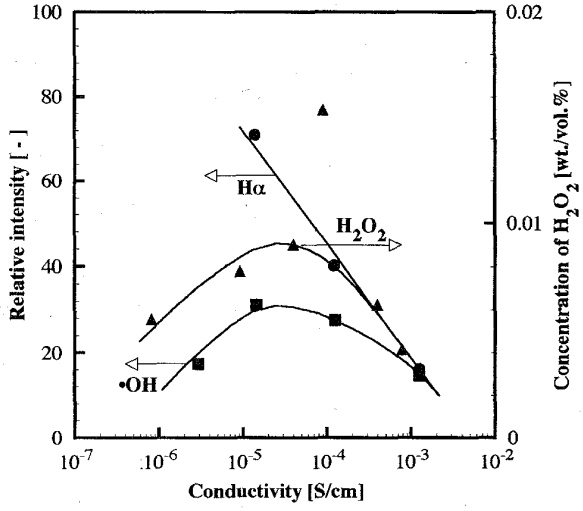

Fig. 6. Effect of conductivity of aqueous KCl solution on the emission intensity of Hα and ·OH, and concentration of H_2O_2 (applied voltage: 19 kV).

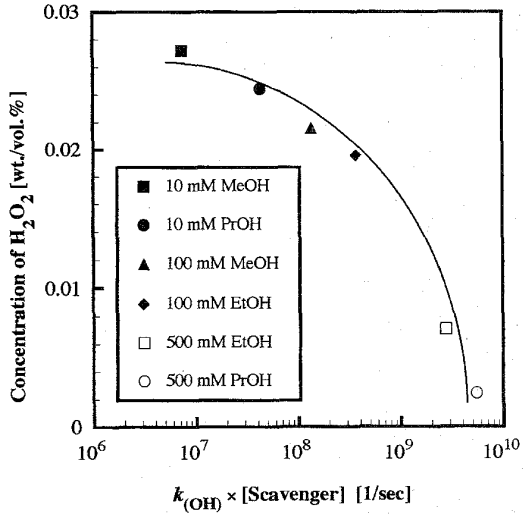

Fig. 7. Concentration of H_2O_2 in 1 mM KCl aqueous solution plotted versus the radical scavenger reaction rate (pulse voltage: 19 kV; treatment time: 30 min).

TABLE I
SPECIFIC RATE CONSTANT OF OH RADICAL REACTION

Reactant	pH	Rate constant [1/Ms]
Ethanol	7.0	1.0×10^9
Methanol	7.0	5.0×10^8
2-Propanol	7.0	2.0×10^9

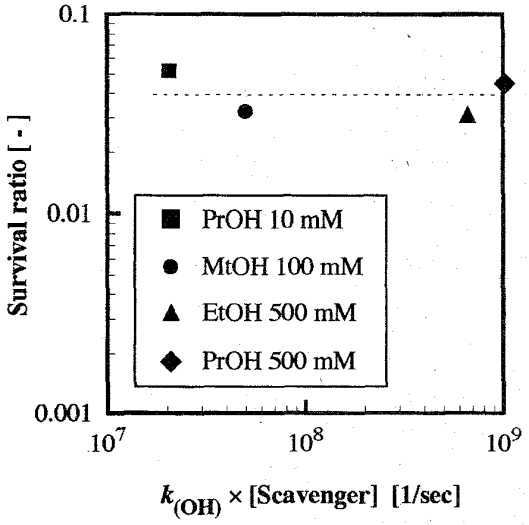

Fig. 8. Relationship between cell survival ratio and OH radical reaction rate obtained by adding radical scavengers (pulse voltage: 19 kV; pulse energy: 180 J/ml).

electrodes, especially in the more conductive water samples. Therefore, tests were conducted in which water samples containing 1 mM KCl (conductivity 100 μS/cm) were exposed to a +19 kV pulse discharge for 30 min. (The K^+ and Cl^- ions have negligibly small reaction rates with ·OH, i.e., $<10^{-3}$ 1/Ms). Several ·OH scavengers with different ·OH reaction rate constants (see Table I) were added to the water samples in various concentrations. In Fig. 7, the amount of H_2O_2 present after each treatment period is plotted versus the "scavenging power," i.e., the product of the scavenger concentration and reaction rate constant (k). The H_2O_2 concentration decreased dramatically with increasing "scavenging power." In another test, O_2 was dissolved in the water sample and pulses with voltage below the streamer production threshold (12.5 kV) were applied. (A larger diameter needle was used to raise the threshold voltage.) No H_2O_2 could be detected in this case, even though the application of these pulses could still result in a dc current flow. The results of these two experiments strongly suggest that almost all the H_2O_2 is formed by recombination of OH radicals and not by an electrolytic reaction.

C. Lethal Effects of Active Species on Yeast Cells

Effects of OH radical—The lethal effects of OH radicals on microorganisms were studied by treating a water sample containing 1 mM KCl and 10^7 yeast cells with +19 kV pulses for 5.5 min. The effects on yeast cells were determined with the addition of ·OH scavengers to the water sample. The yeast survival ratios for these conditions are shown versus "scavenging power" in Fig. 8. The yeast survival ratios were all less than 10%, most likely due to the effects of the pulsed electric field. However, the survival curve was "flat," i.e., there was no correlation between "scavenging power" and yeast survival. Since the ·OH concentration is inversely proportional to the "scavenging power," this indicates that the ·OH had no significant lethal effect at the concentrations produced by the pulsed discharge.

The yeast cells may not have been affected by the ·OH because the ·OH was generated outside the cells. Streamers

should not pass through cells because the cell membrane has a high dielectric constant which greatly lowers the electric field inside the cell (the field inside a sphere with infinite dielectric constant (conductor) is zero). The situation is quite different when radiation is used for sterilization. The radiation passes through the cell and breaks up H_2O molecules forming ·OH, which then damages the DNA leading to the death of the cell [24].

Another possible reason that the ·OH did not kill the yeast cells was that ·OH reacts very quickly with a large variety of molecules (nonspecific), and therefore would not be able to travel to the cell membrane without already reacting. For example, if the concentration of molecules that can react with ·OH and the reaction rate constant are assumed to be 10 mM and 10^9 l/Ms, respectively, then the average ·OH lifetime would be about 70 ns. In this time period, the ·OH would diffuse only about 20 nm, so that it would not be able to reach the cell. There may also be additional molecules present which would react with the ·OH before it reached the cell, i.e., intracellular protein or DNA which had been released from the cell due to the applied pulses [4], [29].

Effects of hydrogen peroxide—The effect of H_2O_2 on yeast cells was not determined *in situ* because it was difficult to distinguish the electric field effect from the H_2O_2 effect. In addition, the buffer solution necessary to support cell life contains electrolytes and could change the characteristics of the streamer discharge, and the H_2O_2 and catalase could be scavenged by ·OH ($k = 2.6 \times 10^{11}$ l/Ms). For these reasons, treated water samples containing H_2O_2 generated by the streamer discharge were added to a buffer solution containing yeast cells (cell suspension), and the cell survival ratio was determined. Other measurements were made in which the catalase was added to the treated water (to scavenge the H_2O_2) before it was mixed with the cell suspension. Also, a known H_2O_2 solution was prepared by adding commercial H_2O_2 reagent to distilled water at the same concentration as the pulse-generated H_2O_2. This solution was mixed with a cell suspension and the survival ratio determined. In Fig. 9, both the generated H_2O_2 concentration and the cell survival ratio are plotted as a function of the total pulse energy (+19 kV pulses applied at 50 Hz for various treatment times). As the accumulated pulse energy (treatment time) increased, the H_2O_2 concentration increased and the cell survival ratio (after mixing) decreased: the logarithm of the survival ratio decreased linearly with increasing H_2O_2 concentration. Adding the commercial H_2O_2 solution to the cell suspension had the same lethal effect as the treated water. In addition, when the catalase was used to scavenge the pulse-generated H_2O_2, no H_2O_2 was detected and no significant reduction in cell survival occurred. Taken together, these results indicate that a stable, long-lived chemical generated by the discharge was effective in killing the yeast cells, and that this chemical was H_2O_2.

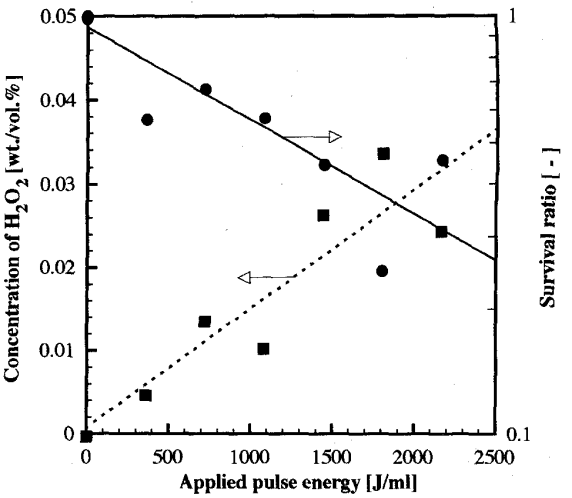

Fig. 9. Cell survival ratio and H_2O_2 concentration in distilled water as a function of total pulse energy applied (pulse voltage: 19 kV).

IV. Conclusion

1) The H and OH radicals were detected in the streamer discharge region using *in-situ* emission spectroscopic analysis.
2) The emission intensity of atomic hydrogen (H) decreased with increasing water conductivity. On the other hand, the OH radical intensity went through a maximum at a conductivity of about 10^{-5} S/cm. The maximum H_2O_2 concentration was obtained at 10^{-4} to 10^{-5} S/cm, which correlates well with that for the OH radical.
3) A stable, long-lived oxidizing agent was generated by the discharge. The absorption spectra with and without catalase (scavenger) addition indicated that hydrogen peroxide was the only oxidizing agent generated in significant amounts, and that its concentration increased linearly with increasing total pulse energy (treatment time).
4) Test results strongly suggest that the hydrogen peroxide was generated by the recombination of ·OH and not by an electrolytic reaction of dissolved oxygen on the negative electrode.
5) The H and OH radicals generated by the pulsed streamer discharge were not effective in killing yeast cells (*in situ*).
6) Treated water samples containing H_2O_2 generated by the discharge were lethal when added to yeast cell suspensions, and cell survival decreased logarithmically with increasing H_2O_2 concentration.

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