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Sterilization Effects of HO_2/O_2^- Radicals Produced by H_2O-O_2 Plasma

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In order to realize the sterilization of the medical implements packed by a sterilization bag or medical implements of a complicated structure with tiny gaps, application of the long-lifetime oxygen active species were investigated using H_2O-O_2 plasma.

By the sterilization experiment using BI with the spore of *Geobacillus stearothermophilus* ATCC 7953 with high heat resistance, it was found that the more the partial pressures of H_2O and O_2 increased, the more the shorter the required irradiation time for sterilization became. Sterilization time for BI enclosed in the sterilization bag was 15 minutes, when the partial pressure of H_2O and O_2 were made equal and the total pressure was set to 150 Pa. From the emission spectrum diagnostic result, the reduction of emission intensity based on ・OH or ・O was confirmed with pressure increase, and it suggested that the formation of ・O_2^- was dominant with the total pressure of 150 Pa or more.

Keywords: Sterilization, H_2O-O_2 plasma, *Geobacillus*, Biological Indicator, Plasma Chemical Indicator

1. Introduction

The process of sterilization is mainly divided into two categories, the process for hospitals, and the process for industry. In the former, the uses of hot-steam autoclave or dry-heat and ethylene oxide gas or low-temperature steam formaldehyde are the most commonly used methods. In the latter, steam, ethylene oxide, and ionizing radiation sterilization, either gamma irradiation (Co-60) or electron beam, are the most commonly used methods of sterilizing medical devices [1]. However, these methods have a drawback [1]. For example, high temperature sterilization processes are not available to heat-sensitive medical implements which are increasing these days. In this case, there is no means besides using strong toxic ethylene oxide or formaldehyde. Therefore the vent after processing is needed in order to ensure the safety of the surrounding people, so that the extension of processing time is not avoided.

In the case of radiation sterilization, equipment cost is one important issue. All of these issues lead to the need for a rapid, low-temperature (below 75°C), low-cost terminal sterilization process that presents no toxicological hazard to the surrounding people and that do not negatively affect the environment or the device on which it is used. This is the background of the development of low-temperature gas plasma sterilization technology since a patent assigned to the Arthur D. Little Company in 1968. Much research on plasma sterilization technology with a variety of plasma gas such as H_2O_2 [2], O_2 [3], air [4], O_2/H_2O [5] and with a different type of plasma conditions such as low-pressure [2,6] or atmospheric pressure [4] has been reported so far. However, there are few examples of a report put into practical use. This means that all the above issues have not been sufficiently dealt with yet, and that generation of the activated species with a long lifetime seems to be a special key when aiming at sterilization of the medical implements of a complicated structure with tiny gaps packed by a sterilization bag.

The gas to be used is generally harmless when it is not a plasma state, and as for the activated species generated by plasma, ideally, disappears in

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an instant, when the plasma is stopped, and does not leave harmful gas. As described above, we focused our attention on the $\cdot HO_2$ ($E°= +1.7$ V [5]) and $\cdot O_2^-$ ($E° = +0.07$ V for the $O_2|\cdot O_2^-$ couple and $E° = +0.36$ V for the $\cdot O_2^-|H_2O_2$ couple [5]) as activated species ($\cdot HO_2 \Leftrightarrow \cdot O_2^- + H^+$, pK = 4.8). Although the oxidation potential of these activated species is lower than $\cdot$ OH, the sterilization effect inside the medical implements of a complicated structure or narrow space such as inside the catheter is expectable because of their relatively long-lifetime; the lifetimes of $\cdot O_2^-$, $\cdot HO_2$ and $\cdot OH$ are roughly several hours, several minutes and hundreds of micro seconds, respectively. The weakness of oxidization power could be conquered by the lifetime and the population, or through short-time sterilization. It seems that it is not necessary to adhere to $\cdot OH$ with the strongest oxidization power if it is sufficiently sterilized. Moreover, what is necessary is to just use water and oxygen for plasma source (H_2O-O_2 plasma) in order to generate these activated species. These plasma sources are predominant in respect to safety and cost.

2. Experiment

2.1. Materials

The sterilization effect by radical was confirmed by biological indicator (BI, Fukuzawa) with spores of *Geobacillus stearothermophilus* ATCC 7953 with high heat resistance. The sectional view of BI is shown in Figure 1. This BI consists of a tubular container and a cap with six small square windows, made of resin respectively. In this container, the ampoule in which the culture solution with a pH indicator was enclosed was inserted together with the nonwoven fabric strip on which strain was fixed. A spore was fixed and snugly fit in a crevice between a container and an ampoule that was less than 1 mm, in a fabric strip of 0.6 mm thickness. The entrance of the container was sealed by a nonwoven fabric filter to avoid microbial contamination. Finally, the top was covered with a cap over the nonwoven fabric filter. The cap is equipped with six square shaped small windows from which gas molecules can trespass.

In order to confirm the existence of radicals, a prototype plasma chemical indicator (PCI, SAKURA) was used. The color of PCI shifted toward green when the PCI made contact with the radicals such as $\cdot OH$, $\cdot O$ and $\cdot O_2^-$. Whereas it is shifted toward red when the PCI made contact with $O_2(^1\Delta_g)$ [7, 8].

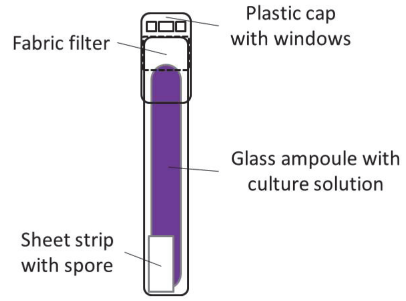

Figure 1. The sectional view of BI with 8.3 mm outer diameter and 45.5 mm height

2.2. H_2O-O_2 Plasma Sterilization Experiment

H_2O-O_2 plasma sterilization experiment with BI was carried out using a bell-jar type of plasma reactor shown in Figure 2.

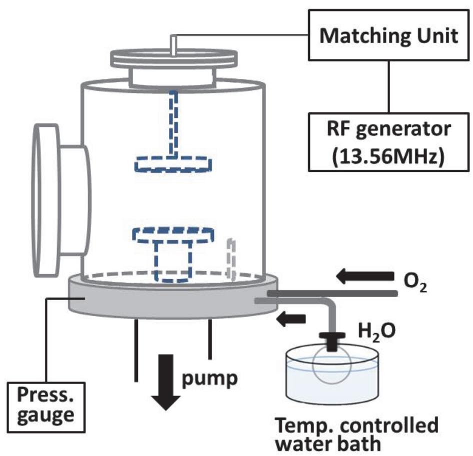

Figure 2. Schematic diagram of plasma reactor.

The reactor is equipped with a 8.8-L bell-jar made of stainless steel in which parallel disk-electrodes are set. H_2O vapor was supplied from a 50 mL glass ampoule which can keep a steady temperature between 25 and 35°C, and gas flow stability was maintained using a needle valve. These connecting lines made of stainless steel pipe were kept warm enough to prevent coagulation of these liquid sources. Pure O_2 was supplied from O_2 gas cylinder and controlled by mass flow controller (STEC). The pressure inside the reactor was monitored by capacitance gauge (MKS Baratron). H_2O-O_2 plasma was generated and controlled by rf generator (ADTEC, AX-1000) and automatic matching unit (ADTEC, AM-1000S).

BI was placed on the lower electrode, and then H_2O-O_2 plasma sterilization was carried out at a predetermined system pressure and 75 W of rf power for given periods of time. System pressure was set by controlling the partial pressure of H_2O and O_2.

When the direction of PCI discoloration was compared with and without a sterilization bag, a Plasma 200 sterilization bag (KAO) fabricated with DuPont Tyvek® was used. In this experiment, PCI was enclosed in the sterilization bag and the opening was sealed by a Heat Sealer (HAKKO, FV-803) before use.

2.3. Measurements

2.3.1. Sterilization Effect

After H_2O-O_2 plasma processing, BI was taken out promptly, The ampoule with culture medium inside BI container was destroyed in order to immerse the sheet strip with the spore into the culture medium. Then BI was kept in a cultivation apparatus at 60°C. After a 24-hour cultivation, the color of the culture solution was observed and judged. When no color change was observed from the original purple color it was judged to be an imperfect sterilization. When the color turned yellow, it was judged to be perfect sterilization.

2.3.2. Optical Emission Spectroscopy

Optical emission spectra are detected through an optical fiber by an optical spectrometer (Ocean Optics USB2000+) in the entire range from 200 nm to 900 nm.

2.3.3 PCI Color measurement

Discolorations of PCI after exposure to plasma were compared using a CIELAB color indicating system obtained from feflectance measurements results measured by the color analyzer NE-2000 (Nihon Denshoku).

3. Results and Discussion

3.1. Sterilization Effect

The results of an investigation of sterilization effect of H_2O-O_2 plasma treatment onto BI with different partial pressures of H_2O and O_2 and the processing time are summarized in Table 1.

When BI was irradiated with H_2O or O_2 plasma independently, sterilization did not occur at low pressure, and it turns out that the pressure of 80 Pa or more and 100 Pa or more are required for H_2O and O_2 respectively to acquire the sterilization effect within 60 min of the irradiation time.

On the other hand, when H_2O and O_2 were mixed, the sterilization effect increased notably with the increase in both partial pressures. It turned out that full sterilization can be attained by the plasma treatment for 15 minutes when the total pressure exceeded 150 Pa. However, when the total pressure exceeded 200 Pa, the container deformed because it exceeded the heat-resistant temperature (130 ℃) of BI container. From these results, it was found that H_2O-O_2 plasma treatment demonstrated higher sterilization effect than that by independent treatment of H_2O and O_2. 15 minutes after a plasma exposure at the pressure of 200 Pa, the temperature was less than 100 ℃. In addition, it was preliminary confirmed that the used *bacillus* cannot be inactivated by heat treatment for 1 hour at 120 ℃.

Table 1. Sterilization effect of H_2O-O_2 plasma treatment onto BI with different partial pressures of H_2O and O_2 and the processing time. The numbers in the frame express plasma irradiation time in minutes required for perfect sterilization to be confirmed.

		H_2O partial pressure (Pa)						
		0	**20**	**50**	**80**	**100**	**200**	**300**
O_2 partial pressure (Pa)	**0**	NR	+	+	60	60	30	D
	20	+	+	+	30	30	NR	UE
	50	+	+	30	30	15	D	UE
	80	+	30	30	15	15	D	UE
	100	60	30	15	15	15	D	UE
	200	30	NR	D	D	D	D	UE
	300	UE	UE	UE	UE	UE	UE	UE

D: BI container deformed with heat within 15 min.
+: Imperfect sterilization within 60 min.
UE: Unexecuted

3.2. Sterilization experiment using the sterilization bag

In order to investigate the H_2O-O_2 plasma sterilization effect on medical implements packed by the sterilization bag or medical implements of a complicated structure with tiny gaps, an H_2O-O_2 plasma sterilization experiment to BI enclosed with the sterilization bag was carried out. As a comparison, experiments with H_2O_2 plasma and H_2O_2 vapor were also carried out (results summarized in Table 2).

Table 2. Comparison of sterilization effects for BI with and without the sterilization bag.

	Total pressure (Pa)				
	50	**80**	**100**	**150**	**150***
H_2O-O_2 plasma	NR	NR	30	15	15
H_2O_2 plasma**	60	30	30	15	15
H_2O_2 gas**	UE	UE	UE	+	+

*: enclosed with the sterilization bag.
**: 30% H_2O_2 solution was used
+: Imperfect sterilization within 60 min.
UE: Unexecuted

The total pressure of 100 Pa took 30 minutes to sterilize in both plasma, but it only took 15 minutes to sterilize at 150 Pa. The temperatures at 15 minutes after plasma treatment by 150 Pa were below 75℃ in both case. It turned out that the sterilization effect of H_2O-O_2 plasma gives almost equal effect of the H_2O_2 plasma. Moreover, H_2O_2 vapor treatment without plasma showed no sterilization effect at all within this pressure range.

3.3. Optical Emission Spectroscopy

The emission spectra observed by H_2O and O_2 plasmas emerged by 75 W of rf power at 200 Pa are shown in Figures 3 and 4, respectively.

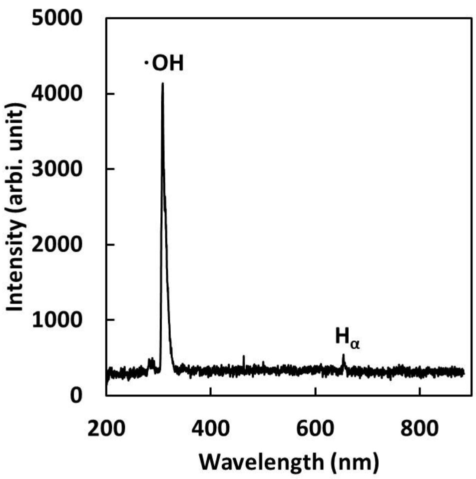

Figure 3. The emission spectrum of H_2O plasma emerged by 75W of rf power at 200 Pa.

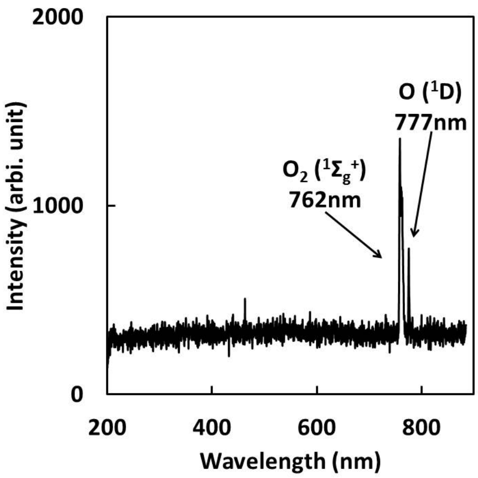

Figure 4. The emission spectrum of O_2 plasma emerged by75W of rf power at 200 Pa.

From the spectrum profiles shown in Figure 3 and 4, the peak which belongs ·OH was observed at 308 nm in the case of H_2O_2 plasma, and the peaks which belong to $O(^1D)$ and $O_2(^1\Sigma_g^+)$ were observed at 762 nm and 777 nm, respectively.

Next, the relations between H_2O pressure and the peak intensity of 308 nm that belongs to ·OH in H_2O plasma were plotted and shown in Fig. 5.

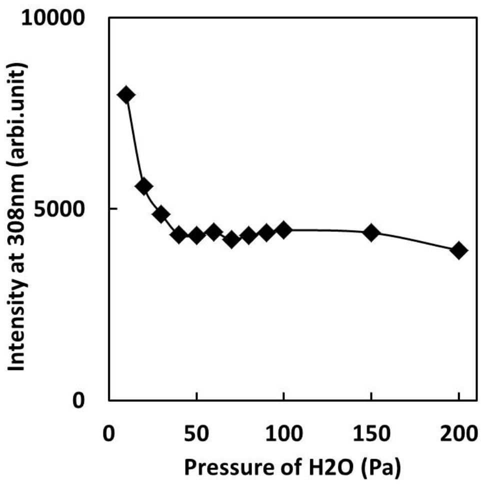

Figure 5. The relationship between H_2O pressure and the peak strength of ·OH at 308 nm.

About 7 eV of excitation energy is needed for generating ·OH from a H_2O molecule according to the potential curve of H_2O. From the results of Figure 5, ·OH peak intensity decreases with the increase in the pressure of H_2O, and it seems that the absolute quantity of ·OH has also declined with it.

In figures 6 and 7, the relations between O_2 pressure and the peak intensities of 777 nm and 767 nm that belong to $O(^1D)$ and $O_2(^1\Sigma_g^+)$ in O_2 plasma were plotted.

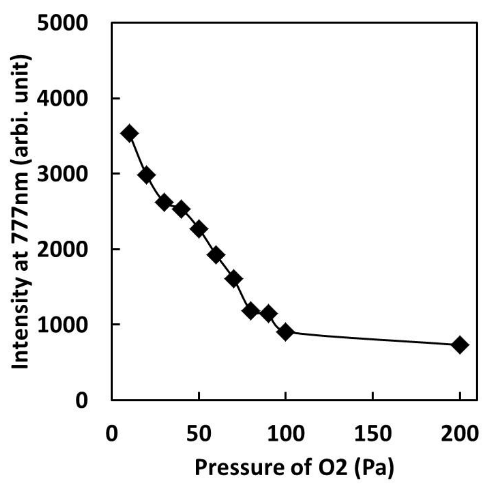

Figure 6. The relationship between O_2 pressure and the peak intensity of 777 nm that belongs to $O(^1D)$ in O_2 plasma.

About 6.0 or 8.4 eV excitation energy is needed for generating ·O from a O_2 molecule from the potential curve of O_2. From the result of Figure

6, it can be seen that ･O peak intensity decreases with the increase in the pressure of O_2, and it seems that the absolute quantity of ･O has also declined with it.

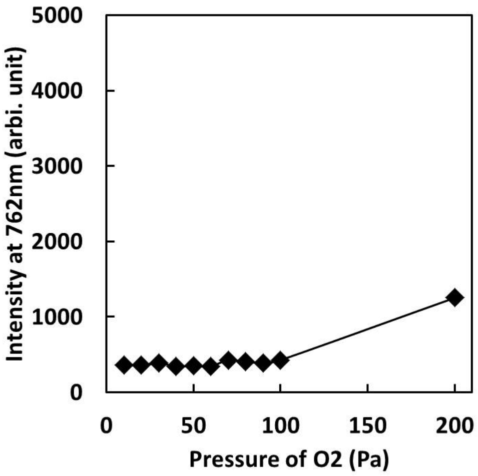

Figure 7. The relationship between O_2 pressure and the peak intensity of 767 nm that belongs to $O_2(^1\Sigma_g^+)$ in O_2 plasma.

From the result of Fig. 7, $O_2(^1\Sigma_g^+)$ peak intensity is seen to increase with the increase in the pressure of O_2, and it seems that the absolute quantity of active oxygen molecules such as $O_2(^1\Sigma_g^+)$, $O_2(^1\Delta_g)$ and $･O_2^-$ have increased along with it. In other words, at high pressure the action of ･O or ･OH which requires relatively large excitation energy for generating become inferior in strength. Instead, the action of molecular active oxygen species generable with relatively low excitation energy become superior.

3.4. Plasma Diagnosis by PCI

In our previous report [7,8], in order to confirm the plasma processing effect at a required point by visual observation, PCI was fabricated. The relation between the resultant color of PCI and interactions with H_2O and O_2 plasmas are shown in Table 3. The original color of PCI is purple.

Table 3. The resultant color of PCI with original color of purple irradiated by different gas plasma with different pressure, and the activated species assumed to be involved with discoloration.

Plasma Gas	10 Pa	Main Active Species	100 Pa	Main Active Species
H_2O	Green	･OH	Green	･OH
O_2	Green	･O	Red	$O_2(^1\Delta_g)$

In the case of H_2O plasma, PCI discolored in the green direction from original color of purple. In comparison with 10 Pa and 100 Pa at H_2O plasma irradiation PCI, the 10 Pa showed much more clear green compared to 100 Pa, indicating that 10 Pa has produced more ･OH than 100 Pa, which was thought to be the main active specie of discoloration in H_2O plasma. And it is in good agreement with the result of Figure 5.

In the case of O_2 in Table 3, it discolored in the green direction from original purple in 10 Pa. However, discoloration in the direction of red was confirmed in 100 Pa.

The resultant color of green in both case of H_2O and O_2 plasma is considered to be the action of ･OH for H_2O plasma and ･O for O_2 plasma, and it is explained by the ease of decomposing of red dye in PCI against radical [7, 8]. On the other hand, the resultant color of red in 100 Pa for O_2 plasma is explained by the ease of decomposing of green pigment in PCI against the action of $O_2(^1\Delta_g)$ [7, 8]. As for the action of $O_2(^1\Sigma_g^+)$, it is omitted here since concentration of it is about 3 order of magnitude lower than the $O_2(^1\Delta_g)$ and the quenching rate is also about 10^5 times more rapid than $O_2(^1\Delta_g)$ [9].

The Passage of discoloration of PCI by H_2O_2-O_2 plasma at 200 Pa was expressed by CIELAB, and shown in Figure 8. In this figure *a* and *b* are the chromaticity coordinates (+*a* is for red, -*a* for green, +*b* for yellow, -*b* for blue).

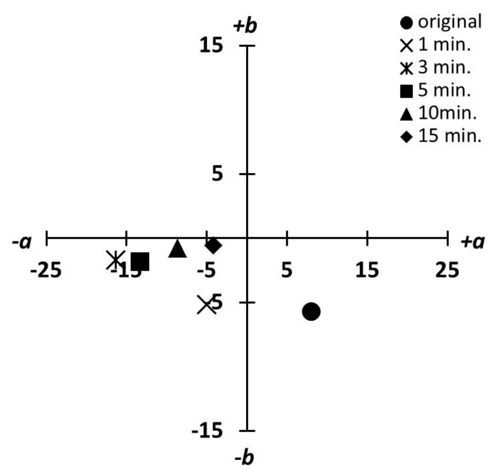

Figure 8. The relationship between H_2O-O_2 plasma irradiation time and color change in PCI at 200 Pa.

As shown in Figure 8, the color of PCI after H_2O_2-O_2 plasma irradiation start is shifting in the green direction of –a, and it turns out that the time which showed the clearest green is 3 minutes after the start of plasma irradiation. Then, the plot is shifted to the right and the center of an axis of coordinates, i.e., turned white, indicating decomposition of all coloring matter. At 400 Pa, this tendency became still more remarkable.

3.5. Plasma Diagnosis by PCI using sterilization bag

Table 4 indicates the resultant color of PCI discoloration compared with and without sterilization bag by O_2 plasma at different pressure.

Table 4. The resultant color of PCI discoloration was compared with and without a sterilization bag by O_2 plasma at different pressure, and the activated species assumed to be involved with discoloration.

Plasma Gas	100 Pa	Main Active Species	200 Pa	Main Active Species
O_2 (Without Sterilization bag)	Red	$O_2(^1\Delta_g)$	Red	$O_2(^1\Delta_g)$
O_2 (With Sterilization bag)	Green	$\cdot O_2^-$ HOO$\cdot$	Green	$\cdot O_2^-$ HOO$\cdot$

As shown in Table 4, PCI without a sterilization bag is discolored red from original purple because of the action of $O_2(^1\Delta_g)$ as described in Table 4. However, it discolored green when the PCI was enclosed in the sterilization bag. This change can be explained as follows. DuPont Tyvek®, which is a material of the sterilization bag, is a kind of filter. That is, it is permeable for small molecules like a gas molecule, but not for a microbe. Although it is permeable to a gas molecule, activated species with short-lifetime could be thinned out with this filter. Therefore, in this experiment, the species which can pass along a sterilization bag were seen to be restricted to relatively long-lifetime active species. Moreover, as the resultant color of PCI is green this suggests that the kind of activated species should be a radical. In Table 4, it is hard to consider the $\cdot$O from the result of Fig. 6 as an activated species which turned the PCI to green by the O_2 plasma at 100 Pa and 200 Pa of pressure in spite of the PCI being enclosed in a sterilization bag. Therefore, $\cdot O_2^-$ can be considered as the main active specie formed with such comparatively high oxygen pressure.

$\cdot O_2^-$ has an important role in the living body. Since the oxidization power is not so powerful (+0.07 to +0.36 V), we do not know whether the bacterial spore of a *bacillus* can be destroyed or not. On the other hand, it is also necessary to take account of the generation of hydrogen peroxide and $\cdot HO_2$ from $\cdot O_2^-$ if a H^+ exists. Since the sterilization bag is made of high-density polyethylene, the source of hydrogen exists in plasma. Moreover, it is thought that generation of $\cdot HO_2$ becomes easier since sufficient hydrogen will be supplied from water if H_2O_2-O_2 plasma is used even if it does not take a sterilization bag into consideration.

4. Conclusion

In order to realize the sterilization of the medical implements packed by the sterilization bag or medical implements of a complicated structure with tiny gaps, application of the long-lifetime oxygen active species such as HO_2/O_2^- radicals formed by H_2O-O_2 plasma were confirmed to be effective. This conclusion was supported by the following three experimental results. The first is that the sterilization time became short with the increase in the pressure in H_2O-O_2 plasma with equal partial pressures of H_2O and O_2. The second is that the activated species which were created by this H_2O-O_2 plasma have passed two filters, the filter of a sterilization bag, and the filter of BI. The third is that the radicals which can discolor PCI green were detected in the O_2 plasma of 100 Pa or more pressure, at which the actions of $O_2(^1\Sigma_g^+)$ and $O_2(^1\Delta_g)$ become dominant.

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