

Sporicidal action of ozone and hydrogen peroxide: a comparative study

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Received 4 October 2000; received in revised form 10 May 2001; accepted 11 June 2001

Abstract

Elimination of contaminating spores on packaging materials and food-contact surfaces remains a challenge to the food industry. Hydrogen peroxide and chlorine are the most commonly used sanitizers to eliminate these contaminants, and ozone was recommended recently as an alternative. Hence, we compared the sporicidal action of ozone and hydrogen peroxide against selected foodborne spores of *Bacillus* spp. Under identical treatment conditions, 11 $\mu\text{g}/\text{ml}$ aqueous ozone decreased spore counts by 1.3 to 6.1 \log_{10} cfu/ml depending upon the bacterial species tested. Hydrogen peroxide (10%, w/w), produced only 0.32 to 1.6 \log_{10} cfu/ml reductions in spore counts. Thus, hydrogen peroxide, at $\sim 10,000$ -fold higher concentration, was less effective than ozone against *Bacillus* spores. Resistance of spores to ozone was highest for *Bacillus stearothermophilus* and lowest for *B. cereus*. Therefore, spores of *B. stearothermophilus* are suitable for testing the efficacy of sanitization by ozone. Electron microscopic study of ozone-treated *B. subtilis* spores suggests the outer spore coat layers as a probable site of action of ozone. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ozone; Hydrogen peroxide; Sporicidal; *Bacillus*

1. Introduction

The bacterial endospore is resistant to a variety of harsh treatments including heat, irradiation, chemicals and desiccation. Spores can survive for long periods in the absence of moisture and exogenous nutrients. Bacterial spores survive treatments with commercial sterilants and disinfectants (Sagripanti and Bonifacino, 1999). Spores also possess a swift and highly efficient mechanism for reverting to the vegetative state when nutrients, in aqueous solutions, become available (Gould et al., 1994). Therefore,

presence of *Bacillus* and *Clostridium* spores in food constitutes a challenge to the industry.

Clostridium botulinum spores are widely distributed in the environment (Smith and Sugiyama, 1988); these spores are occasionally isolated from food (Franciosa et al., 1999). Bacterial spores, present as contaminants in food, may survive processing, grow during storage, and cause spoilage of food or diseases to consumers. Meer et al. (1991) noted that *Bacillus cereus* survives adverse environmental conditions, adapts and eventually multiplies in foods. Some strains of *B. cereus* grew to $\sim 10^6$ cfu/g and produced toxin in refrigerated foods (Dufrenne et al., 1995). Sporeforming bacilli were reported to cause spoilage of pasteurized, aseptically packed apple juice (Cerny et al., 1985; Splittsoesser et al., 1994). Con-

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concentrated orange juice from different suppliers has been recently found to contain spores of *Alicyclobacillus* spp. (Eiroa et al., 1999). Additionally, Komitopoulou et al. (1999) reported the ability of *Alicyclobacillus acidoterrestris* to grow in orange juice, grapefruit juice and apple juice, and the resistance of its spores under normal juice pasteurization conditions. Elimination of such spores from equipment surfaces, packaging materials and the food itself is a prerequisite for successful production of aseptically packaged products.

To inactivate contaminating spores in the processing environment, hydrogen peroxide (Yokoyama, 1990) and chlorine (Marriott, 1999) are commonly used. Ozone was recommended recently as an alternative to chlorine (Kim, 1998) and hydrogen peroxide (Khadre and Yousef, 2001). Ozone use in the processing environment may become feasible if the sporicidal action of this sanitizer is demonstrated. Therefore, this study was initiated to compare the effectiveness of ozone and H₂O₂ against a variety of foodborne bacterial spores.

2. Materials and methods

2.1. Ozone

Ozone demand-free glassware was prepared as described previously (Kim et al., 1999). Aqueous ozone was produced by bubbling ozone gas into sterile deionized water at controlled flow rates. Ozone gas was produced from purified extra dry oxygen by an ozone generator (U.S. Filter/Polymetrics T-816, San Jose, CA). The desired ozone concentration in water was attained by adjusting the flow rate of gaseous ozone. Approximate concentration of ozone solubilizing in water was monitored by measuring absorbance at 258 nm (A_{258}), using a spectrophotometer (Spectronic 1201, Milton Roy, Rochester, NY), as indicated in a previous study (Kim and Yousef, 2000). Ozonation of water continued until the targeted ozone concentration ($\sim 10 \mu\text{g/ml}$) was attained. Final ozone concentration in water was measured using the indigo method (Bader and Hoigne, 1981). The resulting aqueous ozone solution (11 $\mu\text{g/ml}$) was tested against spores of eight *Bacillus* spp. This concentration was chosen based on

preliminary experiments on the sensitivity of spores of *B. subtilis* OSU494 to varying concentrations of ozone (0.2 to 14 $\mu\text{g/ml}$). All experimental work with ozone was done under a chemical hood. Excess ozone was neutralized by diverting the gas stream into a reservoir containing 2% potassium iodide solution or to an ozone-decomposing catalytic column. Protective masks and ozone-resistant gloves were worn during the experiments.

2.2. Hydrogen peroxide

Hydrogen peroxide solution (30% w/w) (Sigma, St. Louis, MO) was stored at 4 °C, as recommended by the manufacturer. Lower concentrations of hydrogen peroxide were prepared by dilution in sterile deionized water, and kept at 4 °C until used.

2.3. Catalase enzyme

Lyophilized catalase enzyme (Sigma) contained 3260 units/mg, and it was stored at -18 °C. Catalase enzyme solutions were prepared according to the manufacturer's specifications and used within 30 min, during which it was kept at 4 °C.

2.4. Bacterial cultures

Eight *Bacillus* spp. were obtained from the culture collection of the Department of Microbiology at the Ohio State University and tested in this study. These strains were *B. subtilis* OSU494, *B. subtilis* OSU848, *B. subtilis* var niger ATCC 9372, *B. subtilis* ATCC 19659, *B. cereus* OSU11, *B. polymyxa* OSU443, *B. megaterium* OSU125 and *B. stearothermophilus* OSU24. Stock cultures of these bacteria were grown in nutrient broth (Difco Laboratories, Detroit, MI) at 37 °C for 24 h, and their spores were prepared as indicated later.

2.5. Spore suspensions

Spore suspensions were prepared as described by Sala et al. (1995). Briefly, cultures of *Bacillus* spp. were spread onto sporulation agar medium and inoculated plates were incubated for 6–8 days at 37 °C. The sporulation medium consisted of nutrient agar supplemented with 500 ppm Bacto-dextrose (Difco

Laboratories) and 3 ppm manganese sulfate (Mol-linckrodt, Paris, KY). Sporulation was verified by microscopic inspection of the growth under phase contrast. Spores were harvested and treated in a sonicator (FS-28, Fisher, Pittsburgh, PA) to disperse clumps. The sonicated suspensions were washed six times by centrifugation ($8000 \times g$ for 20 min at 4 °C) and resuspension in sterile deionized water. After an additional centrifugation, the spore pellet was resuspended in 0.1% sodium chloride solution to obtain $\sim 10^9$ spores/ml. The spore suspension was stored at 4 °C until used.

2.6. Ozone treatment

A portion of the spore suspension (0.2 ml) was dispensed in a 4-oz stomacher bag and 20 ml, 11 $\mu\text{g/ml}$ aqueous ozone (22 °C) was added. The mixture was stomached immediately for 1 min, and 1.0-ml aliquot was transferred to a test tube containing 9-ml sterile peptone water to neutralize excess ozone. In some experiments, 2 ml sodium thiosulfate solution (0.206 g/l) (Fisher Scientific, Fair Lawn, NJ) was added to the contents of the stomacher bag to neutralize excess ozone before counting the survivors. These two methods were equally effective in neutralizing excess ozone. Additionally, sodium thiosulfate, at the amount used, had no effect on the viability of the treated spores (data not shown).

2.7. Hydrogen peroxide treatment

Spores of the eight *Bacillus* spp. were treated with 10% hydrogen peroxide solution (i.e., 100,000 $\mu\text{g/ml}$) as follows. A spore suspension aliquot (0.2 ml) was dispensed in a sterile 500-ml Erlenmeyer flask and 20 ml hydrogen peroxide solution (22 °C) was added. The mixture was stirred for 1 min using a magnetic stirrer. A solution (2 ml) containing enough catalase enzyme to neutralize excess hydrogen peroxide was added to the flask with continuous stirring until frothing stopped and most of the bubbles dissipated. Catalase enzyme at the concentrations used did not have any sporicidal effect. A 1.0-ml aliquot was transferred to a test tube containing 9-ml sterile peptone for dilution and plating. A similar procedure was used to test the activity of 1% to 30% hydrogen peroxide against spores of *B. subtilis* OSU494.

2.8. Microbiological analysis

For enumerating surviving bacterial spores, sanitizer-treated and untreated spore suspensions were heat-shocked at 80 °C for 30 min, and counts were determined in plate count agar using the pour-plating technique. Plates were incubated for 48 h at 35 °C and colonies were counted.

2.9. Electron microscopy

A spore suspension (0.2 ml) was mixed with 20 ml ozone-water (22 °C) in a 4-oz stomacher bag and the mixture was stomached immediately for 1 min. Sodium thiosulfate (2 ml, 0.206 g/l) was added to the bag contents to neutralize excess ozone. The control treatment was exposed to 20 ml deionized water instead of ozone-water. The following procedure was recommended by the Department of Imaging and Microscopy, the Ohio State University. Spores were centrifuged at $8000 \times g$ for 20 min, the pellet was suspended in 1.5 ml, 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and kept at 4 °C overnight for fixation. Spores were centrifuged and rinsed three times in 0.1 M cacodylate buffer, pH 7.2 (referred to as buffer hereafter), at 25 °C. Spores were fixed in 1% osmium tetroxide in buffer for 1.5 h, and rinsed twice in buffer with centrifugation and resuspension. After centrifugation and removal of most of the buffer, spores were suspended in a small quantity of 2% agarose, which was allowed to gel. After the agarose-spores mixture was cooled in an ice-bath, it was cut into pieces not larger than 1 mm^3 and left in buffer overnight at 4 °C. Samples were rinsed twice in distilled water and en bloc stained in 1% uranyl acetate for 90 min. Samples were rinsed twice in distilled water and gradually dehydrated in solutions containing 50% to 100% ethanol. Samples were put into propylene oxide for 20 min and infiltrated in 1:1 propylene oxide/Spurr resin for 24 h. Samples were embedded in Spurr resin in flat embedding molds and polymerized overnight at 60 °C. Sections were cut at 70 nm on a Reichert Ultracut E ultramicrotome and picked up on formvar-coated 200 mesh copper grids. Grids were stained in 2% aqueous uranyl acetate for 15 min, followed by Reynolds lead citrate for 5 min. Grids were examined in a Philips CM 12 transmission electron microscope at 60 kV.

2.10. Data analysis

Population of spores, which was inactivated during the ozone treatment (\log_{10} cfu/ml untreated– \log_{10} cfu/ml treated sample), was analyzed using MINITAB statistical program (Minitab, State College, PA). One-way analysis of variance was performed for the effect of spore strain on the degree of inactivation by ozone. Multiple comparison of means was done using Fisher's range test at an error rate of 0.05.

3. Results

3.1. Relative resistance of spores to ozone

Treatment of spore suspensions with 11 $\mu\text{g/ml}$ aqueous ozone for 1 min followed by neutralization of excess ozone, reduced spore counts by 1.3 to 6.1 \log_{10} cfu/ml depending upon the bacterial strain (Table 1). Resistance of spores to ozone was highest

Table 1

Decrease in spore populations (\log_{10} cfu/ml untreated control– \log_{10} cfu/ml treated sample)^a after exposure of different *Bacillus* spp. to ozone (11 $\mu\text{g/ml}$) or hydrogen peroxide (100,000 $\mu\text{g/ml}$) for 1 min at 22 °C, followed by neutralization with sodium thiosulfate or catalase, respectively

<i>Bacillus</i> spp.	Ozone		Hydrogen peroxide	
	Average ^{b,c}	SD ^d	Average ^{c,e}	SD ^d
<i>B. cereus</i> OSU11	6.1 ^A	1.0	1.6 ^A	0.22
<i>B. megaterium</i> OSU125	2.1 ^C	0.49	0.93 ^{AD}	0.29
<i>B. polymyxa</i> OSU443	1.9 ^C	0.50	0.58 ^D	0.11
<i>B. stearothermophilus</i> OSU24	1.3 ^C	0.07	0.64 ^{CD}	0.19
<i>B. subtilis</i> OSU494	2.7 ^C	0.83	0.32 ^D	0.14
<i>B. subtilis</i> OSU848	4.8 ^B	0.57	1.2 ^{ABC}	0.68
<i>B. subtilis</i> ATCC 19659	6.1 ^{AB}	0.85	0.64 ^{BD}	0.03
<i>B. subtilis</i> var Niger ATCC 9372	5.7 ^{AB}	0.43	1.3 ^A	0.44

^aAverage initial count is 1.3×10^7 spore/ml.

^bData represent averages of two to seven repeats.

^cAverages, within the same column, with the same capital letter are not significantly different (Fisher's LSD at $p = 0.05$).

^dSample Standard Deviation.

^eData represent averages of three repeats.

for *B. stearothermophilus* OSU24, *B. polymyxa* OSU443, *B. megaterium* OSU125 and *B. subtilis* OSU494; differences among these species were insignificant ($p < 0.05$). Spores of *B. subtilis* OSU848 had an intermediate resistance to ozone. Compared to other tested strains, spores of *B. subtilis* ATCC 19659, *B. cereus* OSU11 and *B. subtilis* var Niger ATCC 9372 were the most sensitive to ozone; differences among these three strains were not significant ($p < 0.05$).

3.2. Relative resistance of spores to hydrogen peroxide

When spores of eight *Bacillus* strains were treated with 10% H_2O_2 for 1 min at 22 °C, the counts decreased 0.32 to 1.6 \log_{10} cfu/ml, depending on the bacterial species tested (Table 1). Spores of *B. subtilis* OSU494, *B. polymyxa* OSU443, *B. stearothermophilus* OSU24, *B. subtilis* ATCC 19659 and *B. megaterium* OSU125 were the most resistant to the hydrogen peroxide treatment, and differences among these strains were not statistically significant ($p < 0.05$). Spores of *B. subtilis* OSU848 had intermediate resistance, whereas spores of *B. cereus* OSU11 and *B. subtilis* var Niger ATCC 9373 were the most sensitive to the hydrogen peroxide treatment.

Results in Table 1 illustrate the superiority of ozone to hydrogen peroxide as a sporicidal agent; H_2O_2 , at $\sim 10,000$ -fold higher concentration, was less effective than ozone against *Bacillus* spores. Since *B. subtilis* OSU494 showed the highest resistance to 10% H_2O_2 solution, this strain was tested at a range of H_2O_2 concentrations. The count of *B. subtilis* OSU494 spores decreased modestly when the concentration of H_2O_2 increased from 1% to 15%, and appreciably at 20% to 30% (Fig. 1).

3.3. Mechanism of action of ozone on spores

Correlation between susceptibility of spores to ozone and hydrogen peroxide may reflect similarity in the mechanism of spore inactivation by these two oxidizing agents. Spores, treated or untreated with ozone, were examined by transmission electron microscope (TEM). Inspecting these micrographs re-

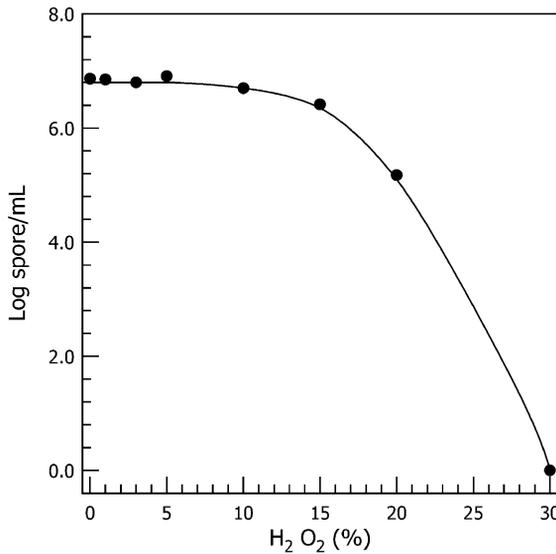


Fig. 1. Inactivation of spores of *B. subtilis* OSU494, 7.3×10^6 initially, when treated with a varying concentration of hydrogen peroxide (1% to 30%) at 22 °C for 1 min.

vealed damage to the surface layer, the outer spore coat, and to some extent to the inner spore coat layer in ozone-treated spores, which may have lead to exposing the cortex to the action of ozone (Fig. 2). Spore structure designations followed that of Henrique and Moran (2000).

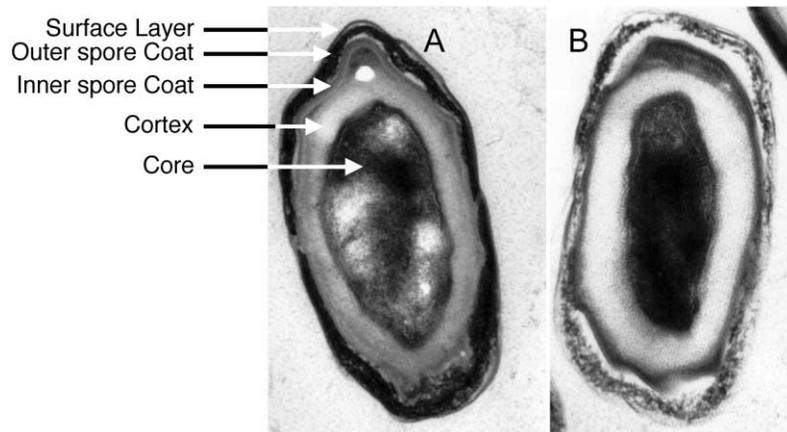


Fig. 2. Transmission electron microscopic micrograph of *B. subtilis* OSU494 spores, untreated (A), or treated (B) with ozone. Ozone-treated spores were exposed to aqueous ozone (10 $\mu\text{g}/\text{ml}$) at 22 °C for 1 min followed by neutralization with sodium thiosulfate. Note that the surface layer and the outer spore coat are the structures most apparently damaged by ozone treatment.

4. Discussion

4.1. Spores and ozone

Our study demonstrates the ability of ozone in water at low concentrations to produce significant reduction in spore counts, compared to hydrogen peroxide. Sensitivity of bacterial spores to ozone, compared to other sanitization factors, is of interest to food processors who are also interested in identifying an indicator microorganism for this sanitizer. *B. stearothermophilus* may serve as a suitable indicator for ozone sanitization. In addition to its resistance to ozone (Table 1), spores of *B. stearothermophilus* also are extremely resistant to heat (Russell, 1982). Spores of *B. subtilis* var niger ATCC 9372 are used as indicators in dry heat and ethylene oxide sterilization (Anonymous, 1995, 1999). Spores of *B. subtilis* ATCC 19659 and *B. subtilis* var niger ATCC 9372 are used commercially in sterility testing of aseptic fillers (e.g., the spore-strip kit of North American Science Associates, Northwood, OH). These two strains, however, are sensitive to ozone (Table 1).

4.2. Spores and hydrogen peroxide

Compared to ozone in water, hydrogen peroxide was substantially inferior in sporicidal activity. Set-

low and Setlow (1993) found *B. subtilis* spores resistant to treatment with 4 M hydrogen peroxide solution for 20 min. It is of interest to note also that the antimicrobial power of hydrogen peroxide increases as the temperature rises (Toledo, 1975), while that of ozone increases as the temperature decreases below ambient (Herbold et al., 1989). In this study, hydrogen peroxide at a concentration of 15% (22 °C) for 1 min decreased *B. subtilis* spores 0.44 log₁₀ cfu/ml, whereas Shin et al. (1994) observed 4.7 log₁₀ reduction of similar spores using 15% hydrogen peroxide at 60 °C for 30 min. Therefore, for effective sporicidal action in the food processing environment, treatment with H₂O₂ (at 30%) is followed by application of hot air (Yokoyama, 1990). Detectable changes in the physical structure of spores required 10 µg/ml ozone at 22 °C for 1 min (Fig. 2) or 15% hydrogen peroxide at 60 °C for 120 min (Shin et al. 1994). Cerf and Metro (1977) suggested that hydrogen peroxide in the immediate vicinity of spores is destroyed by an associated spore catalase enzyme. Lawrence (1957) indicated that intact spores have demonstrated catalase activity independent of the vegetative residue or the presence of germinated spores.

When spores were compared, *B. cereus* OSU11 and *B. subtilis* var niger ATCC 9372 were the most sensitive, whereas *B. subtilis* OSU494 and *B. polymyxa* OSU443 were the most resistant to hydrogen peroxide, under the conditions tested in this study. Spores of *B. subtilis* ATCC 19659 and *B. subtilis* var niger ATCC 9372, which are commonly used in sterility testing of aseptic fillers, varied in sensitivity to H₂O₂; ATCC 19659 was moderately resistant but ATCC 9372 was sensitive to the sanitizer. *B. stearothermophilus* produces one of the most heat-resistant spores known (Russell, 1982); this bacterium was also fairly resistant to hydrogen peroxide and ozone (Table 1). Resistance of spores to inactivation by hydrogen peroxide and tertiary butyl hydroperoxide has been reported for *B. stearothermophilus*, *B. subtilis* and *B. megaterium* (Marquis et al., 1994). It appears that there is a threshold concentration for the sporicidal action of H₂O₂. According to our data (Fig. 1), 15% was the threshold of action of hydrogen peroxide against *B. subtilis* OSU494. Therefore, in aseptic processing, high concentration of H₂O₂ should be maintained

for effective sanitization of equipment surfaces and packaging materials.

4.3. Mechanism of action of ozone on spores

The precise killing mechanism of spores by ozone and similar oxidizing agents are not fully understood. Setlow and Setlow (1993) found no increase in mutation frequency and no DNA damage among survivors of H₂O₂-treated spores of *B. subtilis*. In contrast, *B. subtilis* spores treated with H₂O₂ showed clear degradation of outer spore layers including spore coats and cortex (Shin et al. 1994). Our present study on ozone supports the notion that oxidizing agents including ozone and H₂O₂ probably kill spores by degrading outer spore components, and exposing the spore core to the action of the sanitizer (Fig. 2).

Coats comprise ~ 50% of the spore volume. These coats contain ~ 80% of the spore proteins and they constitute barriers to damaging enzymes such as lysozyme (Murrell, 1967; Aronson and Horn, 1972; Marquis et al., 1994). Spore coats are probably disrupted by oxidizing sporicidal agents such as hydrogen peroxide and hypochlorite, which may cause extraction of spore coat material, facilitating the penetration of these sanitizers into the cortex and protoplast (Bayliss and Waites, 1976). It is important to note that extracted spores, i.e., spores in which the spore coats have been removed, retain their dipicolinic acid, and refractility. These extracted spores are resistant to heat and radiation, and are fully viable but they become sensitive to lysozyme (Russell, 1982; Marquis et al., 1994). Hydrogen peroxide was shown to remove protein from the spore coats in *B. cereus* and *C. bifementans* (Russell, 1982).

In spite of the evidence that oxidizing agents target spore coats, damage to DNA may partially explain spore inactivation by these agents. Setlow and Setlow (1993) believe that hydrogen peroxide, or possibly the free hydroxyl radicals resulting from its degradation, gained access to the core of spores of certain *B. subtilis* mutants and killed these spores at least in part by DNA damage. Similarly, Shin et al. (1994) found that H₂O₂-treated (15%, at 60 °C for 30 min) spores of *B. megaterium* greatly lost viability (> 5 log₁₀ reduction in viability) with almost no loss in optical density, change in the phase micro-

scopic appearance of the spores, or observable changes in the fine structure of the spores. Ozone, in our study, damaged the outer spore coat but slightly affected the inner coat and spared the cortex (Fig. 2); the vast majority of these spores lost viability. Gerhardt et al. (1972) suggested that molecules greater than 200 Da penetrate ~40% of the spore volume.

5. Conclusion

It is evident that ozone is superior to hydrogen peroxide in killing bacterial spores. The comparatively low concentration needed to eliminate large population of spores at ambient temperature in short-time periods makes ozone best suited for industrial settings. Effectiveness of ozone in disinfecting food-contact surfaces may be tested using spores of *B. stearothermophilus* as indicators.

Acknowledgements

The research in this publication was partially funded by the Center for Advanced Processing and Packaging Studies and the National Science Foundation.

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