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### Research Article

The possibility that neutrophils produce the hydroxyl radical (OH<sup>-</sup>) was studied by examining the ability of these cells to support the release of ethylene from methional, a reaction in which it has been shown that OH<sup>-</sup>, but not O<sub>2</sub><sup>-</sup> or H<sub>2</sub>O<sub>2</sub>, may serve as the oxidizing agent. When neutrophils were exposed to opsonized zymosan in the presence of 0.35 mM methional, ethylene was released in quantities amounting to 44.6±3.6 pmol/10<sup>6</sup> cells/40 min. Ethylene production required the presence of neutrophils, opsonized zymosan, and methional, indicating that it was formed from methional by stimulated but not resting neutrophils. Ethylene was not produced by zymosan-treated cells from patients with chronic granulomatous disease, confirming the requirement for respiratory burst activity in this process. Ethylene production was suppressed by benzoic acid, an OH<sup>-</sup> scavenger. Superoxide dismutase (3 microgram/ml) reduced ethylene production to 21% of control levels, but catalase had no significant effect in this system. These findings indicate that stimulated neutrophils produce a highly reactive oxidizing radical, possibly OH<sup>-</sup>, which releases ethylene from methional, and that the O<sub>2</sub><sup>-</sup> generated during the respiratory burst is involved in the production of this reactive species.

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# Evidence for Hydroxyl Radical Production by Human Neutrophils

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**ABSTRACT** The possibility that neutrophils produce the hydroxyl radical ( $\text{OH}\cdot$ ) was studied by examining the ability of these cells to support the release of ethylene from methional, a reaction in which it has been shown that  $\text{OH}\cdot$ , but not  $\text{O}_2^-$  or  $\text{H}_2\text{O}_2$ , may serve as the oxidizing agent. When neutrophils were exposed to opsonized zymosan in the presence of 0.35 mM methional, ethylene was released in quantities amounting to  $44.6 \pm 3.6$  pmol/ $10^6$  cells/40 min. Ethylene production required the presence of neutrophils, opsonized zymosan, and methional, indicating that it was formed from methional by stimulated but not resting neutrophils. Ethylene was not produced by zymosan-treated cells from patients with chronic granulomatous disease, confirming the requirement for respiratory burst activity in this process. Ethylene production was suppressed by benzoic acid, an  $\text{OH}\cdot$  scavenger. Superoxide dismutase (3  $\mu\text{g}/\text{ml}$ ) reduced ethylene production to 21% of control levels, but catalase had no significant effect in this system. These findings indicate that stimulated neutrophils produce a highly reactive oxidizing radical, possibly  $\text{OH}\cdot$ , which releases ethylene from methional, and that the  $\text{O}_2^-$  generated during the respiratory burst is involved in the production of this reactive species.

## INTRODUCTION

It has been clearly established that oxygen-requiring bactericidal mechanisms play a role in the destruction of pathogens by neutrophils (1). The best characterized of these is the myeloperoxidase-dependent system (1, 2), in which myeloperoxidase, an enzyme abundant in neutrophils, mediates bacterial killing in the presence of  $\text{H}_2\text{O}_2$  and a halide ion (probably  $\text{Cl}^-$ , in the intact

neutrophil). The  $\text{H}_2\text{O}_2$  used by this system arises by the dismutation of  $\text{O}_2^-$  (3–9), the product of an enzyme-catalyzed reaction in which NADPH reduces oxygen by one electron (8, 9). The  $\text{O}_2^-$ -forming system is dormant in resting cells, but is activated on exposure of the cells to suitable stimuli; the changes in oxygen metabolism that result from the activation of this system are designated the “respiratory burst.”

Recently it has become apparent that neutrophils employ additional oxygen-requiring bactericidal mechanisms that do not depend upon myeloperoxidase (1, 10). The possibility that  $\text{O}_2^-$ , a reactive oxygen radical, might function in the neutrophils as a bactericidal agent in such a mechanism was suggested by studies showing that  $\text{O}_2^-$  was produced by activated but not resting neutrophils (3–6), and by observations on the distribution of superoxide dismutase among bacterial species which were consistent with the notion that aerotolerant organisms but not obligate anaerobes survived in air by protecting themselves enzymatically against  $\text{O}_2^-$  (11).<sup>1</sup> When this possibility was examined directly with artificial  $\text{O}_2^-$ -generating systems, it was found that  $\text{O}_2^-$  by itself was only weakly bactericidal (14–17). However,  $\text{O}_2^-$  in combination with  $\text{H}_2\text{O}_2$  was found to be highly toxic to a number of bacterial species, much more so than either compound alone (10, 14, 15). These results, which were obtained not only with artificial  $\text{O}_2^-$ -generating systems, but also with intact neutrophils engaged in the destruction of bacteria (10), were interpreted to indicate that a very potent antimicrobial agent was formed by a reaction between  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ . The compound proposed to account for the efficient bactericidal activity of the  $\text{O}_2^-$ - $\text{H}_2\text{O}_2$  system was the hydroxyl radical ( $\text{OH}\cdot$ ), which was postulated to arise by the transfer of an electron from  $\text{O}_2^-$  to  $\text{H}_2\text{O}_2$  (the Haber-Weiss reaction):

<sup>1</sup> Superoxide dismutase has now been reported to occur in a number of obligate anaerobes (12, 13).

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Additional evidence for the participation of  $\text{OH}\cdot$  in bacterial killing by  $\text{O}_2^-$ -generating systems has been obtained through the use of  $\text{OH}\cdot$  scavengers such as mannitol and benzoic acid (14). These compounds provide weak but reproducible protection against bacterial destruction by such systems. This protection has been attributed to their ability to react with  $\text{OH}\cdot$ .

The foregoing observations, which constituted persuasive but indirect evidence for the participation of  $\text{OH}\cdot$  in neutrophil function, suggested that a more direct approach to this question might be of value. We have therefore sought to obtain evidence for  $\text{OH}\cdot$  production by neutrophils by examining whether the incubation of these cells with methional results in ethylene production. Studies with chemical systems have shown that  $\text{OH}\cdot$  is capable of mediating the formation of ethylene from methional (18). The ability of neutrophils to support this reaction may thus be taken as evidence for the production by these cells of an unusually powerful oxidizing agent:  $\text{OH}\cdot$  itself, or a species of similar oxidizing potency.

## METHODS

Catalase (Sigma C-40, thymol free), methional (3-methylthiopropional), bovine serum albumin, mannitol, and zymosan were obtained from Sigma Chemical Co., St. Louis, Mo. Superoxide dismutase was purchased from Truett Laboratories, Dallas, Tex. Hanks' balanced salts solution (HBSS, 1× without phenol red) was obtained from Grand Island Biological Co., Grand Island, N. Y. Other reagents were the best grade commercially available, and were used without further purification.

Catalase was assayed spectrophotometrically (19), then dissolved in HBSS at a concentration of 98,000 Sigma U/ml (10 mg/ml). Superoxide dismutase was dissolved in water at a concentration of 3 mg/ml. Methional was stored at 4°C. Stock solutions of aqueous methional (3.5 mM) were prepared fresh daily and stored at 0°C until use.

Neutrophils (4) and opsonized zymosan (20) were prepared according to published methods and suspended in HBSS. Oxygen uptake by neutrophils stimulated with opsonized zymosan, measured by respirometry as previously described (3), was not affected by 0.35 mM methional alone or in the presence of ethanol (0.01 M), mannitol (0.05 M), or benzoic acid (2 mM).

**Ethylene production.** Ethylene production by neutrophils was measured by gas chromatography using a modification of the method described by Beauchamp and Fridovich (18). Incubations were conducted in 3.5 ml siliconized tubes. Neutrophils ( $4 \times 10^6$  cells, unless otherwise noted), together with superoxide dismutase, catalase, or radical scavenger as indicated, in a combined volume of 0.7 ml, were placed in the tubes and incubated for 3 min at 37°C in a Dubnoff shaking incubator. Opsonized zymosan, 2 mg in 0.2 ml of HBSS, was then added, and the incubation continued for an additional 10 min. Finally, 0.1 ml of the stock solution of methional was added, and the tubes were stoppered with serum ports sealed with silicone grease. The incubations were continued for the time intervals noted, after which the reactions were terminated by placing the tubes in

melting ice. Using a Hamilton gas-tight syringe (1.0 ml volume), 0.5-ml samples of the gas overlying the reaction mixtures were withdrawn and subjected to gas chromatography at a temperature of 100°C on Chromosorb 102 packed in a 6-foot by 1/4-inch glass column (Varian Associates, Palo Alto, Calif.), using  $\text{N}_2$  at 40 psi as the carrier gas. The instrument was a Varian Aerograph model 1740 gas chromatograph with a flame ionization detector. Ethylene in  $\text{N}_2$  (18 ppm) obtained from Supelco, Inc. (Bellefonte, Pa.) was used as standard. The retention time of standard ethylene under the conditions employed was 45 s, and the peak height was proportional to the mass injected. The instrument was calibrated each day with the standard gas. The quantity of ethylene released by each incubation was calculated from the height of the peak observed on the chromatogram tracing, using the calibration chromatograms obtained on the same day to establish the relationship between the peak height and the mass of ethylene injected, with appropriate corrections for temperature and pressure in the sampled gas phase.

## RESULTS

The incubation of methional with neutrophils in the presence of opsonized zymosan resulted in the appearance of ethylene in the gas phase overlying the reaction mixture, as determined by gas chromatography. In 14 experiments, ethylene production amounted to  $44.6 \pm 3.6$  SE pmol/ $10^6$  cells/40 min. Ethylene formation was all but abolished when either cells or methional was omitted (Table I), indicating that the reaction observed by gas chromatography was in fact the production of ethylene from methional by neutrophils. Ethylene production was also greatly reduced if opsonized zymosan was omitted from the reaction mixture (Table I), demonstrating that for ethylene to be formed the neutrophils had to be activated, presumably because oxidizing agents generated during the respiratory burst were required for ethylene production. The importance of the respiratory burst to ethylene production was confirmed in experiments

TABLE I  
*The Production of Ethylene from Methional  
by Stimulated Neutrophils*

Reaction conditions	Ethylene production
	pmol $\pm$ SE/ $10^6$ cells/40 min
Complete	$39.1 \pm 4.8$
No cells	$4.0 \pm 1.2$
No zymosan	$2.5 \pm 0.6$
No methional	$0.5 \pm 0.5$

The experiments were conducted as described in Methods, except with the omissions noted, using  $4 \times 10^6$  cells per ml. The incubation with methional (or its replacement) was carried out for 40 min. Where omitted, cells or zymosan were replaced with equivalent volumes of HBSS; omitted methional was replaced with water. The results presented are the means  $\pm$  SE of three experiments, each using cells from a different donor.

with cells from patients with chronic granulomatous disease. Two such experiments were carried out on separate days, each experiment being conducted with cells from a different patient. In each case, a control incubation with normal cells was performed at the same time. The value for ethylene production by zymosan-treated cells from the patients with chronic granulomatous disease was 0 in both cases, compared with control values of 48.4 and 50 pmol. These findings show that ethylene is produced from methional by neutrophils engaged in respiratory burst activity, but not by resting cells. Neither  $O_2^-$  nor  $H_2O_2$  is able to oxidize methional to ethylene (18); these results therefore demonstrate the production by neutrophils of an oxidizing species more powerful than either of the foregoing.

As shown in Fig. 1, ethylene production was proportional to cell numbers at concentrations lower than  $6 \times 10^6$  cells/ml, but fell off sharply at higher concentrations. The reasons for this decline are not known. For present purposes, however, it is sufficient to note that, apart from the results presented in Fig. 1, all data were obtained with cell concentrations of less than  $6 \times 10^6$  cells/ml, values within the region where ethylene production and cell numbers are linearly related.

Measurement of ethylene production against time (Fig. 2) revealed a small but consistent acceleration in the rate of release of the hydrocarbon into the gas phase. This was indicated by the slight upward bowing

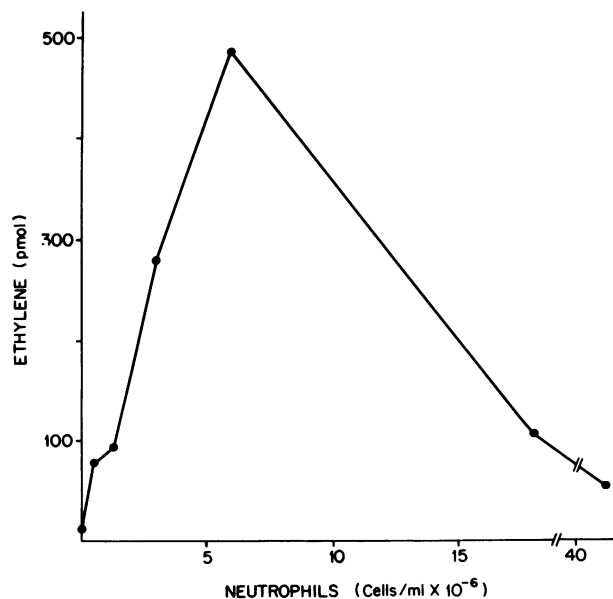


FIGURE 1 Ethylene production by stimulated neutrophils as a function of cell concentration. The experiments were conducted as described in Methods. The cell concentrations were as shown, and the incubation with methional was carried out for 40 min.

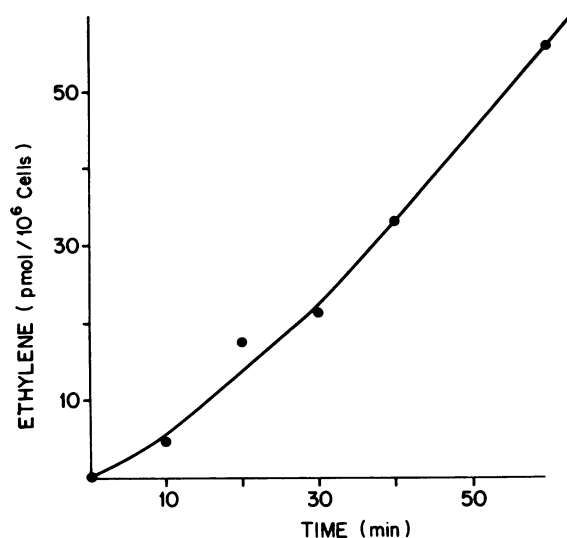


FIGURE 2 Ethylene production by stimulated neutrophils as a function of time. The experiments were conducted as described in Methods. The cell concentration was  $4 \times 10^6$ /ml, and the incubations with methional were carried out for the times noted.

of the curve in the figure. Evidence against the possibility that this acceleration was the result of the accumulation of  $H_2O_2$  over the period of the incubation was provided by experiments conducted in the presence of added  $H_2O_2$ , in which ethylene production was seen to follow the same time course as in the absence of  $H_2O_2$  (see below). The reason for the non-linear time-course is not apparent. Further investigation will be required to provide explanations for both the time-course of ethylene production and the unusual dependence of ethylene release on the cell concentration.

To obtain evidence as to the nature of the oxidizing species, experiments were performed to determine the effects of benzoate, ethanol, and mannitol on ethylene production in this system. All three of these compounds are known to be  $OH\cdot$  scavengers (21), and are presumably able to scavenge other reactive free radicals as well. If the oxidizing species is such a radical, then these compounds, by competing with methional for this radical, should diminish ethylene production. The results of these experiments (Table II) show that, at concentrations that had no effect on oxygen uptake, benzoic acid was able to inhibit ethylene production by zymosan-stimulated neutrophils. Mannitol and ethanol, however, showed little effect. The results with benzoate indicate that a compound that can intercept reactive radical species is able to attenuate ethylene production in this system. The failure of mannitol and ethanol to affect ethylene production may reflect several factors, including the inability to employ these agents at sufficiently high concentrations owing

**TABLE II**  
*The Effect of Radical Scavengers on Ethylene Production by Stimulated Neutrophils*

Scavenger	Ethylene production
	% of control
None	100
Benzoic acid (2 mM)	52.4±4.2*
Ethanol (0.1 M)	92.1±5.7
Mannitol (0.05 M)	90.0±9.3

The experiments were conducted as described in Methods, using  $5.6 \times 10^6$  cells/ml and incubating with methional for 40 min. The inhibitors, dissolved in HBSS, were added in quantities such that their concentrations in the complete reaction mixture (i.e., the reaction mixture after the addition of methional) were as shown. The final volume of each mixture was 1.0 ml. The data shown represent the results of four experiments, each conducted with a different neutrophil preparation. Control values were  $109 \pm 24$  SE pmol/ $10^6$  cells/40 min.

\* Mean±SE.

to nonspecific toxicity, and the possibility that the secondary radicals produced by the reaction between these alcohols and the oxidizing species might themselves release ethylene from methional. In any case, these results suggest that the oxidizing species is an unusually reactive free radical.

Because respiratory burst activity was essential for the production of the newly demonstrated oxidizing radical, the question arose as to whether either  $O_2^-$  or  $H_2O_2$  could be implicated in its formation. This question was of particular interest because of the conjectures on the part of previous workers that stimulated neutrophils generate  $OH^\cdot$ , an agent whose properties are comparable to those of the oxidizing radical, and that this  $OH^\cdot$  is produced by way of the Haber-Weiss reaction, in which both  $O_2^-$  and  $H_2O_2$  are participants (10, 14, 15, 22). The roles of  $O_2^-$  and  $H_2O_2$  in oxidizing radical formation were examined by testing the effect of superoxide dismutase and catalase on the release of ethylene from methional by zymosan-stimulated neutrophils. Table III shows that active, but not autoclaved, superoxide dismutase was an effective inhibitor of ethylene production by this system, 3  $\mu$ g/ml of the active enzyme reducing ethylene levels to <25% of control. This finding implicates  $O_2^-$  in oxidizing radical formation. Catalase, however, was much less effective. Levels of 980 U/ml (100  $\mu$ g/ml) caused ethylene production to decrease by only 40% (Fig. 3), and significant production was seen at levels ten times as great. This contrasts with the results of Beauchamp and Fridovich (18), who showed that the release of ethylene from methional by the xanthine-xanthine oxidase system was totally abolished by catalase at only

50 U/ml. Furthermore, as shown in Table IV, substantial inhibition was seen with both boiled catalase and albumin. These findings imply that such inhibition as was seen with active catalase represented a non-specific effect of protein plus the heme residue rather than a manifestation of the catalytic activity of the enzyme. The specific activity of the catalase was not affected by incubation for 40 min at 37°C under the experimental conditions described in Table IV, so the inefficacy of catalase cannot be accounted for by destruction of the enzyme during the course of the incubation. Rather, it suggests that the  $H_2O_2$  produced during the respiratory burst—or at least that fraction of  $H_2O_2$  to which catalase has access—does not participate in the production of the oxidizing radical. This conclusion is supported by the results presented in Table V, which show that the addition of exogenous  $H_2O_2$  to the reaction mixture did not augment ethylene production to any extent either early or late in the incubation.

## DISCUSSION

The findings reported here show that during the respiratory burst, neutrophils produce a powerful oxidizing agent capable of releasing ethylene from methional. Similar results have been obtained independently by another group using the monocyte as the phagocytic cell (23). The inhibition of ethylene release by  $OH^\cdot$  scavengers suggests that the oxidizing agent is a radical whose reactivity is similar to that of

**TABLE III**  
*The Effect of Superoxide Dismutase on Ethylene Production by Stimulated Neutrophils*

Reaction conditions	Ethylene production	
	Experiment 1	Experiment 2
	pmol/ $10^6$ cells/40 min	
No addition	43.0	32.0
Active dismutase	6.9	9.1
Autoclaved dismutase	40.8	31.0

The experiments were conducted as described in Methods, using  $4 \times 10^6$  cells/ml and incubating with methional for 40 min. Inactive dismutase was prepared by autoclaving 1 ml of active enzyme for 15 min, then dialyzing the autoclaved enzyme overnight at 4°C against 400 ml of HBSS. A 1-ml portion of the active enzyme to be used in this experiment was dialyzed simultaneously in the same bath. After dialysis, the two enzyme preparations were diluted 10-fold with fresh HBSS. 10- $\mu$ l portions of the diluted preparations were added to the incubation mixtures, as indicated, to give a final concentration of superoxide dismutase of 3  $\mu$ g/ml. The control incubation received 10  $\mu$ l of HBSS. The final volume of each incubation mixture was 1.0 ml. The two experiments were carried out with cells from two different donors.

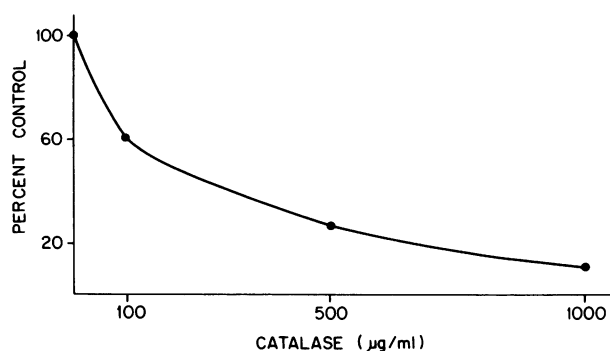


FIGURE 3 Ethylene production by stimulated neutrophils as a function of catalase concentration. The experiments were conducted as described in Methods. The cell concentrations were  $4 \times 10^6$ /ml, and the incubation with methional was carried out for 40 min. Catalase was added as a solution of 10 mg/ml of HBSS to bring the final concentrations to the values indicated. The final volume of each incubation mixture was 1.0 ml. Control ethylene production was 51.8 pmol/ $4 \times 10^6$  cell per 40 min.

OH $\cdot$ . The observation that superoxide dismutase in low concentrations strongly inhibited ethylene formation from methional implicates O $_2^-$  in the process by which the oxidizing radical is generated. The role of H $_2$ O $_2$  in this process, however, remains unclear, because the inefficacy of catalase as an inhibitor of ethylene production could as reasonably be explained by postulating a lack of access of the enzyme to the site of radical formation as by proposing that H $_2$ O $_2$  is not a reactant in the radical-forming reaction.

TABLE IV  
The Effect of Catalase on Ethylene Production by Stimulated Neutrophils

Reaction conditions	Ethylene production	
	Experiment 1	Experiment 2
	pmol/ $10^6$ cells/40 min	
No addition	83.0	51.8
Active catalase	26.9	13.7
Boiled catalase	43.4	40.5
Bovine serum albumin	50.1	38.8

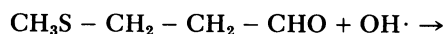
The experiments were conducted as described in Methods, using  $4 \times 10^6$  cells/ml and incubating with methional for 40 min. Inactive catalase was prepared by heating a solution of the active enzyme (10 mg/ml) for 2 min in boiling water, centrifuging to remove precipitated protein and withdrawing the supernate for use in the experiment. 50- $\mu$ l portions of active catalase, boiled catalase, or bovine serum albumin (10 mg/ml in HBSS) were added to the incubation mixtures as indicated. The control incubation received 50  $\mu$ l of HBSS. The final volume of each incubation mixture was 1.0 ml. The two experiments were carried out with cells from two different donors.

TABLE V  
The Effect of Exogenous H $_2$ O $_2$  on Ethylene Production by Stimulated Neutrophils

Conditions	Ethylene production	
	Experiment 1	Experiment 2
	pmol/ $10^6$ cells/40 min	
Incubation (10 min)		
Control	8.0	11.7
+ H $_2$ O $_2$	10.0	7.7
Incubation (40 min)		
Control	30.2	55.1
+ H $_2$ O $_2$	24.1	63.6

The experiments were conducted as described in Methods, using  $4 \times 10^6$  cells/ml and incubating with methional for the times noted. The incubation mixtures also received 10  $\mu$ l of 3 mM H $_2$ O $_2$  in HBSS or 10  $\mu$ l of HBSS, as indicated, at the time the methional was added. The final volume of each incubation mixture was 1.0 ml. The two experiments were carried out with cells from two different donors.

Could the oxidizing radical be OH $\cdot$  itself? Chemical evidence suggests that it could. A recent study demonstrated that xanthine plus xanthine oxidase, a system which, like the stimulated neutrophil, produces both O $_2^-$  and H $_2$ O $_2$ , was capable of oxidizing methional to ethylene (18). In this study it was shown that neither O $_2^-$  nor H $_2$ O $_2$  was by itself a sufficiently powerful oxidizing agent to react with methional. The conclusion from the xanthine-xanthine oxidase study was that the agent responsible for methional oxidation was the hydroxyl radical, which oxidized methional according to the following reaction sequence:



The production of ethylene from methionine, a compound closely related to methional, by Fenton's reagent (Fe $^{++}$  plus H $_2$ O $_2$ ; reference 24), a well-characterized source of OH $\cdot$  (25), lends further support to the claim that methional was oxidized to ethylene by OH $\cdot$ .

The similarities between the observations in the xanthine-xanthine oxidase system and the findings reported here make the concept of OH $\cdot$  generation by neutrophils a very appealing one. The only substantive difference between the two sets of findings was that catalase inhibited ethylene production by the xanthine-xanthine oxidase system but not by stimulated neutrophils. This difference, however, does not rule out the possibility that the oxidizing radical in neutrophils is OH $\cdot$ . For one thing, it is possible that in the neutro-

phil system, the Haber-Weiss reaction is occurring at a site from which catalase is excluded. Alternatively, the neutrophil might possess a mechanism for  $\text{OH}\cdot$  production that requires  $\text{O}_2^-$  but not  $\text{H}_2\text{O}_2$ . Thus, the production of  $\text{OH}\cdot$  by stimulated neutrophils is a reasonable interpretation of the present results. However, in a system as complex as an intact neutrophil, it is conceivable that the potential exists for generating a variety of reactive oxidizing radicals, any one of which might display the properties described here. Therefore, the conclusion that the observations reported above are accounted for specifically by the production of  $\text{OH}\cdot$  must be regarded at present as provisional.

In summary, our results show that working but not resting neutrophils produce a highly reactive oxidizing radical with properties similar to those of  $\text{OH}\cdot$ . These findings provide substantial, though indirect, support for the participation of such a radical as a bactericidal agent in one of the oxygen-dependent antimicrobial systems of this cell.

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