ETHYLENE FORMATION BY POLYMORPHONUCLEAR LEUKOCYTES
Role of Myeloperoxidase*

BY SEYMOUR J. KLEBANOFF AND HENRY ROSEN
(From the Department of Medicine, University of Washington, Seattle, Washington 98195)

Micro-organisms ingested by polymorphonuclear leukocytes (PMNs) are exposed to a variety of anti-microbial systems; some require oxygen, whereas others are effective under anaerobic conditions (1, 2). The importance of the oxygen-dependent anti-microbial systems is emphasized by the presence of a major microbicidal defect associated with an impairment in the phagocytosis-induced respiratory burst in the PMNs of patients with chronic granulomatous disease (CGD). One of the oxygen-dependent anti-microbial systems of the PMN consists of myeloperoxidase (MPO), H2O2, and a halide. After phagocytosis, MPO is released into the phagocytic vacuole from adjacent lysosomal granules where it interacts with H2O2 generated either by leukocytic or microbial metabolism and a halide such as chloride or iodide to form an agent or agents toxic to the ingested organisms. The evidence in support of a role for the MPO-mediated anti-microbial system in the microbicidal activity of the intact cell is substantial (1, 2). Of particular pertinence is the finding of a major fungicidal and bactericidal defect in leukocytes which lack MPO, i.e., from patients with hereditary MPO deficiency (3, 4).

Oxygen-dependent anti-microbial agents which do not require MPO also are present in the PMN; among these may be the hydroxyl radical (OH·), generated by the interaction of the superoxide anion (O2-) and H2O2. The superoxide anion is formed by the univalent reduction of oxygen and its dismutation to form H2O2 readily occurs either spontaneously or catalyzed by superoxide dismutase (SOD). A mechanism for the formation of OH· was proposed by Haber and Weiss (5):

\[ \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^- + \text{O}_2. \]  

(a)

When a chemical reaction is induced by a O2- H2O2 generating system, the inhibition of that reaction by SOD, catalase, and OH· scavengers such as mannitol, ethanol, and benzoate and its stimulation by H2O2, have been employed as evidence for the involvement of OH· generated by the Haber-Weiss reaction (6). The Haber-Weiss reaction has recently been questioned as an efficient mechanism for the generation of OH· (7, 8); however a number of reactions respond to inhibitors in a manner suggestive of the participation of O2-, H2O2 and OH·, leaving open the possibility that OH· is generated by an, as yet, unknown mechanism requiring O2- and H2O2.

Both O2- (9) and H2O2 (10) are generated by PMNs during phagocytosis and the formation of OH· by phagocytosing PMNs might therefore be anticipated.

* Supported in part by grants AI07763 and CA18364 from the U. S. Public Health Service.

1 Abbreviations used in this paper: CGD, chronic granulomatous disease; DABCO, 1,4-diazabicyclo [2,2,2] octane; D2O, deuterium oxide; KMB, 2-keto-4-thiomyethylbutyric acid; MPO, myeloperoxidase; O2-, superoxide anion; O2, singlet oxygen; OH-, hydroxyl radical; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase.

490 J. Exp. Med. © The Rockefeller University Press · 0022-1007/78/0801-0490$1.00
The inhibition of the microbicidal activity of the PMN by certain OH· scavengers and by the cophagocytosis with the bacteria of either catalase or SOD bound to latex particles (11) has implicated OH· as an antimicrobial agent in the intact cell. Recently a method for the detection of OH· in PMNs (12, 13) and monocytes (14) has been proposed based on the formation of ethylene from β-methylthiopropionaldehyde (methional) or 2-keto-4-thiomethylbutyric acid (KMB), a reaction which can occur by an OH· mechanism (6, 15). In this paper, ethylene formation by intact PMNs is shown to be largely dependent on MPO, and a cell-free, SOD-sensitive, MPO-dependent system with properties similar to those of the intact cell is described. Evidence for the participation of singlet molecular oxygen (100) in ethylene formation by this system is presented.

Materials and Methods

Isolation of Leukocytes. Blood for isolation of human PMNs was collected from healthy laboratory personnel and two siblings with hereditary MPO deficiency, one male (B. F.) and one female (J. F.). MPO deficiency was indicated by the absence of MPO from neutrophils and monocytes on histochemical examination, low peroxidase activity in extracts of isolated PMNs, and by a marked inhibition of phagocytosis-induced iodination (16). The blood was drawn in heparin (20 U/ml) and the leukocytes were isolated by centrifugation on Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.-Hypaque (Winthrop Laboratories, New York) gradients according to Böyum (17). The leukocyte preparations contained >97% PMNs.

Guinea pig eosinophils were obtained by peritoneal lavage of animals receiving a weekly intraperitoneal injection of polymyxin (18). The peritoneal exudate contained an average of 5 × 109 leukocytes of which 55-65% were eosinophils and the remainder mononuclear cells. If neutrophil contamination was observed, the preparation was discarded. Further purification of the eosinophils was performed by centrifugation on a layer of sodium diatrizoate (Hypaque) at a density of 1.143. Erythrocytes were removed by hypotonic lysis if necessary. The final preparations contained 85–95% eosinophils with the remainder mononuclear cells. The human PMNs and guinea pig eosinophils were suspended in 0.154 M sodium chloride at a concentration of 5 × 107 cells/ml and used immediately.

Ethylene Formation. The components of the reaction mixture in a total vol of 1.0 ml (see legends to figures and tables) were incubated in 10-ml serum vials capped with a sleeve-type rubber stopper for 60 min unless otherwise indicated, in a shaking water bath maintained at 37°C. N-Ethylmaleimide (0.1 ml of 0.01 M) was added through the rubber stoppers to vials containing intact cells and all vials were placed on ice at the end of the incubation period. A 0.5-ml sample of the gas phase was obtained with an air-tight gas syringe (Pressure-Lok Series A 0.5 ml; Precision Sampling Corp., Baton Rouge, La.) and the ethylene content determined by analysis in a Hewlett-Packard 5803A flame ionization gas chromatography machine (Hewlett-Packard Co., Avondale, Pa.). An ½ inch × 6 foot stainless steel column packed with Poropak Q 80–100 was used. The injector, detector, and column temperatures were 160°, 300°, and 101°C, respectively, and the gas flow rates were 210 ml/min air, 28 ml/min hydrogen, and 25 ml/min nitrogen. Under these conditions, the retention time for ethylene was 0.9 min. Ethylene standards were included in each experiment and the ethylene content of the unknown was determined by comparison of the area of the experimental ethylene peak with those of the standards of known ethylene content. The amount of ethylene formed during the incubation of the thioether with buffer alone was subtracted from each experimental value.

Statistical Analyses. Statistical differences were determined by using Student's two tailed t test for independent means (not significant, NS, P > 0.05).

Special Reagents. MPO was prepared from canine granulocytes by the method of Agner (19) to the end of step 6, and peroxidase activity was determined on the day of each experiment by the o-dianisidine method (20). 1 U of enzyme is the amount decomposing 1 μmol of H2O2 per min at 26°C. Catalase (bovine liver, × 2 crystallized, 50,000 U/mg; 6.1 mg/ml) was obtained from Worthington Biochemical Corp., Freehold, N.J., and SOD (bovine erythrocytes, approximately 3,000 U/mg as assayed by SOD-inhibitable cytochrome C reduction) was obtained from Miles.
Ethylene formation by phagocytosing PMNs from patients with hereditary MPO deficiency was 7% of normal \( P < 0.001 \) with either methional or KMB as the substrate under the conditions employed in Table I. Purified MPO reversed this defect; indeed, when KMB was the substrate employed, ethylene formation by MPO-deficient leukocytes was increased by MPO to a level greater than that of similarly treated normal cells. This effect of MPO was largely abolished by heat-treatment.

Since azide and cyanide are potent inhibitors of peroxidase-catalyzed reactions, they would be expected to inhibit ethylene formation by normal neutrophils, if MPO is required. An inhibition of ethylene formation from KMB by azide and cyanide has been reported (13). Under the conditions shown in Fig. 1, cyanide had an inhibitory effect on ethylene formation which was comparable with either methional or KMB as substrate; the inhibition increased progressively with an increase in cyanide concentration above \( 1 \times 10^{-4} \), with complete inhibition at \( 2 \times 10^{-3} \) M. In contrast, the effect of azide varied with the substrate employed. With KMB, the inhibition increased with the azide concentration above \( 2 \times 10^{-6} \) M; however, when methional was employed, an inhibition was observed at relatively low and high azide concentrations which was not seen when the intervening concentrations were used.

The effect of azide and cyanide on ethylene formation by MPO-deficient leukocytes is shown in Table II. At a concentration of \( 2 \times 10^{-4} \) M, azide strongly stimulated ethylene formation from methional without affecting formation from KMB. In contrast, cyanide inhibited the residual ethylene formation from methional and had a small but significant stimulatory effect on formation from KMB.

These findings suggest the presence in MPO-deficient and presumably also in normal PMNs of MPO-independent mechanisms for the formation of ethylene from methional, which are stimulated by azide. Two such mechanisms are described here, one involving eosinophils, and the other \( \text{H}_2\text{O}_2 \) with or without catalase.

Ethylene is formed from methional or KMB by guinea pig eosinophils and preopsonized zymosan (Table III) although to a lesser degree than by human PMNs. Ethylene formation is largely prevented by the addition of SOD (but not heated SOD), whereas catalase had little or no effect. Of particular interest was the marked stimulation of ethylene formation by \( 2 \times 10^{-4} \) M azide when
TABLE I
Ethylene Formation by MPO-Deficient PMNs

| Myeloperoxidase | Methional Normal | MPO-deficient | KMB Normal | MPO-deficient |
|-----------------|-----------------|---------------|------------|---------------|
|                 | mU              | %             | mU         | %             |
| Normal          |                 |               | 9,63 (10)  | %             |
| MPO-deficient   |                 | 13,196 (21)   | 963 (10)   |
| 8               | 17,487 (21)*    | 1,311 (10)    | 7*         |
| 20              | 18,644 (3)      | 4,338 (5)     | 163*       |
| 80              | 19,758 (3)      | 16,737 (5)    | 85*        |
| 20-Heated       | 20,515 (3)      | 2,218 (4)     | 11*        |

The reaction mixture contained 5 x 10^{-2} M sodium chloride, 6 x 10^{-3} M potassium chloride, 1 x 10^{-2} M magnesium chloride, 5 x 10^{-4} M calcium chloride, 5 x 10^{-3} M sodium phosphate buffer pH 7.4, 1 x 10^{-3} M glucose, 2.5 x 10^{-6} normal or MPO-deficient PMNs, 2 mg preopsonized zymosan; 1 x 10^{-6} M of either methional or KMB and MPO in the amounts indicated.

* pmol/10^7 PMNs per hour; mean of (n) experiments.
† MPO-deficient value as per cent of normal.

FIG. 1. Effect of azide and cyanide on ethylene formation from methional (O—O) or KMB ( — — ) by normal PMNs. The reaction mixture was as described in Table I except that normal PMNs were employed and either azide or cyanide was added at the concentrations indicated. Single circles and bars are the mean value ± 1 SD of the conversion in the absence of inhibitor. The P value for the difference between the presence and absence of inhibitor is shown, where P < 0.05.

methional was employed. Cyanide at this concentration inhibited ethylene formation from methional, whereas ethylene formation from KMB was not significantly altered by either azide or cyanide under the conditions employed.

Incubation of either methional or KMB with H_2O_2 resulted in the formation
TABLE II

Effect of Azide and Cyanide on Ethylene Formation by MPO-Deficient PMNs

| Inhibitor | Ethylene formation (pmol/10⁷ PMNs per h) |
|-----------|----------------------------------------|
|           | Methional                               | KMB              |
| ~          | 1,012 ± 116 (7)*                         | 551 ± 25 (7)     |
| Azide      | 6,433 ± 395 (7)                          | <0.001†          |
| Cyanide    | -980 ± 658 (4)                           | <0.01            |

The reaction mixture was as described in Table I except that 2 × 10⁻⁴ M azide or cyanide was added where indicated and MPO-deficient PMNs were employed.

* Mean ± SE of (n) experiments.
† P value for the difference between the presence and absence of inhibitor.

TABLE III

Formation of Ethylene by Guinea Pig Eosinophils

| Additions                  | Ethylene formation (pmol/10⁷ eosinophils per h) |
|---------------------------|-----------------------------------------------|
|                           | Methional                                    | KMB               |
| Eosinophils + zymosan     | 1,485 ± 203*                                 | 1,676 ± 315       |
| SOD Added                 | 131 ± 124                                    | 218 ± 80          |
| Heated SOD added          | 1,344 ± 216                                   | 1,345 ± 301       |
| Catalase added            | 1,079 ± 117                                   | 1,410 ± 196       |
| Heated catalase added     | 1,512 ± 233                                   | 1,470 ± 464       |
| Azide added               | 9,607 ± 1,790                                 | 1,244 ± 165       |
| Cyanide added             | 763 ± 155                                     | 1,326 ± 460       |

The reaction mixture was as described in Table I except that 2.5 × 10⁶ guinea pig eosinophils replaced PMNs and 5 μg SOD (approximately 15 U), 60 μg catalase (3,600 U), 2 × 10⁻⁴ M azide or 2 × 10⁻⁴ M cyanide was added where indicated.

* Mean ± SE of five to seven experiments.
† P value for the difference from the result with eosinophils + zymosan.

of ethylene (Fig. 2). With methional, ethylene formation increased sharply as the pH was increased above 5.0 to reach a maximum at pH 7.5–8.0. Ethylene formation from KMB was relatively low over the entire pH range. However formation at pH 6.5 (674 ± 94 pmol, n = 3), 7.0 (696 ± 108 pmol, n = 8), 7.5 (1,006 ± 175 pmol, n = 3), and 8.0 (1,125 ± 204 pmol, n = 3) was significantly greater (P < 0.05) than that at pH 5.0 (412 ± 41 pmol, n = 7). At pH 5.0, azide stimulated the formation of ethylene from methional by H₂O₂ while inhibiting formation from KMB under the conditions in Table IV. EDTA markedly inhibited ethylene formation by H₂O₂ and abolished the stimulatory effect of azide. Catalase also inhibited ethylene formation by H₂O₂; however, a marked stimulation of ethylene formation from either methional or KMB by azide was observed in the presence of catalase which was unaffected by EDTA. Because of the many complexities introduced by the use of methional as the substrate (14), KMB was employed in the remainder of the study.

The formation of ethylene from KMB by phagocytosing normal PMNs was stimulated by EDTA with activity increasing to a maximum at an EDTA concentration of 2.5 × 10⁻⁴ M (Fig. 3). Ethylene formation required both PMNs and zymosan in the presence of EDTA as it did in its absence. A potential complication of the use of EDTA with intact PMNs is the inhibition of
FIG. 2. Ethylene formation by \( \text{H}_2\text{O}_2 \) at various pH levels. The reaction mixture contained \( 4 \times 10^{-3} \) M potassium phosphate buffer at the pH indicated, \( 1 \times 10^{-3} \) M \( \text{H}_2\text{O}_2 \) and either \( 1 \times 10^{-3} \) M methional (●—●) or \( 1 \times 10^{-3} \) M KMB (○—○). Results are the mean ± SE of three to eight experiments.

**TABLE IV**

*Effect of Azide on Ethylene Formation by \( \text{H}_2\text{O}_2 \) with or without Catalase*

| Additions            | Methional       | KMB          |
|----------------------|-----------------|--------------|
|                      | - Azide | + Azide | \( P \) | - Azide | + Azide | \( P \) |
| \( \text{H}_2\text{O}_2 \)       | 1,958 ± 150 (17)* | 3,808 ± 269 (10) | <0.001 | 455 ± 28 (17) | 281 ± 55 (8) | <0.002 |
| \( \text{H}_2\text{O}_2 \) + EDTA | 209 ± 62 (9) | 133 ± 33 (4) | NS | 126 ± 40 (9) | 63 ± 10 (4) | NS |
| \( \text{H}_2\text{O}_2 \) + Catalase | 80 ± 150 (11) | 4,310 ± 545 (9) | <0.001 | 31 ± 36 (9) | 6,024 ± 542 (8) | <0.001 |
| \( \text{H}_2\text{O}_2 \) + Catalase + EDTA | 23 ± 32 (4) | 3,625 ± 540 (4) | <0.001 | 29 ± 11 (4) | 5,914 ± 535 (4) | <0.001 |

The reaction mixture contained \( 4 \times 10^{-3} \) M potassium phosphate buffer pH 5.0 and where indicated, \( 1 \times 10^{-3} \) M methional, \( 1 \times 10^{-3} \) M KMB, \( 1 \times 10^{-3} \) M \( \text{H}_2\text{O}_2 \), \( 1 \times 10^{-4} \) M azide, \( 1 \times 10^{-4} \) M EDTA, and 60 µg catalase.

* Mean ± SE of (n) experiments.

† Significance of the difference between the presence and absence of azide.

phagocytosis which may result from the chelation of essential divalent cations. However, the \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \) concentrations in the reaction mixture (\( 2 \times 10^{-2} \) and \( 10^{-3} \) M, respectively) were in excess and microscopic examination revealed intracellular zymosan in amounts which were indistinguishable from preparations incubated without EDTA.

**MPO-Model System.** The above findings suggest that ethylene formation by normal PMNs occurs largely by an MPO-dependent mechanism. Table V demonstrates the formation of ethylene by a model system which consists of MPO, \( \text{H}_2\text{O}_2 \), chloride, and EDTA. Ethylene-generating activity was lost on the deletion of \( \text{H}_2\text{O}_2 \) and greatly reduced on the omission of MPO or on its heat-
Fig. 3. Effect of EDTA on ethylene formation by PMNs. The reaction mixture was as described in Table I (normal PMNs, KMB) except that EDTA was added at the concentrations indicated. The results are the mean of five experiments. The P value for the difference between the presence and absence of EDTA is shown, where P < 0.05.

TABLE V

| Additions | Ethylene formation |
|-----------|-------------------|
| Complete system (MPO + H₂O₂ + chloride + EDTA) | 2,227 ± 135 (4)* |
| H₂O₂ Omitted | 9 ± 33 (3) |
| MPO Omitted | 336 ± 16 (4) |
| MPO Heated | 399 ± 21 (3) |
| EDTA Omitted | 804 ± 35 (3) |
| Chloride omitted | 1,425 ± 75 (3) |
| Chloride omitted, bromide added | 2,628 ± 71 (3) |
| Chloride omitted, iodide (10⁻³ M) added | 120± 16 (3) |
| Chloride omitted, iodide (10⁻⁴ M) added | 675 ± 57 (3) |
| Chloride omitted, iodide (10⁻⁵ M) added | 1,162 ± 105 (3) |
| Iodide (10⁻³ M) added | 106 ± 9 (3) |
| Iodide (10⁻⁴ M) added | 870 ± 79 (3) |
| Iodide (10⁻⁵ M) added | 1,945 ± 150 (3) |

The reaction mixture contained 4 x 10⁻² M sodium phosphate buffer pH 7.5, 1 x 10⁻³ M KMB and where indicated, 80 mU MPO, 2.5 x 10⁻⁴ M H₂O₂, 5 x 10⁻⁵ M EDTA, 5 x 10⁻³ M sodium chloride, 1 x 10⁻³ M sodium bromide and iodide at the concentrations indicated.

* Mean ± SE of (n) experiments.

inactivation (all P < 0.001). EDTA also was required for optimum activity (P < 0.001) and deletion of chloride resulted in a small (35%) but significant (P < 0.01) decrease in ethylene formation. Chloride could be replaced by bromide but not by iodide at concentrations ranging from 10⁻³ to 10⁻⁵ M. Indeed, iodide at 10⁻³ and 10⁻⁴ M strongly inhibited ethylene formation both in the presence and absence of chloride (P < 0.001). The stimulation of ethylene formation by MPO was highly significant at all the concentrations employed (Fig. 4) and was most
Fig. 4. Effect of MPO concentration. The reaction mixture was as described for the complete system in Table V except that the amount of MPO added was varied as indicated. Results are the mean ± SD of three to four experiments. The P values for the difference between the presence and absence of MPO are shown.

Fig. 5. Effect of pH. The reaction mixture was as described for the complete system in Table V except that the pH of the phosphate buffer was varied as indicated and ethylene formation was measured in the presence (●—●) or absence (○—○) of MPO. The results are the mean of four experiments except pH 5.5 where n = 1. The P values for the difference between the presence and absence of MPO are shown, where P < 0.05.

striking at neutral or alkaline pH with optimum activity at about pH 8.0 (Fig. 5).

The requirement for EDTA for optimum activity prompted a study of the effect of metal ions. As shown in Fig. 6, Mn²⁺ inhibited ethylene formation by the MPO system at concentrations which were related to the EDTA concentration. When the EDTA concentration was 5 × 10⁻⁵ M, Mn²⁺ was inhibitory at concentrations of 5 × 10⁻⁵ M or greater. In contrast, when the EDTA concentration was decreased to 5 × 10⁻⁶ M, an inhibition was observed at Mn²⁺ concentrations as low as 2.5 × 10⁻⁶ M. Table VI compares the effect of a number of divalent cations at twice the EDTA concentration on ethylene formation by the MPO system. Mn²⁺, Zn²⁺, and Co²⁺ were strongly inhibitory, Cu²⁺ was less inhibitory and Ca²⁺ and Mg²⁺ had no effect. Fe²⁺ markedly increased ethylene
Fig. 6. Effect of Mn²⁺ on the ethylene formation by the MPO system. The reaction mixture was as described for the complete system in Table V except that either 5 x 10⁻⁵ or 5 x 10⁻⁶ M EDTA was employed and MnSO₄ was added at the concentrations indicated. The P values for the difference between the presence and absence of Mn²⁺ is shown, where P < 0.05.

TABLE VI
Effect of Divalent Cations on Ethylene Formation by the MPO System

| Divalent cation | Ethylene formation pmol |
|-----------------|-------------------------|
|                 |                         |
| —               | 2,227 ± 135 (4)*        |
| Mn²⁺            | 410 ± 53 (4)            | <0.001† |
| Mg²⁺            | 2,294 ± 180 (8)         | NS      |
| Ca²⁺            | 2,427 ± 103 (3)         | NS      |
| Zn²⁺            | 507 ± 40 (3)            | <0.001  |
| Cu²⁺            | 1,318 ± 112 (3)         | <0.002  |
| Co²⁺            | 685 ± 37 (3)            | <0.001  |
| Fe²⁺            | 7,833 ± 1,035 (5)       | <0.01   |

The reaction mixture was as described for the complete system in Table V except that divalent cations (1 x 10⁻⁴ M as the sulfate salt) were added as indicated.

* Mean ± SE of (n) experiments.
† P value for the difference between the presence and absence of divalent cation.

formation which was largely independent of MPO (data not shown) and presumably resulted from the interaction of Fe²⁺ with H₂O₂ to form OH⁻ (Fenton's reagent). The inhibition by Mn²⁺, Zn²⁺, Co²⁺, or Cu²⁺ was abolished by an increase in the EDTA concentration to twice that of the divalent cation.

Table VII indicates the effect of a number of inhibitors on ethylene formation by the MPO-H₂O₂-chloride-EDTA system. Of particular interest was the strong inhibition by SOD at low concentration (5 μg/ml), which was abolished by heat-treatment. The inhibition by SOD also was observed in the absence of MPO (−SOD 405 pmol ± 25 [SE]; +SOD 93 ± 5; P < 0.001), chloride (−SOD 1,230 ± 93; +SOD 140 ± 14; P < 0.001), or EDTA (−SOD 729 ± 16; +SOD 588 ± 49; P < 0.05). Unheated catalase also was inhibitory, as would be expected from the H₂O₂ requirement. Ethylene formation was strongly inhibited by azide and
SEYMOUR J. KLEBANOFF AND HENRY ROSEN

TABLE VII
Effect of Inhibitors on the MPO System

| Inhibitors           | Ethylene formation |
|----------------------|--------------------|
|                      | Change ± SE        | P*     |
|                      | %                  |        |
| SOD 5 µg/ml          | -92 ± 1            | <0.001 |
| Heated SOD 5 µg/ml   | +12 ± 14           | NS     |
| Catalase 60 µg/ml    | -95 ± 0.3          | <0.001 |
| Heated catalase 60 µg/ml | +13 ± 4        | NS     |
| Cyanide 10⁻³ M       | -96 ± 1            | <0.001 |
| Cyanide 10⁻⁴ M       | -27 ± 9            | NS     |
| Cyanide 10⁻⁵ M       | -8 ± 3             | NS     |
| Azide 10⁻³ M         | -85 ± 0.1          | <0.001 |
| Azide 10⁻⁴ M         | -74 ± 5            | <0.01  |
| Azide 10⁻⁵ M         | -55 ± 3            | <0.01  |
| Azide 10⁻⁶ M         | -17 ± 6            | NS     |
| Mannitol 0.1 M       | -50 ± 3            | <0.01  |
| Mannitol 10⁻² M      | -33 ± 0.1          | <0.001 |
| Mannitol 10⁻³ M      | -6 ± 8             | NS     |
| Benzoate 10⁻² M      | -45 ± 3            | <0.01  |
| Benzoate 10⁻³ M      | -19 ± 6            | NS     |
| DABCO 0.1 M          | -102 ± 1           | <0.001 |
| DABCO 10⁻² M         | -48 ± 3            | <0.01  |
| DABCO 10⁻³ M         | +16 ± 7            | NS     |
| Histidine 10⁻³ M     | -78 ± 2            | <0.001 |
| Histidine 10⁻⁴ M     | -27 ± 4            | <0.02  |
| Histidine 10⁻⁵ M     | -5 ± 2             | NS     |

The reaction mixture was as described in Table V except that the inhibitors were added in the amounts indicated. The results are the mean of three to four experiments.

* Significance of the difference between the presence and absence of the inhibitor.

cyanide, with cyanide effective at a concentration of 10⁻³ M and azide at 10⁻⁵ M. The OH· scavengers, mannitol and benzoate at high concentration (10⁻² M), also inhibited ethylene formation, although the extent of the inhibition (<50%) was not as great as that observed with the other inhibitors tested. Finally the ¹O₂ quenchers, DABCO, and histidine at concentrations of 10⁻² M or greater, strongly inhibited ethylene formation by the MPO system.

Formation of Ethylene by Dye-Sensitized Photo-Oxidation. The possibility of ¹O₂ involvement in ethylene formation by the MPO system prompted a study of the dye-sensitized photo-oxidation of KMB. Fig. 7 demonstrates the formation of ethylene from KMB by rose bengal in the presence of light and oxygen, the inhibition of this reaction by the substitution of nitrogen for air or by the removal of light and the stimulation of the conversion by the substitution of D₂O for H₂O. The inhibition of this reaction by azide at a concentration of 10⁻³ M, by DABCO at 0.1 M and by histidine at 10⁻² M, is shown in Table VIII. Histidine at 10⁻³ M significantly increased ethylene formation (Table VIII). Ethylene formation from KMB was increased from 532 ± 51 (SE; n = 5) to 8,454 ± 477 (n = 4) pmol by 60 min incubation with rose bengal at 10 times the
Fig. 7. Dye-sensitized formation of ethylene from KMB. The control reaction mixture (○—○) consisting of $4 \times 10^{-2}$ M sodium phosphate buffer pH 7.5; $1 \times 10^{-3}$ M KMB and $5 \times 10^{-4}$ M rose bengal in a total vol of 1.0 ml was irradiated at 37°C for the periods indicated, with a standard fluorescent bulb (Sylvania F48T12-CW-VHO) positioned 15 inches from the samples. Where indicated, reaction vessels were covered with black tape (dark; placed in comparable locations elsewhere in legend ▲—▲); the reaction components were gassed with N₂ before the initiation of incubation (N₂; ▲—▲) or the water was replaced with D₂O at pL 7.5 (D₂O; ○—○). The results are the mean of three to five experiments. The $P$ values for the difference from the control (rose bengal + light + oxygen in water) are shown, where $P < 0.05$.

Table VIII

| Inhibitor       | Ethylene formation (pmol) |
|-----------------|---------------------------|
| None            | $4,256 \pm 236^*$         |
| Azide $10^{-4}$ M | $16 \pm 20$              |
| Azide $10^{-3}$ M | $671 \pm 92$             |
| Azide $10^{-4}$ M | $4,004 \pm 421$          |
| DABCO 0.1 M     | $1,112 \pm 78$           |
| DABCO $10^{-3}$ M | $4,474 \pm 321$         |
| DABCO $10^{-3}$ M | $4,756 \pm 337$          |
| Histidine $5 \times 10^{-4}$ M | $353 \pm 23$             |
| Histidine $2.5 \times 10^{-3}$ M | $739 \pm 73$             |
| Histidine $10^{-3}$ M | $2,091 \pm 52$          |
| Histidine $10^{-4}$ M | $8,455 \pm 225$         |
| Histidine $10^{-5}$ M | $4,352 \pm 502$          |

The reaction mixture was as described for the control system in Fig. 7 except that the inhibitors were added as indicated. Incubation 120 min.

* Mean $\pm$ SE of five experiments.
‡ $P$ value for the difference between the presence and absence of inhibitor.
§ Stimulation.
concentration employed in Fig. 7; however, the substitution of D$_2$O for water inhibited ethylene formation ($6,495 \pm 563; n = 5; P < 0.05$) under these conditions.

Discussion

The use of ethylene formation from methional or KMB as a method for the detection of OH$^-$ was based initially on studies with the xanthine oxidase system. Xanthine oxidase, acting on its substrate (xanthine, hypoxanthine, and acetaldehyde) generates both O$_2^-$ and H$_2$O$_2$; it also catalyzes the formation of ethylene from methional (6). Ethylene formation by xanthine oxidase is inhibited by SOD, catalase, and OH$^-$ scavengers and is stimulated by H$_2$O$_2$, suggesting a requirement of OH$^-$ generated by the Haber-Weiss reaction (equation a) (6). Ethylene is formed from methional by monocytes (14) and from methional (12) and KMB (13) by PMNs during phagocytosis. Its formation is strongly inhibited by SOD and to a lesser degree by catalase (however see 12), and OH$^-$ scavengers. This suggests that an OH$^-$ mechanism is, in part, responsible for the ethylene formation and implies the generation of OH$^-$ by phagocytic cells during phagocytosis.

The findings reported here indicate that, in the phagocytosing PMN, ethylene formation from methional or KMB is dependent to a large degree on MPO. The evidence is as follows. (a) Ethylene formation is markedly decreased when MPO-deficient PMNs are employed. (b) Ethylene formation by normal PMNs is inhibited by the peroxidase inhibitors, azide, and cyanide. (c) Ethylene is formed by a model MPO-dependent system which has many of the properties of the predominant ethylene forming system of the PMN.

Ethylene formation by MPO-deficient PMNs was less than 10% of normal with either methional or KMB as the substrate. This defect in ethylene formation was reversed by purified MPO; indeed, with KMB as substrate, ethylene formation was increased by MPO to a level greater than that of normal cells (Table I). MPO-deficient PMNs, in contrast to CGD leukocytes, do not have an impaired phagocytosis-induced respiratory burst; indeed, the burst appears to be greater than that of normal cells (16, 22, 23). Thus, when MPO is added in excess, ethylene formation by MPO-deficient PMNs may reflect the high activity of the respiratory burst in these cells.

Azide and cyanide inhibited ethylene formation from methional or KMB by normal PMNs (13; Fig. 1), although with methional as substrate, the effect of azide was variable, with an inhibition at relatively low and high concentrations but not at the intervening concentrations (Fig. 1). Azide stimulated ethylene formation by phagocytosing monocytes (14) and MPO-deficient PMNs (Table II). These findings are compatible with two opposing actions of azide: an inhibition of MPO-dependent ethylene formation and a stimulation of MPO-independent ethylene formation. Two MPO-independent ethylene-forming systems which are stimulated by azide were identified; one involves eosinophils and the other H$_2$O$_2$, alone or in the presence of catalase.

Azide stimulated ethylene formation from methional by phagocytosing guinea pig eosinophils (Table III). Since PMN suspensions contain small numbers of eosinophils, the stimulation by azide of ethylene formation by MPO-deficient leukocytes and the paradoxical effect of azide on normal leukocytes may be due
ETHYLENE FORMATION BY POLYMORPHONUCLEAR LEUKOCYTES

in part to an effect on eosinophils. The eosinophil peroxidase, which differs from the peroxidase of neutrophils and monocytes (MPO), is present in normal amounts in the eosinophils of patients with hereditary MPO deficiency (24).

The formation of ethylene from methional by H\textsubscript{2}O\textsubscript{2} or from methional and KMB by H\textsubscript{2}O\textsubscript{2} + catalase also is stimulated by azide at acid pH (Table IV). Ethylene formation by H\textsubscript{2}O\textsubscript{2} and its stimulation by azide was strongly inhibited by EDTA, suggesting a trace metal requirement; the stimulation by azide of the H\textsubscript{2}O\textsubscript{2}-catalase system, however, was unaffected by EDTA. Azide binds to the heme iron of catalase (25) and, in the presence of low H\textsubscript{2}O\textsubscript{2} concentrations, is oxidized to form nitrous oxide, nitric oxide, and nitrogen (26, 27), with an associated reduction of the heme iron (25). A product of this reaction may initiate or facilitate the formation of ethylene under the conditions employed.

Ethylene was formed from KMB by a model system which required MPO, H\textsubscript{2}O\textsubscript{2}, chloride (or bromide) and EDTA for optimum activity (Table V). This system was similar in a number of respects to the predominant ethylene-forming system of the PMN.

(a) Ethylene formation by both the intact cell (Table I) and the model system (Table V) required MPO for optimum activity.

(b) Both were stimulated by EDTA (Fig. 3; Table V). A number of divalent cations (Mn\textsuperscript{2+}, Zn\textsuperscript{2+}, Co\textsuperscript{2+}, Cu\textsuperscript{2+}) had an inhibitory effect on ethylene formation by the MPO system which was abolished by excess EDTA (Fig. 6; Table VI). The stimulation by EDTA therefore may result from the removal of trace metal inhibitors from the reaction mixture. EDTA or the EDTA-metal chelate also may participate directly in the ethylene-forming reaction. Thus, the participation of an iron-EDTA chelate in the formation of OH\textsuperscript{-} from O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2} has recently been described (28).

(c) Ethylene formation by both phagocytosing PMNs (12, 13) and the MPO system (Table VII) was strongly inhibited by SOD.

(d) Catalase strongly inhibited ethylene formation by the MPO system (Table VII), whereas its effect on PMNs was less striking (12, 13) and, in the case of formation from methional, could be explained in part by a nonspecific effect of protein (12). The discrepancy between the effect of catalase on the MPO model system and the intact PMN may reflect a lesser role for H\textsubscript{2}O\textsubscript{2} in the intact cell or the less efficient interaction of H\textsubscript{2}O\textsubscript{2} with added catalase because of competing cellular reactions and/or structural barriers to catalase.

(e) Hydroxyl radical scavengers partially inhibit ethylene formation by both the MPO system (Table VII) and intact PMNs (12, 13).

Ethylene formation from either methional or KMB is initiated by electron abstraction from the sulfur atom to form a radical cation which subsequently degrades under nucleophilic attack by OH\textsuperscript{-} (29). Methional yields ethylene, methyl-disulfide and formic acid, while KMB yields ethylene, methyldisulfide and carbon dioxide (29). A number of one electron oxidants would be expected to initiate ethylene formation. Hydroxyl radicals are particularly effective in this regard; a second order rate constant for methional of 8.2 × 10\textsuperscript{9} M\textsuperscript{-1} s\textsuperscript{-1} has been reported (15). The superoxide anion also reacts with methional to yield ethylene although more sluggishly (first order rate constant 5.2 × 10\textsuperscript{3} s\textsuperscript{-1}) (15). Ethylene is also formed from KMB by dye-sensitized photo-oxidation (Fig. 7). Two general mechanisms for photo-oxidation have been described (30). In type I
photo-oxidation reactions, the light-activated sensitizer (dye) reacts directly with the target molecule, whereas in type II photo-sensitized reactions, the light-activated dye reacts first with oxygen to form \(^1\text{O}_2\) which then can initiate subsequent reactions. The lifetime of singlet oxygen in solution is considerably prolonged by the substitution of D\(_2\)O for H\(_2\)O (31, 32) and agents such as azide (33), DABCO (34) and histidine (35) can quench \(^1\text{O}_2\) and thus inhibit \(^1\text{O}_2\)-dependent reactions. The generation of ethylene from KMB by rose bengal (5 \(\mu\)g/ml) in the presence of light and oxygen is stimulated by the substitution of D\(_2\)O for water (Fig. 7) and is inhibited by DABCO, histidine and azide (Table VIII). These findings suggest a type II (i.e., \(^1\text{O}_2\)-mediated) photo-oxidation mechanism. Initiation of ethylene formation in this way implies electron abstraction by \(^1\text{O}_2\) either directly or via the intermediate formation of another oxidant. A type I photooxidation reaction in which the dye itself acts as a one electron oxidant for KMB seems unlikely under the conditions employed in Fig. 7, although it cannot be entirely excluded. If the rose bengal concentration is increased 10-fold (50 \(\mu\)g/ml), ethylene formation from KMB is no longer stimulated by D\(_2\)O but is inhibited; a type I photo-oxidation mechanism may be operative under these conditions.

What is the mechanism of the MPO-dependent ethylene formation by the model system described here and by the PMN? This question cannot be answered definitively at the present time. In a previous study, horseradish peroxidase was found to catalyze the conversion of methional or KMB to ethylene in the presence of sulfite, a monophenol, Mn\(^{2+}\) and oxygen; Mn\(^{2+}\) and oxygen could be replaced by H\(_2\)O\(_2\) (36). The initiation of ethylene formation by an oxidant such as \(\text{O}_2^-\) or OH\(^-\) generated during peroxidase-catalyzed sulfite oxidation was proposed. Hydroxyl radical scavengers inhibit ethylene formation by the MPO-H\(_2\)O\(_2\)-chloride-EDTA system (Table VII) and by PMNs (12, 13). However, the inhibition of the MPO system by benzoate and mannitol was less than 50% at concentrations 10 times and 100 times that of KMB, respectively (Table VII), and under our experimental conditions, ethylene formation by phagocytosing PMNs was inhibited only 30% by mannitol at 100 times the KMB concentration (data not shown). Similarly, Weiss et al. (13) reported a 38 and 33% inhibition of ethylene formation from KMB by ethanol and benzoate, and Tauber and Babior (12) a 48, 8 and 10% inhibition by benzoate, ethanol, and mannitol, respectively. Although these studies are compatible with a contribution by OH\(^-\) to the formation of ethylene by the MPO system and PMNs, they suggest that this contribution, if present, is not large. Thus, caution should be exercised in the interpretation of ethylene formation by PMNs from KMB and methional as a measure of OH\(^-\) generation.

SOD markedly inhibits ethylene formation by the MPO-H\(_2\)O\(_2\)-chloride-EDTA system and by PMNs. The primary action of SOD is the dismutation of \(\text{O}_2^-\), suggesting an as yet undefined role for \(\text{O}_2^-\) in MPO-dependent ethylene formation. SOD also can react with H\(_2\)O\(_2\) at alkaline pH with accompanied chemiluminescence, \(\text{O}_2^-\) generation and peroxidatic activity (37, 38), and a direct quenching effect of SOD on \(^1\text{O}_2\) has been reported (39-43), although the latter finding has been questioned by a number of investigators (35, 44-46). The inhibition of MPO-dependent ethylene formation by SOD is of particular interest in view of the inhibition by SOD of PMN microbicidal activity (11), a
function which is dependent to a considerable degree on MPO.

Ethylene formation from KMB by the MPO-H₂O₂-halide-EDTA system was strongly inhibited by the 'O₂ quenchers, DABCO, histidine and azide (Table VII). Although the specificity of the inhibitors is not absolute, these findings, together with the evidence for both the generation of 'O₂ by the MPO-H₂O₂-halide system (21) and the initiation of ethylene formation from KMB by 'O₂ (Fig. 7; Table VIII), raise the possibility that ethylene formation may be initiated in part by 'O₂ generated by MPO-dependent reactions.

Summary

Ethylene formation from the thioethers, β-methylthiopropionaldehyde (methional) and 2-keto-4-thiomethylbutyric acid by phagocytosing polymorphonuclear leukocytes (PMNs) was found to be largely dependent on myeloperoxidase (MPO). Conversion was less than 10% of normal when MPO-deficient PMNs were employed; formation by normal PMNs was inhibited by the peroxidase inhibitors, azide, and cyanide, and a model system consisting of MPO, H₂O₂, chloride (or bromide) and EDTA was found which shared many of the properties of the predominant PMN system. MPO-independent mechanisms of ethylene formation were also identified. Ethylene formation from methional by phagocytosing eosinophils and by H₂O₂ in the presence or absence of catalase was stimulated by azide. The presence of MPO-independent, azide-stimulable systems in the PMN preparations was suggested by the azide stimulation of ethylene formation from methional when MPO-deficient leukocytes were employed. Ethylene formation by dye-sensitized photooxidation was also demonstrated and evidence obtained for the involvement of singlet oxygen ('O₂). These findings are discussed in relation to the participation of H₂O₂, hydroxyl radicals, the superoxide anion and 'O₂ in the formation of ethylene by PMNs and by the MPO model system.

We would like to thank Ann Waltersdorph and Joanne Fluvog for their excellent technical assistance and Kay Tisdel for her valuable help in the preparation of the manuscript.

Received for publication 27 March 1978.

References
1. Klebanoff, S. J. 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Semin. Hematol.* 12:117.
2. Klebanoff, S. J., and R. A. Clark. 1978. The Neutrophil: Function and Clinical Disorders. North-Holland Publishing Co., Amsterdam.
3. Lehrer, R. I., and M. J. Cline. 1969. Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to Candida infection. *J. Clin. Invest.* 48:1478.
4. Lehrer, R. I., J. Hanifin, and M. J. Cline. 1969. Defective bactericidal activity in myeloperoxidase-deficient human neutrophils. *Nature (Lond.)*, 223:78.
5. Haber, F., and J. Weiss. 1934. The catalytic decomposition of hydrogen peroxide by iron salts. *Proc. R. Soc. Lond. A.* 147:332.
6. Beauchamp, C., and I. Fridovich. 1970. Mechanism for the production of ethylene from methional. Generation of the hydroxyl radical by xanthine oxidase. *J. Biol. Chem.* 245:4841.
7. McClune, G. J., and J. A. Fee. 1976. Stopped flow spectrophotometric observation of superoxide dismutation in aqueous solution. FEBS (Fed. Eur. Biochem. Soc.) Lett. 67:294.

8. Halliwell, B. 1976. An attempt to demonstrate a reaction between superoxide and hydrogen peroxide. FEBS (Fed. Eur. Biochem. Soc.) Lett. 72:8.

9. Babior, B. M., R. S. Kipnes, and J. T. Curnutte. 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. 52:741.

10. Iyer, G. Y. N., D. M. F. Islam, and J. H. Quastel. 1961. Biochemical aspects of phagocytosis. Nature (Lond.). 192:533.

11. Johnston, R. B. Jr., B. B. Keele, Jr., H. P. Misra, J. E. Lehmeyer, L. S. Webb, R. L. Baehner, and K. V. Rajagopal. 1975. The role of superoxide anion generation in phagocytic bactericidal activity. Studies with normal and chronic granulomatous disease leukocytes. J. Clin. Invest. 55:1357.

12. Tauber, A. I., and B. M. Babior. 1977. Evidence for hydroxyl radical production by human neutrophils. J. Clin. Invest. 60:374.

13. Weiss, S. J., P. K. Rustagi, and A. F. LoBuglio. 1978. Human granulocyte generation of hydroxyl radical. J. Exp. Med. 147:316.

14. Weiss, S. J., G. W. King, and A. F. LoBuglio. 1977. Evidence for hydroxyl radical generation by human monocytes. J. Clin. Invest. 60:370.

15. Bors, W., E. Lengfelder, M. Saran, C. Fuchs, and C. Michel. 1976. Reactions of oxygen radical species with methional: a pulse radiolysis study. Biochem. Biophys. Res. Commun. 70:81.

16. Rosen, H., and S. J. Klebanoff. 1976. Chemiluminescence and superoxide production by myeloperoxidase-deficient leukocytes. J. Clin. Invest. 58:374.

17. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21(Suppl. 97):77.

18. Pincus, S. J. 1978. Blood. In press.

19. Agner, K. 1958. Crystalline myeloperoxidase. Acta Chem. Scand. 12:89.

20. Worthington Enzyme Manual. 1972. Worthington Biochemical Corp., Freehold, N. J. p. 43.

21. Rosen, H., and S. J. Klebanoff. 1977. Formation of singlet oxygen by the myeloperoxidase-mediated antimicrobial system. J. Biol. Chem. 252:4803.

22. Klebanoff, S. J., and S. H. Pincus. 1971. Hydrogen peroxide utilization in myeloperoxidase-deficient leukocytes: a possible microbial control mechanism. J. Clin. Invest. 50:2226.

23. Klebanoff, S. J., and C. B. Hamon. 1972. Role of myeloperoxidase-mediated antimicrobial systems in intact leukocytes. J. Reticuloendothel. Soc. 12:170.

24. Grignaschi, V. J., A. M. Sperperato, M. J. Etcheverry, and A. J. L. Macario. 1963. Un nuevo cuadro citoquímico: negatividad espontanea de las reacciones de peroxidasa, oxidasas y lipidio en la progenie neutrofila y en los monocitos de dos hermanos. Rev. Asoc. Med. Argent. 77:218.

25. Keilin, D., and E. F. Hartree. 1945. Properties of azide-catalase. Biochem. J. 39:149.

26. Theorell, H., and A. Ehrenberg. 1952. Magnetic properties of some peroxide compounds of myoglobin, peroxidase and catalase. Arch. Biochem. Biophys. 41:442.

27. Keilin, D., and E. F. Hartree. 1955. Catalase, peroxidase and metmyoglobin as catalysts of coupled peroxidatic reactions. Biochem. J. 60:310.

28. McCord, J. M., and E. D. Day, Jr. 1978. Superoxide-dependent production of hydroxyl radical catalyzed by iron-EDTA complex. FEBS (Fed. Eur. Biochem. Soc.) Lett. 86:139.

29. Yang, S. F. 1969. Further studies on ethylene formation from α-keto-γ-methylthiolobutyric acid or β-methylthiopropyialdehyde by peroxidase in the presence of sulfite.
and oxygen. *J. Biol. Chem.* 244:4360.
30. Foote, C. S. 1976. Photosensitized oxidation and singlet oxygen: consequences in biological systems. In Free Radicals in Biology. W. A. Pryor, editor. Academic Press, Inc., New York. II:85.
31. Merkel, P. B., R. Nilsson, and D. R. Kearns. 1972. Deuterium effects on singlet oxygen lifetimes in solutions. A new test of singlet oxygen reactions. *J. Am. Chem. Soc.* 94:1030.
32. Kajiwara, T., and D. R. Kearns. 1973. Direct spectroscopic evidence for a deuterium solvent effect on the lifetime of singlet oxygen in water. *J. Am. Chem. Soc.* 95:5886.
33. Hasty, N., P. B. Merkel, P. Radlick, and D. R. Kearns. 1972. Role of azide in singlet oxygen reactions: reaction of azide with singlet oxygen. *J. Am. Chem. Soc.* 94:1030.
34. Ouannes, C., and T. Wilson. 1968. Quenching of singlet oxygen by tertiary aliphatic amines. Effect of DABCO. *J. Am. Chem. Soc.* 90:5527.
35. Hodgson, E. K., and I. Fridovich. 1974. The production of superoxide radical during the decomposition of potassium peroxochromate (V). *Biochemistry.* 13:3811.
36. Yang, S. F. 1967. Biosynthesis of ethylene. Ethylene formation from methional by horseradish peroxidase. *Arch. Biochem. Biophys.* 122:481.
37. Hodgson, E. K., and I. Fridovich. 1975. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: inactivation of the enzyme. *Biochemistry.* 14:5294.
38. Hodgson, E. K., and I. Fridovich. 1975. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: chemiluminescence and peroxidation. *Biochemistry.* 14:5299.
39. Paschen, W., and U. Weser. 1973. Singlet oxygen decontaminating activity of erythrocuprein (superoxide dismutase). *Biochim. Biophys. Acta.* 327:217.
40. Finaazzi Agrò, A., C. Giovagnoli, P. De Sole, L. Calabrese, G. Rotilio, and B. Mondovi. 1972. Erythrocuprein and singlet oxygen. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 21:183.
41. Paschen, W., and U. Weser. 1975. Problems concerning the biochemical action of superoxide dismutase (erythrocuprein). *Hoppe-Seyler's Z. Physiol. Chem.* 356:727.
42. Richter, C., A. Wendel, U. Weser, and A. Azzi. 1975. Inhibition by superoxide dismutase of linoleic acid peroxidation induced by lipoxidase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 51:300.
43. Weser, U., W. Paschen, and M. Younes. 1975. Singlet oxygen and superoxide dismutase (cuprein). *Biochem. Biophys. Res. Commun.* 66:769.
44. Goda, K., T. Kimura, A. L. Thayer, K. Kees, and A. P. Schaap. 1974. Singlet molecular oxygen in biological systems: non-quenching of singlet oxygen-mediated chemiluminescence by superoxide dismutase. *Biochem. Biophys. Res. Commun.* 58:660.
45. Schaap, A. P., A. L. Thayer, G. R. Faler, K. Goda, and T. Kimura. 1974. Singlet molecular oxygen and superoxide dismutase. *J. Am. Chem. Soc.* 96:4025.
46. Michelson, A. M. 1974. Is singlet oxygen a substrate for superoxide dismutase? No. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 44:97.