Formation of Singlet Oxygen by the Myeloperoxidase-mediated Antimicrobial System*

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Myeloperoxidase, H₂O₂, and a halide form a potent antimicrobial system which is operative in the polymorphonuclear leukocyte (PMN). The formation of singlet oxygen (¹⁰₂) by this system is suggested by (a) the conversion of 2,5-diphenylfuran to its specific singlet oxygen product cis-dibenzoylethylene (cis-DBE); (b) the inhibition of this reaction by the ¹⁰₂ quenchers β-carotene, bilirubin, histidine, and 1,4-diazabicyclo[2,2,2]octane (DABCO); and (c) the stimulation of conversion by D₂O, which prolongs the lifetime of ¹⁰₂ in solution. Diphenylfuran conversion by the myeloperoxidase system has a pH optimum of 4.5 and an optimum H₂O₂ concentration of 30 μM and the halides vary in effectiveness in the order Br⁻ > Cl⁻ > I⁻. Reagent H₂O₂ could be replaced by the H₂O₂-generating system, glucose + glucose oxidase.

Hypochlorous acid, which is formed by the myeloperoxidase/H₂O₂/halide system, also converts diphenylfuran to cis-DBE. As with the myeloperoxidase system, this conversion has an acid pH optimum, is inhibited by ¹⁰₂ quenchers, and is stimulated by D₂O. Diphenylfuran conversion by HOCl is increased by chloride, but not by H₂O₂, in the concentrations employed in the myeloperoxidase system. Our studies suggest that ¹⁰₂ is formed by the myeloperoxidase/H₂O₂/halide system and that the mechanism involves the initial oxidation of chloride to HOCl and the subsequent decomposition of HOCl, particularly in the presence of excess chloride, to form ¹⁰₂. The singlet oxygen so formed may participate in the microbicidal activity of the isolated myeloperoxidase system and of the intact PMN.

Among the antimicrobial systems of the polymorphonuclear leukocyte is one which consists of myeloperoxidase, H₂O₂, and a halide cofactor (for review see Ref. 1). Following particle ingestion by the PMN, myeloperoxidase, which is present in high concentration in the lysosomal granules of the resting cell, is discharged into the phagocytic vacuole where it reacts with H₂O₂, generated by the phagocytosis-induced respiratory burst, and with a halide such as chloride or iodide to kill the ingested organism. The pH optimum of the myeloperoxidase-mediated antimicrobial system is distinctly acid (pH 4.5 to 5.0) as is the pH within the vacuolar space. The isolated system is toxic not only to a variety of bacterial, fungal, viral, and mycoplasmal species but also to certain mammalian cells, namely, spermatozoa (2), erythrocytes (3), leukocytes (4), platelets (4), and tumor cells (5–7).

The mechanism of action of the myeloperoxidase/H₂O₂/halide system is complex (1). The halide cofactor is oxidized by myeloperoxidase and H₂O₂ and it is presumed that this leads to the formation of a toxic agent or agents. The nature of this agent(s) is not known; it may vary with halide employed as well as with other environmental factors. Recently, interest has focused on the possibility that singlet molecular oxygen (singlet oxygen, ¹⁰₂) is involved (8, 9).

Singlet oxygen is an excited state of molecular oxygen in which an electron has been shifted to an orbital of higher energy with an inversion of spin (for review see Refs. 10 to 12). Its excess energy can be dissipated by thermal decay, by the emission of light, or by its participation in characteristic chemical reactions. The latter are generally oxygenation reactions at areas of high electron density such as unsaturated carbon to carbon bonds. A relatively unique product may be formed and its formation thus can be employed as a method for the detection of singlet oxygen. One such reaction is the conversion of 2,5-diphenylfuran to cis-dibenzoylethylene (13, 14). Another property of singlet oxygen is the prolongation of its lifetime in solution by the substitution of D₂O for H₂O with a corresponding stimulation of ¹⁰₂-mediated reactions (15). This property forms the basis of an additional test for ¹⁰₂ participation in a chemical reaction (15, 16).

This paper deals with the generation of singlet oxygen by the myeloperoxidase/H₂O₂/halide system. We report the conversion of diphenylfuran to cis-DHE by the myeloperoxidase system, the stimulation of this conversion by D₂O, and its inhibition by several singlet oxygen quenchers. A mechanism for ¹⁰₂ generation by the myeloperoxidase system is proposed which involves the intermediate formation of hypochlorous acid.

EXPERIMENTAL PROCEDURES

Special Reagents

2,5 Diphenylfuran was obtained from Eastman Organic Chemicals, Rochester, N. Y., trans-dibenzoylethylene was obtained from Aldrich Chemical Co., Milwaukee, Wis., and cis-dibenzoylethylene was...
was synthesized from diphenylfuran by nitric acid oxidation (17). Each was recrystallized from ethanol until judged to be pure by thin layer chromatography. [3H]Diphenylfuran was prepared from unlabeled diphenylfuran by tritium exchange labeling (New England Nuclear Co., Boston, Mass.) and was purified by thin layer chromatography. [3H]Diphenylfuran was mixed with carrier diphenylfuran to a specific activity of approximately 0.02 μCi/nmol except for the radioautographic studies where the specific activity was 0.2 μCi/nmol.

Sodium hypochlorite (Mallinckrodt, St. Louis, Mo.) was standardized either by reaction with ascorbic acid or by reaction with NaCl and spectrophotometric measurement of the \( I_2 \) formed using \( E_{1\%}=2.64 \times 10^4 \) m\(^{-1}\) cm\(^{-1}\) (19, 20). 1,4-Diazabicyclo[2.2.2]octane was obtained from Eastman Organic Chemicals, Rochester, N. Y. and was used as received or recrystallized from acetone with comparable results. β-Carotene (type I, Sigma Chemical Co., St. Louis, Mo.) was used as a 0.5 mM solution in ethanol containing 0.1% ammonium hydroxide.

Myeloperoxidase was prepared from cane granulocytes to the end of Step 6 by the method of Agner (21) and assayed by the orthodianisidine method (22). One unit of enzyme is the amount decomposing 1 μmol of \( H_2O_2 \) per min at 25°. Catalase (beef liver, crystalline, 6.56 mg/ml; 39,132 units/mg) was obtained from Worthington Biochemical Corp., Freehold, N. J. and glucose oxidase (type V, Aspergillus niger, 1,239 units/ml) from Sigma Chemical Co., St. Louis, Mo.

**Conversion of Diphenylfuran to cis-DBE**

The components indicated in the legends were incubated in test tubes (13 x 100 mm) for 30 min at 37° in a shaking water bath oscillating 80 times/min. The reaction was terminated by the addition in each of 1.0 ml of chloroform and mixed vigorously for 20 s with a glass rod with 80 strokes/min. The reaction was terminated by the addition of 2.0 ml of 95% ethanol. After centrifugation at 250 g for 5 min, the components of the residue were separated and quantitated by either Method A or B.

**Method A** - When unlabeled diphenylfuran was employed, the residue was transferred as completely as possible to a specific activity of approximately 0.02 μCi/nmol except for the radioautographic studies where the specific activity was 0.2 μCi/nmol. Each was recrystallized from ethanol until judged to be pure by thin layer chromatography. [3H]Diphenylfuran was prepared from unlabeled diphenylfuran by tritium exchange labeling (New England Nuclear Co., Boston, Mass.) and was purified by thin layer chromatography. [3H]Diphenylfuran was mixed with carrier diphenylfuran to a specific activity of approximately 0.02 μCi/nmol except for the radioautographic studies where the specific activity was 0.2 μCi/nmol.

**Method B** - When [3H]diphenylfuran was employed, the residue was dissolved in 100 μl of 95% ethanol. An aliquot (10 μl) was transferred directly to a scintillation vial for determination of total radioactivity and 10 μl were co-chromatographed with carrier diphenylfuran and cis-DBE as described under "Method A." The silica gel containing the cis-DBE spot was removed by scraping and placed directly into a scintillation vial. Fifteen milliliters of Aquasol scintillation mixture (New England Nuclear Co., Boston, Mass.) were added. The vials were vigorously shaken and stored in the dark overnight to decrease background chemiluminescence prior to counting in a Beckman LS-100C liquid scintillation counter. The per cent conversion was determined by comparison of the radioactivity of the cis-DBE spot to that of the total sample and the number of moles formed was calculated. Each experiment contained a control consisting of diphenylfuran in buffer only and the amount of cis-DBE present in the control was subtracted from the experimental value. The cis-DBE contamination of the diphenylfuran was generally 2 to 4%. In some experiments, the diphenylfuran and trans-DBE spots were also removed for determination of radioactivity.

**Radioautography** - [3H]Diphenylfuran at 10 times the usual specific activity was used. Following separation of the reaction products as described under "Method A," the plates were exposed directly to Kodak No-Screen x-ray film for 5 days and the film was developed. Qualitatively similar radioautographs were obtained when a thin plastic sheet (Handi-Wrap, Dow Chemical Co., Midland, Mich.) was interposed between the film and plate.

**D.O Experiments**

Preparation of D.O/H.O Mixtures - Sodium acetate buffer (0.05 M) was prepared in D.O (>99.8%), Stober Isotope Chemical, Rutherford, N. J.) at a final concentration of 0.99% D.O and a pH of 5.0, where pH represents the negative log of the summed hydrogen and deuterium ion concentrations. pH was determined according to the relationship \( pH = pHD + 0.3314 n + 0.0766 n^2 \), where \( pHD \) is the pH of the mixture as measured by a standard pH electrode and \( n \) is the mole fraction of D.O in the mixture (23). Dilution of the D.O buffer with aqueous 0.05 M sodium acetate buffer, pH 5.0, resulted in D.O/H.O mixtures of constant pH (range 4.96 to 5.02). Where indicated, NaCl was added at a final concentration of 0.1 M.

**Calculation of \( \Delta \Gamma \), Lifetime** - The approximate lifetimes (\( \Gamma \)) of singlet oxygen in mixtures of D.O and H.O were calculated from the expression

\[
\frac{1}{\tau} = \frac{1}{\tau_D} - \frac{1}{\tau_O} = \frac{1}{0.5x + 0.05y} \quad (3)
\]

where \( x \) is the mole fraction of D.O in a D.O/H.O mixture. This expression was derived from the relationship of the lifetime of singlet oxygen to the intensity of the infrared absorption of the solvent proposed by Merkel et al. (15, 16)

\[
\frac{1}{\tau} = \frac{1}{\tau_D} - \frac{1}{\tau_O} = \frac{1}{0.5x + 0.05y} \quad (3)
\]

Assuming that changes in absorbances of D.O/H.O mixtures are proportional to the concentration of D.O, then

\[
x = x_2 - n(x_2 - x_1) \quad and \quad y = y_2 - n(y_2 - y_1) \quad (4)
\]

**Statistical Analyses**

Statistical differences were determined using Student’s two tailed t test for independent means (not significant, N.S., p > 0.05).
Singlet Oxygen Formation by Myeloperoxidase

chromatography and the spots identified either by ultraviolet illumination (Fig. 2a) or radioautographically (Fig. 2b). When $[^3H]$diphenylfuran was employed, quantitation was by elution and measurement of radioactivity. Under the conditions employed in Fig. 2, 24.6 ± 4.6 (S.D., n = 12) nmol of cis-DBE were formed from 50 nmol of diphenylfuran (49% conversion). In contrast, only 0.2 ± 0.0 (S.D., n = 3) nmol of trans-DBE were detected. Each component of the myeloperoxidase system (myeloperoxidase, H$_2$O$_2$, chloride) was required. Conversion of diphenylfuran to cis-DBE could also be quantitated by elution of the cis-DBE spot with ethanol and comparison of the absorbance at 260 nm to a standard curve prepared with synthesized standard. This technique was employed in Table I to measure the conversion of diphenylfuran to cis-DBE by the peroxidase system with glucose and glucose oxidase as the source of H$_2$O$_2$. Radioactivity was employed for quantitation in all subsequent studies described here.

The conversion of diphenylfuran to cis-DBE by myeloperoxidase, H$_2$O$_2$, and chloride was optimal at a H$_2$O$_2$ concentration of approximately 30 μM with conversion diminishing at both lower and higher H$_2$O$_2$ concentrations (Fig. 3). The fall in activity at high H$_2$O$_2$ concentrations may be due to the inactivation of myeloperoxidase by excess H$_2$O$_2$ (25). The halide requirement is shown in Fig. 4. The most effective halide was bromide with activity increasing sharply at concentrations above 0.1 mM. Activity also was high when chloride was employed, however, concentrations greater than 10 mM were required. With iodide, activity was low and limited to a narrow range of concentrations (difference from zero: 10$^{-4}$ M iodide, p < 0.01, n = 4; 10$^{-3}$ M iodide, p < 0.05, n = 4). Conversion was optimal at pH 4.5 and fell sharply as the pH was increased to neutrality under the conditions employed in Fig. 5.

**Conversion of diphenylfuran to cis-DBE by HOCI** — The formation of hypochlorous acid by myeloperoxidase, H$_2$O$_2$, and chloride (20) and the now well established generation of singlet oxygen by hypochlorite and H$_2$O$_2$ (10-12) prompted a study of the conversion of diphenylfuran to cis-DBE by HOCl and its comparison to conversion by the peroxidase system. Diphenylfuran is converted to cis-DBE at pH 5.0 by HOCl at concentrations greater than 20 μM under the conditions employed in Fig. 6. The further addition of H$_2$O$_2$ at the 50 μM concentration routinely employed in the myeloperoxidase/H$_2$O$_2$/chloride system did not increase diphenylfuran conversion; indeed, it appeared to be inhibitory. Sodium chloride however markedly enhanced HOCl-dependent diphenylfuran conversion; indeed, it appeared to be inhibitory. Sodium chloride however markedly enhanced HOCl-dependent diphenylfuran conversion. Fig. 7 demonstrates the effect of chloride concentration on the conversion of diphenylfuran to cis-DBE by 20 μM HOCl. Under the conditions employed, activity was maximal at 0.1 mM chloride. Either sodium or potassium chloride could be employed; however, 0.1 M sodium nitrate and 0.067 M
sodium sulfate were ineffective (data not shown).

The effect of pH on the conversion of diphenylfuran to cis-
DBE by HOCl in the presence and absence of chloride is shown
in Fig. 8. Conversion by HOCl alone fell continuously as the
pH was increased from 4.0 to 8.5. In contrast, when 0.1 mM
chloride was added, conversion was high between pH 4.0 and
7.0 and then fell sharply as the pH was increased above that
range. This latter pattern was observed at two HOCl concen-
trations, 200 μM where conversion was essentially complete
between pH 4.0 and 7.0 and 30 μM where only partial conver-
sion was observed.

Effect of Singlet Oxygen Quenchers—The conversion of di-
phenylfuran to cis-DBE, but not to trans-DBE, by the myelo-
peroxidase system or by HOCl in the presence or absence of
chloride is compatible with a singlet oxygen-mediated mecha-
nism. This is supported by the inhibitory effect of the singlet
oxygen quenchers β-carotene (26), bilirubin (27), DABCO (28),
and histidine (29) (Table II). The solubility properties of β-
and histidine (29) (Table II). The solubility properties of β-
carotene and bilirubin required the addition of ethanol at a
final concentration of 20%. This concentration of ethanol alone
had no adverse effect on conversion; however, 9μM β-carotene
in 20% ethanol, 25 μM bilirubin in 20% ethanol, 1 mM DABCO,
and 0.1 mM histidine inhibited conversion by all three systems
employed.

Azide is an additional quencher of singlet oxygen (30); it also
is a potent inhibitor of myeloperoxidase. As shown in Table
III, azide inhibits diphenylfuran conversion by the myelo-
peroxidase/H₂O₂/chloride system at a concentration (10⁻³ M) 2
orders of magnitude lower than that required for inhibition of
diphenylfuran conversion by HOCl either in the presence or
absence of chloride. Cyanide inhibits myeloperoxidase but is
not generally recognized as a singlet oxygen quencher. Cy-

TABLE I

Diphenylfuran conversion by the myeloperoxidase/glucose/glucose
oxidase/chloride system

The reaction mixture was as described in Fig. 2 (complete system)
except that 200 nmoI of unlabeled diphenylfuran were employed,
H₂O₂ was replaced by 0.1 mM glucose and 12.5 millimots/ml of glucose
oxidase, and the incubation period was 90 min. The cis-DBE formed
was measured spectrophotometrically (Method A).

<table>
<thead>
<tr>
<th>Supplement</th>
<th>cis-Dibenzylyethylene (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase + glucose + glucose oxidase + chloride</td>
<td>37.2 ± 3.5 (4) a</td>
</tr>
<tr>
<td>Myeloperoxidase omitted</td>
<td>3.0 ± 2.0 (2) &lt;0.01 b</td>
</tr>
<tr>
<td>Glucose omitted</td>
<td>2.5 ± 0.5 (2) &lt;0.01 b</td>
</tr>
<tr>
<td>Glucose oxidase omitted</td>
<td>2.0 ± 0.0 (2) &lt;0.01 b</td>
</tr>
<tr>
<td>Chloride omitted</td>
<td>3.5 ± 1.5 (2) &lt;0.01 b</td>
</tr>
<tr>
<td>Catalase (850 units) added</td>
<td>1.5 ± 0.5 (2) &lt;0.01 b</td>
</tr>
<tr>
<td>Heated catalase (100°C, 15 min) added</td>
<td>25.0 ± 2.0 (2) N.S. c</td>
</tr>
</tbody>
</table>

a Mean ± S.E. of (n) experiments.
b p value for the difference from the complete system.
c Not significant.

Fig. 3 (left). Effect of H₂O₂ concentration. The reaction mixture
was as described in Fig. 2 (complete system) except that the H₂O₂
concentration was varied as indicated.

Fig. 4 (center). Effect of halide concentration. The reaction mix-
ture was as described in Fig. 2 (complete system) except that the
halide concentration was varied as indicated (sodium chloride,
- - -●, sodium bromide, ■ - ■, sodium iodide, ▲ - ▲).

Fig. 5 (right). Effect of pH. The reaction mixture was as de-
scribed in Fig. 2 (complete system) except that the pH was varied as
indicated using either acetate (●) or phosphate (▲) buffer.
Singlet Oxygen Formation by Myeloperoxidase

FIG. 6 (left). Conversion of diphenylfuran to cis-DBE by hypochlorous acid. The reaction mixture contained 0.05 M sodium acetate buffer, pH 5.0, 25 μM diphenylfuran (50 nmol), and HOCI at the concentrations indicated in a total volume of 2.0 ml. The HOCI was either employed alone (●) or in conjunction with 0.1 M sodium chloride (□) or 50 μM H₂O₂ (●) as indicated.

FIG. 7 (center). Effect of sodium chloride concentration on diphenylfuran conversion by HOCI. The reaction mixture was as described in Fig. 6 except that the HOCI concentration was 20 μM and the sodium chloride concentration was varied as indicated.

FIG. 8 (right). Effect of pH on diphenylfuran conversion by HOCI. The reaction mixture was as described in Fig. 6 except that either 200 μM HOCI (○ - - - ○), 200 μM HOCI + 0.1 M sodium chloride (△ - - - △), or 20 μM HOCI + 0.1 M sodium chloride (□ - - - □) was added and the pH was varied as indicated using 0.05 M acetate buffer (open symbols) or 0.05 M phosphate buffer (closed symbols).

TABLE II

Effect of singlet oxygen quenchers on diphenylfuran conversion

The reaction mixture was as described in Fig. 2 for the myeloperoxidase/H₂O₂/chloride system and in Fig. 6 for the hypochlorous acid (200 μM) and the hypochlorous acid (20 μM) + chloride (0.1 M) systems except that 20% ethanol, 9 μM β-carotene in 20% ethanol, 25 μM bilirubin in 20% ethanol, 1 mM DABCO, and 0.1 mM histidine were added where indicated.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>cis-Dibenzoylethylene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPO + H₂O + Cl⁻</td>
</tr>
<tr>
<td></td>
<td>nmol</td>
</tr>
<tr>
<td>None</td>
<td>22.4 ± 0.9 (16)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>27.1 ± 4.0 (4)</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>2.5 ± 1.1 (3)</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>6.0 ± 3.0 (4)</td>
</tr>
<tr>
<td>DABCO</td>
<td>2.3 ± 0.4 (6)</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.2 ± 0.5 (3)</td>
</tr>
</tbody>
</table>

a Mean ± S.E. of (n) experiments.

b Not significant.
c p values for the difference from the system without inhibitor.

FIG. 9. Effect of D₂O on diphenylfuran conversion. The myeloperoxidase (MPO)/H₂O₂/chloride system (a) was as described in Fig. 2 and the HOCI systems (b and c) as described in Fig. 6 with 75 μM HOCI employed in b and 10 μM HOCI employed in c. The concentration of D₂O was varied as indicated under “Experimental Procedures.” The results are expressed as the mean percent of control activity (100% H₂O) ± S.E. in 3 to 11 experiments. The asterisk indicates a significant difference from control (p < 0.05).
Singlet Oxygen Formation by Myeloperoxidase

The reaction mixture was as described in Table II except that hypochlorous acid concentrations of 100 µM and 10 µM were employed in the absence and presence of chloride respectively, and sodium azide and sodium cyanide were added at the concentrations indicated.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Myeloperoxidase + H₂O₂ + Cl⁻</th>
<th>HOCI</th>
<th>HOCI + Cl⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azide</td>
<td>18.1 ± 1.4 (12)*</td>
<td>18.2 ± 1.3 (20)</td>
<td>9.0 ± 1.2 (13)</td>
</tr>
<tr>
<td>10⁻²</td>
<td>-0.8 ± 0.1 (3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10⁻³</td>
<td>-0.6 ± 0.2 (3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>-0.1 ± 0.2 (3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>9.8 ± 1.0 (3)</td>
<td>&lt;0.02</td>
<td>24.0 ± 5.7 (4)</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>90.9 ± 9.5 (3)</td>
<td>N.S.</td>
<td>23.3 ± 5.3 (4)</td>
</tr>
<tr>
<td>Cyanide</td>
<td>10⁻²</td>
<td>-0.1 ± 0.01 (3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10⁻³</td>
<td>-0.3 ± 0.05 (3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0.4 ± 0.02 (3)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>17.9 ± 2.8 (3)</td>
<td>N.S.</td>
<td>17.4 ± 3.6 (4)</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>21.0 ± 3.6 (3)</td>
<td>N.S.</td>
<td>17.5 ± 3.1 (4)</td>
</tr>
</tbody>
</table>

* Mean ± S.E. of (n) experiments.
* p value for the difference from the system without inhibitor.
* Not significant.

**TABLE III**

**Effect of azide and cyanide on diphenylfuran conversion**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Diphenylfuran conversion</th>
<th>Light emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azide</td>
<td>10⁻²</td>
<td>50%</td>
</tr>
<tr>
<td>10⁻³</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>50%</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

This study was undertaken to determine whether singlet oxygen is generated by a system consisting of myeloperoxidase, H₂O₂, and a halide and, if so, to gain some insight into the mechanism of its formation. Evidence in favor of singlet oxygen formation by the myeloperoxidase system is as follows.

1. Light is emitted by myeloperoxidase, H₂O₂, and a halide (32-34). The emission of light indicates the formation of an electronically excited state; however, without spectral analysis it does not indicate the nature of the excited species.

2. The myeloperoxidase system converts diphenylfuran to cis-DBE (Fig. 2). This reaction has been reported to be specific for singlet oxygen (14). Diphenylfuran conversion and light emission by the myeloperoxidase/H₂O₂/halide system have a number of properties in common. In both, bromide is the most effective halide, with activity also high with chloride. When iodide is used, both diphenylfuran conversion and light emission are low and limited to a narrow range of iodide concentrations (Fig. 4) (32). Both diphenylfuran conversion and light emission are optimal at pH 4.5 to 5.0 and decrease sharply as the pH is increased to neutrality (Fig. 5) (33).

3. The conversion of diphenylfuran to cis-DBE by the myeloperoxidase/H₂O₂/chloride system is inhibited by β-carotene, bilirubin, DABCO, and histidine (Table II). These agents are all singlet oxygen quenchers (26-29). Azide, which also inhibits diphenylfuran conversion by the peroxidase system (Table III), is both a quencher of singlet oxygen (30) and a potent inhibitor of myeloperoxidase.

4. The conversion of diphenylfuran to cis-DBE by the peroxidase system is stimulated by D₂O (Fig. 9). This stimulation increases with an increase in D₂O concentration to reach a maximum at 40 to 75% and then decreases as the D₂O concentration is increased further. The substitution of D₂O for H₂O prolongs the lifetime of a singlet oxygen 10-fold (Fig. 1) and generally stimulates O₂-dependent reactions (15, 16).

These findings, taken together, suggest that singlet oxygen is formed by the myeloperoxidase/H₂O₂/halide system, particularly with bromide and chloride as the halide component. A well described mechanism for the formation of singlet oxygen is the interaction of hypochlorite and H₂O₂ (10-12) as follows.

\[
\text{HOCI} + \text{H}_2\text{O}_2 + \text{Cl}^- \rightarrow \text{Cl}^- + \text{H}_2\text{O} + 1/2\text{O}_2
\]

It might be expected therefore that the HOCI formed by the myeloperoxidase system would react with excess H₂O₂ to yield singlet oxygen. This hypothesis was tested using diphenylfuran conversion as an indicator of singlet oxygen formation.

The myeloperoxidase system routinely employed in studies of diphenylfuran conversion contained in addition to myeloperoxidase, 50 µM H₂O₂ and 0.1 M NaCl in acetate buffer, pH 5.0. Under these conditions, approximately 50% of the diphenylfuran was converted to cis-DBE. The maximum HOCI concentration which could have been generated under these conditions was 50 µM, assuming complete utilization of H₂O₂ for HOCI formation (Equation 10). HOCI alone in acetate buffer pH 5.0 converted diphenylfuran to cis-DBE (Fig. 6); however, a concentration of approximately 200 µM was required for 50% conversion, four times that theoretically possible from the peroxidase system. The further addition of H₂O₂ at the concentration employed in the myeloperoxidase system (90 µM) did not increase diphenylfuran conversion by HOCI at pH 5.0; indeed, a small inhibition was observed. This lack of effect of H₂O₂ may be due in part to the pH employed since O₂ formation by the hypochlorite/H₂O₂ system is strongly pH-dependent with the rate of the reaction falling sharply as the pH is lowered below 8.5 (35). The conversion of diphenylfuran to cis-DBE by HOCI at pH 5.0, however, was strongly stimulated by 0.1 M chloride; under these conditions, 50% conversion occurred at a HOCI concentration of approximately 30 µM. This concentration of HOCI is 60% of that theoretically possible from the myeloperoxidase system; it corresponds well with the 40% efficiency of HOCI formation by the myeloperoxidase/H₂O₂/chloride system under the conditions employed by Harrison and Schultz (20).

As with the myeloperoxidase system, diphenylfuran conversion by HOCI in the presence or absence of chloride was...
inhibited by the singlet oxygen quenchers, DABCO, β-carotene, bilirubin, and histidine (Table II) and was enhanced by D₂O (Fig. 9) suggesting the involvement of singlet oxygen. Azide also was inhibitory (Table III), however, diphenylfuran conversion by the myeloperoxidase system was considerably more sensitive to inhibition by azide than was conversion by HOCI. This suggests that the effect of azide on the peroxidase system is due predominantly to an inhibition of myeloperoxidase rather than to its quenching effect on singlet oxygen. Cyanide inhibited diphenylfuran conversion by the myeloperoxidase system and by HOCI at equivalent concentrations. It is not known to quench singlet oxygen; however, cyanide reacts chemically with HOCI (31). Thus, the inhibitory effect of cyanide on the myeloperoxidase system may be due either to reaction with the product of the myeloperoxidase system, HOCI, or, like azide, to an inhibition of myeloperoxidase through reaction with the iron of the heme prosthetic group.

Hypochlorous acid decomposes at mildly acid pH to yield oxygen (31). Our data suggest that the oxygen evolved under these conditions is in the singlet state. The mechanism of O₂ formation in the presence of chloride appears to differ to some degree from that operative in its absence. With HOCI alone, diphenylfuran conversion fell sharply as the pH was increased above 4, whereas in the presence of chloride, conversion remained high in the pH range 4.0 to 7.0 and then fell sharply as the pH was further increased (Fig. 8). The pH₅₀ of HOCI is 7.5 (31) suggesting that O₂ formation in the presence of chloride requires HOCI in the undissociated form but is not otherwise strongly dependent on H⁺. The more striking pH dependence in the absence of chloride suggests a greater requirement for H⁺ under these conditions.

The effect of D₂O on diphenylfuran conversion by the myeloperoxidase, HOCI, and HOCI + chloride systems is complex. In all three systems, D₂O at relatively low concentrations stimulated diphenylfuran conversion. However, when the D₂O concentration was increased above 60% in the myeloperoxidase system and 30% in the HOCI system, diphenylfuran conversion decreased. In the HOCI + chloride system, diphenylfuran conversion continued to increase throughout the D₂O concentration range employed, although the degree of stimulation by 98% D₂O (173%) was considerably less than the corresponding prolongation of O₂ lifetime (>800%) (Fig. 1).

Diphenylfuran conversion by O₂ depends on the rate of formation of O₂, on its lifetime in the solvent, and on the rate constant for the reaction,

\[ \text{DPF} + \text{O}_2 \rightarrow \text{cis-DBE} \]  

Solvation deuterium is not expected to alter the reaction rate constant (15); however, the lifetime of singlet oxygen is prolonged by D₂O and thus presumably accounts for the increased diphenylfuran conversion by the myeloperoxidase and HOCI systems at relatively low D₂O concentrations and by the HOCI/chloride system over the entire D₂O concentration range. The decrease in diphenylfuran conversion by HOCI at high D₂O concentrations may be due to an inhibition of O₂ formation. D₂O has been reported to decrease the rate of the OC⁻/H₂O₂ reaction (Equation 9) (35) and may have a comparable effect on the decomposition of HOCI in the absence of H₂O. Two mechanisms may be involved. First, the deuterium atoms of D₂O can exchange with the hydrogens of HOCI to form DOCl. It has been proposed that the increased stability of D—O bonds results in slower decomposition rates and perhaps even different decomposition mechanisms (35) and this may apply to DOCl decomposition. Second, since H⁺ appears to be a requirement for HOCI decomposition in the absence of chloride (see above), the substitution of D⁺ for H⁺ may further inhibit the rate of decomposition. Both of these mechanisms could be operative in the absence of chloride, whereas only the former would be expected when chloride is present since the HOCI/chloride system is less dependent on H⁺.

In the myeloperoxidase/H₂O₂/chloride system, HOCI is formed in the presence of excess chloride. One would expect the properties of the myeloperoxidase system therefore to be similar to those of the HOCI/chloride system. However, diphenylfuran conversion by the myeloperoxidase system was considerably more sensitive to inhibition by azide than is the HOCI/chloride system with activity falling at pH levels above 4.5 (Fig. 5). In the myeloperoxidase system, O₂ formation is dependent on the rate of formation of HOCI by myeloperoxidase, as well as on the rate of its degradation, and D⁺ may influence HOCI formation by its participation in the reaction mechanism. Furthermore, deuterium may be incorporated into the myeloperoxidase molecule by exchange with protein hydrogens (36) and may have an effect on enzyme activity in this manner. In this regard, we have observed an inhibition of myeloperoxidase activity by D₂O as measured by the H₂O₂-dependent oxidation of ortho-dianisidine.

Some of the evidence suggesting O₂ formation by the myeloperoxidase- and HOCI-mediated systems taken individually may be subject to other interpretations. Thus, the specificity of the diphenylfuran conversion for O₂ may not be absolute; cis-DBE can be formed from diphenylfuran in reactions not known to be O₂-dependent, e.g. nitric acid oxidation (17), ozone addition (33), and halogen oxidation (37). Chlorine may be formed in the reaction between HOCI and Cl⁻ (31) and may thus contribute to diphenylfuran conversion. Further, as with most experiments employing inhibitors, it is not certain that inhibition occurs solely by the anticipated mechanism, i.e. O₂ quenching. However, all the evidence taken together strongly suggests that O₂ is formed by the myeloperoxidase/H₂O₂/halide system, particularly with chloride or bromide as the halide, and that its formation probably occurs via the formation and decomposition of the hypohalous acid.

The myeloperoxidase/H₂O₂/halide antimicrobial system is present in the phagocytic vacuole of PMNs and appears to contribute significantly to the microbicidal activity of these cells (1). That singlet oxygen-dependent reactions on the surface of the microorganism may be toxic is suggested by the damage to biological systems induced by certain dyes in the presence of light and oxygen (photodynamic action) which appears to be mediated by singlet oxygen (11). The inhibitory effect of the singlet oxygen quencher DABCO on the bactericidal activity of the myeloperoxidase system (38) and the inhibition of the cytotoxic effect of the myeloperoxidase system on mammalian tumor cells by DABCO and diphenylfuran (4) is compatible with the participation of singlet oxygen in the toxicity of this system. Although the formation of O₂ by intact PMNs has not yet been conclusively demonstrated, its involvement in the antimicrobial activity of these cells has been proposed, based on the emission of light by intact PMNs during phagocytosis (8) and on the resistance of organisms rich in carotenoid pigments to destruction by PMNs (9). The study reported here suggests that the myeloperoxidase system may serve as a source of O₂ in the intact PMN.

1 H. Rosen and S. J. Klebanoff, unpublished data.
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