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

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Myeloperoxidase, H$_2$O$_2$, and a halide form a potent antimicrobial system which is operative in the polymorphonuclear leukocyte (PMN). The formation of singlet oxygen (O$_2^+$) 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 O$_2^+$ quenchers p-carotene, bilirubin, histidine, and 1,4-diazabicyclo[2,2,2]octane (DABCO); and (c) the stimulation of conversion by D$_2$O, which prolongs the lifetime of O$_2$, in solution. Diphenylfuran conversion by the myeloperoxidase system has a pH optimum of 4.5 and an optimum H$_2$O$_2$ concentration of 30 $\mu$M and the halides vary in effectiveness in the order Br$^-$ > Cl$^-$ > I$^-$. Reagent H$_2$O$_2$ could be replaced by the H$_2$O$_2$-generating system, glucose + glucose oxidase.

Hypochlorous acid, which is formed by the myeloperoxidase/H$_2$O$_2$/chloride system, also converts diphenylfuran to cis-DBE. As with the myeloperoxidase system, this conversion has an acid pH optimum, is inhibited by O$_2^+$ quenchers, and is stimulated by D$_2$O. Diphenylfuran conversion by HOCl is increased by chloride, but not by H$_2$O$_2$, in the concentrations employed in the myeloperoxidase system. Our studies suggest that O$_2^+$ is formed by the myeloperoxidase/H$_2$O$_2$/chloride 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 O$_2^+$. 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, H$_2$O$_2$, 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 H$_2$O$_2$, 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/H$_2$O$_2$/halide system is complex (1). The halide cofactor is oxidized by myeloperoxidase and H$_2$O$_2$, 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, O$_2^+$) 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 D$_2$O for H$_2$O with a corresponding stimulation of O$_2^+$-mediated reactions (15). This property forms the basis of an additional test for O$_2^+$ participation in a chemical reaction (15, 16).

This paper deals with the generation of singlet oxygen by the myeloperoxidase/H$_2$O$_2$/halide system. We report the conversion of diphenylfuran to cis-DHE by the myeloperoxidase system, the stimulation of this conversion by D$_2$O, and its inhibition by several singlet oxygen quenchers. A mechanism for O$_2^+$ 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, Wisc., and cis-dibenzoylethylene was obtained from Aldrich Chemical Co., Milwaukee, Wisc.
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,0 Mixtures—Sodium acetate buffer (0.05 M) was prepared in D,0 (>98.5%, Stobler Isotope Chemical, Rutherford, N. J.) at a final concentration of 0.99% D,0 and a pH of 5.0, where pL represents the negative log of the summed hydrogen and deuterium ion concentrations. The pH was determined according to the relationship pL = pH,0 + 0.3314 + 0.0766 n, where pH,0 is the pH of the mixture as measured by a standard pH electrode and n is the mole fraction of D,0 in the mixture (23). Dilution of the D,0 buffer with aqueous 0.05 M sodium acetate buffer, pH 5.0, resulted in D,0/H,0 mixtures of constant pL (range 4.96 to 5.02). Where indicated, NaCl was added at a final concentration of 0.1 M.

Calculation of 'O, Lifetime—The approximate lifetimes (t) of singlet oxygen in mixtures of D,0 and H,0 were calculated from the expression

\[ \tau_{\text{D}=0/\text{H}=0} = \frac{1}{0.5 \times 0.45 n} \ \mu \text{sec} \]  

where n is the mole fraction of D,0 in a D,0/H,0 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 Merkle et al. (15, 16)

\[ \tau_{\text{D}=0/\text{H}=0} = 0.5x + 0.05y \]  

Assuming that changes in absorbances of D,0/H,0 mixtures are proportional to the concentration of D,0, then

\[ x = x_2 - n(x_2 - x_1) \]  

\[ y = y_2 - n(y_2 - y_1) \]  

where x is the OD1270 of water, x is the OD1270 of D,0, y is the OD1590 of D,0, and n is the mole fraction of D,0 in the D,0/H,0 mixture. Substituting in Equation 3,

\[ \tau_{\text{D}=0/\text{H}=0} = 0.5(x_2 - n(x_2 - x_1)) + 0.05(y_2 - n(y_2 - y_1)) \]  

or by rearrangement,

\[ \tau_{\text{D}=0/\text{H}=0} = (0.5x_2 - 0.05y_2) + n(0.50x_2 - 0.05y_2) \]  

Since

\[ \tau_{\text{D}=0/\text{H}=0} = \frac{1}{0.5x_2 - 0.05y_2} \]  

and

\[ \frac{1}{0.5x_2 - 0.05y_2} = 0.5x_2 + 0.05y_2 \]  

then

\[ \tau_{\text{D}=0/\text{H}=0} = \frac{1}{0.5x_2 - 0.05y_2} = \frac{1}{0.5x_2 - 0.05y_2} + \frac{1}{0.5x_2 - 0.05y_2} \]  

Assuming \( \tau_{\text{D}=0} = 9 \ \mu \text{sec} \) and \( \tau_{\text{H}=0} = 20 \ \mu \text{sec} \), then

\[ \tau_{\text{D}=0/\text{H}=0} = \frac{1}{0.5 \times 0.45 n} \ \mu \text{sec} \]  

The lifetime of O, in D,0/H,0 mixtures calculated from Equation 1 is shown in Fig. 1.

**Statistical Analyses**

Statistical differences were determined using Student’s two tailed t test for independent means (not significant, N.S., \( p \geq 0.05 \)).

**RESULTS**

Conversion of Diphenylfuran to cis-DBE by the Myeloperoxidase System—Fig. 2 demonstrates the conversion of [3H]diphenylfuran to cis-DBE by the myeloperoxidase/H,0/O, chloride system. The products were separated by thin layer
Singlet Oxygen Formation by Myeloperoxidase

FIG. 1. Calculated lifetime of singlet oxygen in D$_2$O/H$_2$O mixtures.

chromatography and the spots identified either by ultraviolet illumination (Fig. 2a) or radioautographically (Fig. 2b). When [H]$^3$H$diphenylfuran$ 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$^{-4}$ μM iodide, p < 0.01, n = 4; 10$^{-3}$ μM 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 is an effective enhancer of HOCl-dependent diphenylfuran conversion. Fig. 7 demonstrates the effect of chlorite concentration on the conversion of diphenylfuran to cis-DBE by 20 μM HOCl. Under the conditions employed, activity was maximal at 0.1 mM chlorite. Either sodium or potassium chloride could be employed; however, 0.1 mM 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 concentrations. 200 μM where conversion was essentially complete between pH 4.0 and 7.0 and 20 μM where only partial conversion was observed.

Effect of Singlet Oxygen Quenchers—The conversion of diphenylfuran to cis-DBE, but not to trans-DBE, by the myeloperoxidase system or by HOCl in the presence or absence of chloride is compatible with a singlet oxygen-mediated mechanism. 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-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, 9 μM 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 myeloperoxidase/HOCl/chloride system at a concentration (10−3 mM) 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. Cyanide however reacts with HOCl to yield OCN− and the latter also reacts with HOCl to yield CO3−2, N2, and NO3− (31). Under the conditions employed in Table III, cyanide inhibited diphenylfuran conversion by the myeloperoxidase and HOCl systems at approximately the same concentrations.

Effect of D2O—The substitution of D2O for H2O increases the lifetime of Δ singlet oxygen 10-fold (Fig. 1) with a corresponding stimulation of singlet oxygen-dependent reactions (15, 16). Fig. 9 demonstrates the effect of D2O concentration on the conversion of diphenylfuran to cis-DBE by the myeloperoxidase/H2O2/chloride system, by HOCl and by HOCl and chloride. Myeloperoxidase mediated conversion increased with an increase in D2O concentration to reach a maximum of 150 ± 9% (n = 4, p < 0.02) at a D2O concentration of 40%. Conversion remained constant as the D2O concentration was increased from 40 to 75% and then fell to control levels as the D2O concentration was further increased. HOCl-mediated conversion was increased by D2O to a maximum of 133 ± 7% (n = 8, p < 0.002) at a D2O concentration of 30%. A further increase in D2O produced a fall in diphenylfuran conversion to 76 ± 4% (n = 11, p < 0.001) of control levels at the highest D2O concentration employed (>98%). In contrast, diphenylfuran conversion by HOCl and chloride increased progressively with D2O concentration to a maximum of 173 ± 28% (n = 6, p < 0.05) at >98% D2O.

**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 nmol of unlabeled diphenylfuran were employed, H2O2 was replaced by 0.1 mM glucose and 12.5 milliunits/ml of glucose oxidase, and the incubation period was 90 min. The cis-DBE formed was measured spectrophotometrically (Method A).

<table>
<thead>
<tr>
<th>Supplements</th>
<th>cis-Dibenzylylethylene nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloperoxidase + glucose + glucose oxidase + chloride</td>
<td>37.2 ± 3.5 (4)n</td>
</tr>
<tr>
<td>Myeloperoxidase omitted</td>
<td>3.0 ± 2.0 (2) &lt;0.01</td>
</tr>
<tr>
<td>Glucose omitted</td>
<td>2.5 ± 0.5 (2) &lt;0.01</td>
</tr>
<tr>
<td>Glucose oxidase omitted</td>
<td>2.0 ± 0.0 (2) &lt;0.01</td>
</tr>
<tr>
<td>Chloride omitted</td>
<td>3.5 ± 1.5 (2) &lt;0.01</td>
</tr>
<tr>
<td>Catalase (850 units) added</td>
<td>1.5 ± 0.5 (2) &lt;0.01</td>
</tr>
<tr>
<td>Heated catalase (100°, 15 min) added</td>
<td>26.0 ± 2.0 (2) N.S.</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 mixture 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 described 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 (H)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 200 μM and the sodium chloride concentration was varied as indicated.

Table II

Effect of singlet oxygen quenchers on diphenylfuran conversion

<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>

* Mean ± S.E. of (n) experiments.

† Not significant.

‡ p values for the difference from the system without inhibitor.

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 200 μM HOCl + 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).

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

Effect of azide and cyanide on diphenylfuran conversion

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>cis-Dibenzoylethylene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HOCI</td>
<td>HOCI + Cl⁻</td>
</tr>
<tr>
<td></td>
<td>nmol</td>
<td>nmol</td>
</tr>
<tr>
<td>Azide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻²</td>
<td>18.1 ± 1.4 (12)</td>
<td>18.2 ± 1.3 (20)</td>
</tr>
<tr>
<td></td>
<td>&lt;0.001*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>10⁻³</td>
<td>-0.1 ± 0.2 (3)</td>
<td>2.2 ± 0.4 (3)</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>-0.1 ± 0.2 (3)</td>
<td>7.2 ± 1.2 (3)</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>-2.0 ± 1.0 (3)</td>
<td>9.6 ± 2.4 (3)</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>9.8 ± 9.5 (3)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>23.3 ± 5.3 (4)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>9.7 ± 2.6 (3)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Cyanide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻²</td>
<td>-0.1 ± 0.01 (3)</td>
<td>0.1 ± 0.2 (3)</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>-0.3 ± 0.05 (3)</td>
<td>0.0 ± 0.2 (3)</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>0.4 ± 0.2 (3)</td>
<td>0.1 ± 0.2 (3)</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>17.9 ± 2.8 (3)</td>
<td>4.3 ± 2.3 (3)</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>21.0 ± 3.6 (3)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>17.5 ± 3.1 (4)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>9.9 ± 3.2 (3)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

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

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, iodide 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/halide 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.

\[ \text{OCl}^- + \text{H}_2\text{O}_2 \rightarrow \text{Cl}^- + \text{H}_2\text{O} + \text{O}_2 \]  (9)

Hypochlorous acid is a product of the myeloperoxidase/H₂O₂/chloride system, particularly at acid pH (20).

\[ \text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{HOCI} \]  (10)

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 (50 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 is 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 HOCl. 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 HOCl at equivalent concentrations. It is not known to quench singlet oxygen; however, cyanide reacts chemically with HOCl (31). Thus, the inhibitory effect of cyanide on the myeloperoxidase system may be due either to reaction with the product of the myeloperoxidase system, HOCl 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 singlet 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 HOCl 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 pKₐ of HOCl is 7.5 (31) suggesting that O₂ formation in the presence of chloride requires HOCl 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, HOCl, and HOCl + 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 HOCl system, diphenylfuran conversion decreased. In the HOCl + 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,

$$\text{DPF} + O_2 \rightarrow \text{cis-DBE}$$  \hspace{1cm} (11)

Solvent deuteration is not expected to alter the reaction rate constant (15); however, the lifetime of singlet oxygen is prolonged by D₂O and this presumably accounts for the increased diphenylfuran conversion by the myeloperoxidase and HOCl systems at relatively low D₂O concentrations and by the HOCl/chloride system over the entire D₂O concentration range. The decrease in diphenylfuran conversion by HOCl at high D₂O concentrations may be due to an inhibition of O₂ formation. D₂O has been reported to decrease the rate of the OCl⁻/H₂O₂ reaction (Equation 9) (35) and may have a comparable effect on the decomposition of HOCl in the absence of H₂O₂. Two mechanisms may be involved. First, the deuterium atoms of D₂O can exchange with the hydrogens of HOCl to form DOCI. 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 DOCI decomposition. Second, since H⁺ appears to be a requirement for HOCl decomposition in the absence of chloride (see above), the substitution of D⁺ for H⁺ may 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 HOCl/chloride system is less dependent on H⁺.

In the myeloperoxidase/HOCl/halide system, HOCl 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 HOCl/chloride system. However, diphenylfuran conversion by the myeloperoxidase system falls at high D₂O concentration in contrast to conversion by the HOCl/chloride system. Further, diphenylfuran conversion by the myeloperoxidase system is more sensitive to pH than is the HOCl/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 HOCl by myeloperoxidase, as well as on the rate of its degradation, and D⁺ may influence HOCl 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 HO₂⁻-dependent oxidation of ortho-dianisidine.

Some of the evidence suggesting O₂ formation by the myeloperoxidase- and HOCl-mediated systems taken individually may be subject to other interpretations. Thus, the specificity of 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 HOCl 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 suggests strongly 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 microbiocidal 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.

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