Low-Level Chemiluminescence of Hydroperoxide-Supplemented Cytochrome c

Enrique CADENAS, Alberto BOVERIS and Britton CHANCE
Johnson Research Foundation, School of Medicine, G4, University of Pennsylvania, Philadelphia, PA19104, U.S.A.

(Received 10 July 1979)

Ferricytochrome c showed low-level chemiluminescence, with a light-emission measured of about $1 \times 10^3 - 3 \times 10^3$ counts/s, when supplemented with organic hydroperoxides. Tertiary hydroperoxides (cumene hydroperoxide and t-butyl hydroperoxide) showed a saturation behaviour at about 5 mm-hydroperoxide, whereas primary hydroperoxides showed a quadratic dependence on the hydroperoxide concentration. Chemiluminescence depended linearly on cytochrome c concentration, and optimal light-emission was observed at [t-butyl hydroperoxide]/[ferricytochrome c] ratios of 160–500. Hydroperoxide-supplemented ferricytochrome c consumed O$_2$ at a rate of $1.0 \mu$mol/min per $\mu$mol of cytochrome c; the rate of O$_2$ uptake was linearly related to the concentration of cytochrome c. The Soret absorption band of ferricytochrome c decreased about 64% after incubation with t-butyl hydroperoxide, whereas the 530 nm band was almost totally abolished. Light-emission was (a) inhibited competitively by cyanide. (b) inhibited by singlet-oxygen quenchers (e.g. $\beta$-carotene), scavengers (e.g. dimethylfuran) and traps (e.g. histidine and tryptophan) and (c) increased by singlet-oxygen-chemiluminescence enhancer 1,4-diazabicyclo[2.2.2]octane. Superoxide dismutase had no effect on the present system. The participation of free radicals is suggested by the effect of the radical trap 2,5-di-t-butylquinol. Singlet-oxygen dimol emission seems to be mainly responsible for the observed light-emission; a mechanism that can account for the major part of the present experimental observations is proposed.

The ability of cytochrome c to induce lipid peroxidation as well as its involvement in hydroperoxide breakdown, probably through a unimolecular bond scission, to yield alkoxy and hydroxyl radicals has been described (Tappel, 1955; Desai & Tappel, 1963). Haematin and some haemoproteins, among them cytochrome c, were found to induce lipid peroxidation, the rate of which was further increased by hydroperoxides (Kashnitz & Hatfei, 1975).

The participation of haemoproteins in lipid autoxidation and lipid hydroperoxide breakdown is accompanied by damage to the haemoproteins; the chemical mechanism of damage is not exactly known yet, though cross-linking of proteins either by lipid peroxy radical addition or by a process not involving incorporation of lipid radicals has been proposed (Roubal & Tappel, 1966a,b). O’Brien (1968a,b) described the effect of hydrogen peroxide on ferricytochrome c as affecting amino acid composition and producing a decrease in the absorption bands; these spectroscopic changes had been early reported by Banks et al. (1961) for the reaction of cytochrome c with methyl linoleate hydroperoxide. Peroxidized lipids damage cytochrome c, as indicated by its decreased solubility (Desai & Tappel, 1963). Tomoda et al. (1978) showed the formation of haemichrome from haemoglobin subunits by hydrogen peroxide as a decrease in the absorption bands without the intermediate formation of methaemoglobin.

Hawco et al. (1977) showed that chemiluminescence accompanied the decomposition of linoleic acid hydroperoxide by haematin or methaemoglobin; singlet molecular oxygen was suggested as responsible for light-emission.

We have found that cytochrome c, as well as other haemoproteins, when supplemented with organic hydroperoxides, show low-level chemiluminescence; the results point out that free radicals deriving from the hydroperoxide decomposition and singlet molecular oxygen are involved in the process.
Materials and Methods

Chemicals

β-Carotene, cytochrome c (type VI), d-histidine and L-tryptophan were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). t-Butyl hydroperoxide, 2,5-di-t-butylquinol and 1,4-diazabicyclo[2.2.2]octane were obtained from Aldrich Co. (Milwaukee, WI, U.S.A.). Cumene hydroperoxide (αα-dimethylbenzoyl hydroperoxide) and ethyl hydroperoxide were obtained from Matheson, Coleman and Bell (Norwood, OH, U.S.A.) and Ferrosan (Malmo, Sweden) respectively. Other reagents used were of analytical grade. Native cytochrome c was used without further purification and its concentration was determined by using $\varepsilon_{550} = 27.7 \text{ litre mmol}^{-1} \cdot \text{cm}^{-1}$ after reduction with sodium dithionite (Margoliash, 1954). β-Carotene was dissolved in methanol and its concentration was measured by its absorption at 450 nm ($\varepsilon = 13.8 \text{ litre mmol}^{-1} \cdot \text{cm}^{-1}$) (Auclair & Lecomte, 1978).

Photon counting

Chemiluminescence emission was measured in a photon counter essentially as described by Boveris et al. (1978) in a 35 mm × 25 mm × 5 mm (12.25 cm² surface) cuvette. An RCA 8850 photomultiplier responsive in the range 300–650 nm with an applied potential of 1.8 kV was fed into a Princeton Applied Research (Princeton, NJ, U.S.A.) model 1121 amplifier–discriminator adjusted for single photons.

The output was connected to both a Heathkit IB-1100 frequency counter and a recorder. The photomultiplier was sealed and surrounded by solid CO₂, with a tube temperature of approx. −30°C.

Oxygen uptake

$\text{O}_2$ consumption was measured with a Clark-type oxygen electrode in a cylindrical glass chamber (2 ml final volume) with constant stirring.

Cytochrome c absorption spectra

Cytochrome c spectra were monitored in a Perkin–Elmer–Coleman model 124 spectrophotometer. The effect of t-butyl hydroperoxide on cytochrome c was also monitored by the decrease of the Soret band of ferricytochrome c ($\varepsilon_{408} = 109.5 \text{ litre mmol}^{-1} \cdot \text{cm}^{-1}$); when cyanide was present in the assay mixture, readings were monitored at 412 nm ($\varepsilon = 104 \text{ litre mmol}^{-1} \cdot \text{cm}^{-1}$) (Butt & Keilin, 1962).

Incubation conditions

All the assays were carried out in 0.1 M-potassium phosphate buffer, pH 7.0, at 37°C.

Results

Chemiluminescence of hydroperoxide-supplemented cytochrome c

Addition of hydroperoxide to ferricytochrome c in air-saturated buffer shows low-level chemilumines-

![Fig. 1. Chemiluminescence of hydroperoxide-supplemented cytochrome c](image-url)

(a) A mixture of cytochrome c (20 μM) in phosphate buffer was added to 5 mM-hydroperoxide to start the chemiluminescence reaction. (b) Effect of hydroperoxide concentration on chemiluminescence of cytochrome c (20 μM). Assay conditions were as described in the Materials and Methods section. Abbreviations used: $c^{3+}$, ferricytochrome c; Cu-OOH, cumene hydroperoxide; Et-OOH, ethyl hydroperoxide; Bu′OOH, t-butyl hydroperoxide.

1980
CHEMILUMINESCENCE OF CYTOCHROME c

CHEMILUMINESCENCE (Fig. 1a). Light-emission started immediately after addition of 5 mm-hydroperoxide and reached its maximal intensity in either 2-3 min (cumene hydroperoxide) or 12-13 min (t-butyl hydroperoxide and ethyl hydroperoxide); thereafter the signal was stable for about 5-8 min (with t-butyl hydroperoxide and ethyl hydroperoxide). The time necessary to obtain the maximal light-emission increased when lower concentrations of hydroperoxide were utilized; a lag phase was also present at low hydroperoxide concentration (0.1-0.5 mM).

Fig. 1(b) shows the dependence of the light-emission of cytochrome c on hydroperoxide concentration. Maximal chemiluminescence was taken into account without considering the time needed for light-emission to achieve complexion. Cumene hydroperoxide and t-butyl hydroperoxide show a saturation behaviour, reaching half-maximal light-emission at a concentration of about 0.5-1.0 mM, whereas ethyl hydroperoxide-induced chemiluminescence was linearly related to the square of the ethyl hydroperoxide concentration. The mechanism of the reactions to which primary hydroperoxides are subjected was considered beyond the scope of the present paper. The study on hydroperoxide-induced chemiluminescence of cytochrome c was carried out thereafter with t-butyl hydroperoxide because of the advantage of its higher solubility over cumene hydroperoxide.

When t-butyl hydroperoxide concentration was kept high (5 mM), light-emission was a linear function of the concentration of cytochrome c (Fig. 2a), with a slope of $3.3 \times 10^4$ counts/s per pmol of cytochrome c. Chemiluminescence in the t-butyl hydroperoxide-supplemented cytochrome c system depended on the ratio [t-butyl hydroperoxide]/[ferricytochrome c]. Fig. 2(b) shows how chemiluminescence was affected by the [t-butyl hydroperoxide]/[ferricytochrome c] ratio. Banks et al. (1961) have already reported that high concentration of cytochrome c had an anti-oxygenic effect in comparison with its behaviour at low concentrations. Therefore, to obtain a linear light-emission depending on cytochrome c concentration, [t-butyl hydroperoxide]/[ferricytochrome c] ratios equal to or higher than 160 were necessary.

The addition of t-butyl hydroperoxide to cytochrome c solutions induced an O$_2$ consumption that depended linearly on cytochrome c concentration (Fig. 3). O$_2$ uptake was observed at a rate of 1.0 pmol of O$_2$/min per pmol of cytochrome c. When chemiluminescence of cytochrome c and O$_2$ uptake were assayed in parallel and the measurements taken every minute were plotted, it was found that total O$_2$ consumed in the reaction was related linearly to chemiluminescence (Fig. 3).

The results shown in Fig. 3 point to the following conclusions: (a) hydroperoxide-induced chemiluminescence of cytochrome c is an O$_2$-dependent process; (b) light-emission is proportional to the amount of O$_2$ consumed; (c) the ratio of the slopes in Fig. 2(a) ($3.3 \times 10^7$ photons/s per pmol of cytochrome c, after allowance for a photon-counting efficiency of 0.1%) and Fig. 3 ($1.67 \times 10^{-2}$ pmol of O$_2$/s per pmol of cytochrome c gives a value of about $3 \times 10^{-9}$ photons/O$_2$ molecule; this number

---

**Fig. 2. Dependence of chemiluminescence on (a) cytochrome c concentration and (b) the [t-butyl hydroperoxide]/[ferricytochrome c] ratio**

(a) Assay was carried out in the presence of 5 mm-t-butyl hydroperoxide. Different ratios shown in (b) were obtained by varying the hydroperoxide concentration and keeping ferricytochrome c concentration constant at 30 µM. Experimental details are given in the text.
implies that the photoemissive species $[^1\text{O}_2-\text{O}_2]$ is produced in a minor side reaction.

Hydroperoxide-induced spectroscopic changes of ferricytochrome c

Hydroperoxide-supplemented cytochrome c undergoes changes that can be easily monitored through the absorption spectra. The extent of these effects as well as light-emission depended on temperature, time of incubation and the [t-butyl hydroperoxide]/[ferricytochrome c] molar ratio.

The Soret absorption band of ferricytochrome c decreased about 64% after incubation with t-butyl hydroperoxide ([t-butyl hydroperoxide]/[ferricytochrome c] molar ratio 500) during 10 min at 37°C (Fig. 4a), whereas the 530 nm band was almost totally abolished, perhaps suggesting a new spectral species. The reducibility of t-butyl hydroperoxide-treated cytochrome c was restricted to the ferricytochrome c molecules that were not affected by the treatment with hydroperoxide (Fig. 4b).

Fig. 5 shows the effect of t-butyl hydroperoxide on the Soret absorption band (408 nm) of ferricytochrome c as a function of the time of incu-
bation; parallel traces of chemiluminescence by the same system can be seen; a new addition of cytochrome c increases the 408 nm absorption band, and with its further decrease a higher chemiluminescence value can be observed. Neither chemiluminescence nor alterations in the absorption bands could be detected when the [t-butyl hydroperoxide]/[ferricytochrome c] ratio was equal to 1.

O₂ consumption paralleled cytochrome c decrease of the absorption bands: both exhibited apparent first-order decays, of 0.08 min⁻¹ and 0.14 min⁻¹ respectively. The ratio of initial rates (d[O₂]/dt = 10 μM/min and -d[ferricytochrome c]/dt = 1.4 μM/min) indicated that about 7 oxygen molecules were consumed per cytochrome c molecule.

Effect of cyanide on chemiluminescence of cytochrome c

Light-emission was decreased in about 90% in the presence of 0.25 mM-cyanide (Fig. 6). The half-maximal effect was obtained with 0.05 mM-cyanide. Incubation of cytochrome c with cyanide was not needed, and the effect of the inhibitor was always manifested immediately it was when present in the assay mixture either at the beginning or at the midst of the reaction. Double-reciprocal plots showed that the cyanide inhibition of chemiluminescence was competitive with t-butyl hydroperoxide.

The inhibitory effect of cyanide on chemiluminescence of hydroperoxide-supplemented cytochrome c should somehow have a preventing effect on the decrease of the absorption bands of cytochrome c. At a concentration of 0.25 mM-cyanide, chemiluminescence was found to be almost completely inhibited (see Fig. 6), but the decrease of the absorption bands of cytochrome c was only partially prevented (30–40% inhibition) (Fig. 7). The black circles in Fig. 7 shows that light-emission in the absence of cyanide progresses as absorption of cytochrome c decreases (the abscissa indicates remaining cytochrome c; see Fig. 5); in the presence of 0.25 mM-cyanide, a considerable change in the Soret absorption band or ferricytochrome c (up to 50–60%) could still be observed; however, chemi-

---

**Fig. 5. Relationship between hydroperoxide-induced chemiluminescence and spectroscopic changes of cytochrome c**

Cytochrome c (10 μM) was incubated with 5 mM-t-butyl hydroperoxide at 37°C. Spectroscopic changes were followed by the decrease of the Soret band (408 nm). When chemiluminescence and spectroscopic changes reached a maximum, a new addition of 10 μM-cytochrome c was made. Abbreviations used: c³⁺, ferricytochrome c; Bu¹OOH, t-butyl hydroperoxide.

---

**Fig. 6. Inhibitory effect of cyanide on hydroperoxide-induced chemiluminescence of cytochrome c**

Cytochrome c (15 μM) was incubation with 5 mM-t-butyl hydroperoxide in the presence of various concentrations of NaCN. The inset shows the reciprocal plot of chemiluminescence of cytochrome c versus t-butyl hydroperoxide concentration obtained in the presence (O) and absence (●) of 0.25 mM-NaCN.
luminescence in these conditions was only 10% or less the value in the absence of the inhibitor.

**Effect of various agents on chemiluminescence**

*Quenchers.* β-Carotene is known as a physical quencher of singlet molecular oxygen (the carotenoid pigments physically quench singlet oxygen without chemically altering the pigments themselves) (Foote & Denny, 1968), and in the present system behaved like a potent inhibitor of light-emission (85% maximal inhibition) with a half-maximal effect at a concentration of 0.19 mM (Fig. 8a). When the ratio of light-emission in the absence of the quencher to light-emission in its presence ($I_0/I$) was plotted against the quencher concentration, a straight line of slope $k\tau_0$ (Stern–Volmer quenching constant) was obtained (Fig. 8b) according to the Stern–Volmer equation:

$$
\frac{I_0}{I} = 1 + k\tau_0 [Q]
$$

[τ₀, the life-time of the excited species, for singlet oxygen in water, was considered to be $2.0 \times 10^{-8}$ s (Merkel & Kearns, 1973); [Q] is the molar concentration of the quencher]. The slope ($k\tau_0$) of Fig.

---

**Fig. 7. Relationship between cytochrome c breakdown and chemiluminescence**

Cytochrome c (10 μM) was incubated with 5 mM-t-butyl hydroperoxide at 37°C in the absence (●) and presence (○) of 0.25 mM-NaCN. Abscissa indicates the remaining ferricytochrome c during the incubation as measured by its absorption at 408 nm.

**Fig. 8. Effect of β-carotene on chemiluminescence of cytochrome c**

(a) The reaction mixture contained 25 μM-cytochrome c and 5 mM-t-butyl hydroperoxide. Various concentrations of β-carotene were added and maximal chemiluminescence yields, in the presence or absence (control) of β-carotene, were considered for each point of the curve. (b) Stern–Volmer plot. Details are given in the text.
8(b) has the value of $4.9 \times 10^3 \text{M}^{-1}$ and the quenching rate constant $k$ of $2.5 \times 10^9 \text{M}^{-1}\cdot\text{s}^{-1}$. Sugio & Nakano (1976) reported a $k$ value of $4.0 \times 10^9 \text{M}^{-1}\cdot\text{s}^{-1}$ for the quenching effects of $\beta$-carotene on the chemiluminescence of isolated microsomal fraction. We have found a $k$ value of $3.7 \times 10^9 \text{M}^{-1}\cdot\text{s}^{-1}$ for the quenching of t-butyl hydroperoxide-induced chemiluminescence of submitochondrial particles (Cadenas et al., 1980).

On the other hand, 1,4-diazabicyclo[2.2.2]-octane, which enhances singlet-oxygen dimol emission in aqueous media (Denek & Krinsky, 1977), increased twofold the initial value of chemiluminescence of the hydroperoxide-supplemented system under a wide range of cytochrome $c$ concentration (Fig. 9). The enhancement of light-emission by 1,4-diazabicyclo[2.2.2]-octane and other cyclic diamines was explained in terms of a charge-transfer mechanism of singlet-oxygen quenching by the diamines (Ouannes & Wilson, 1968).

---

Table 1. Effect of some agents on chemiluminescence of hydroperoxide-supplemented cytochrome $c$

<table>
<thead>
<tr>
<th>Addition</th>
<th>Chemiluminescence (counts/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1300</td>
</tr>
<tr>
<td>+10 mM-dimethylfuran</td>
<td>270</td>
</tr>
<tr>
<td>+0.1 mM-mannitol</td>
<td>1250</td>
</tr>
<tr>
<td>+30 $\mu$g of superoxide dismutase/ml</td>
<td>1300</td>
</tr>
<tr>
<td>+20 mM-D-histidine</td>
<td>670</td>
</tr>
<tr>
<td>+20 mM-L-tryptophan</td>
<td>750</td>
</tr>
</tbody>
</table>

The reaction mixture contained 10 $\mu$M-ferricytochrome $c$ and 5 mM t-butyl hydroperoxide in 0.1M potassium phosphate buffer, pH 7.0, at $37^\circ$C. Values correspond to the maximal chemiluminescence yields obtained under each condition.

---

Fig. 9. Enhancement of cytochrome $c$ chemiluminescence by 1,4-diazabicyclo[2.2.2]-octane

Different concentrations of cytochrome $c$ and 5 mM t-butyl hydroperoxide were assayed for chemiluminescence in the absence (O) and presence (●) of 10 mM 1,4-diazabicyclo[2.2.2]-octane. Points in the curve represent the maximal chemiluminescence yields obtained under the present assay conditions.

---

Fig. 10. Effect of 2,5-di-t-butylquinol on chemiluminescence of hydroperoxide-supplemented cytochrome $c$

Cytochrome $c$ (20 $\mu$M) was incubated with 5 mM t-butyl hydroperoxide in the presence and absence of 0.15 mM 2,5-di-t-butylquinol. Abbreviations used: $c^3+$, ferricytochrome $c$; Bu'OOH, t-butyl hydroperoxide; DTBHQ, 2,5-di-t-butylquinol.
Scavengers and traps. Table 1 lists the effect of some singlet-oxygen scavengers and traps on the hydroperoxide-induced chemiluminescence of ferri-cytochrome c. Dimethyluran acts as a singlet-oxygen scavenger (Foote et al., 1968), and inhibited the intensity of light-emission of cytochrome c by about 80% with a half-maximal effect at a concentration of 3.6 mM-dimethyluran. The hydroxyl-radical scavenger, mannitol (Halliwell, 1978), at a concentration of 0.1 mM was found to be without effect on the chemiluminescence of cytochrome c supplemented with hydroperoxide. Superoxide dismutase, which scavenges superoxide anion (McCord & Fridovich, 1969; Fridovich, 1975), had no effect on chemiluminescence of the present reaction when used at a concentration as high as 30 μg/ml.

Histidine and tryptophan have been described as singlet-oxygen traps partially accounting for the inhibitory effect of certain proteins on singlet-oxygen formation (Wilson et al., 1972; Matheson et al., 1975); both amino acids were used at a concentration as high as 20 mM, giving a moderate inhibition of the light-emission of hydroperoxide-supplemented cytochrome c (50% inhibition under those conditions). 2,5-Di-t-butylquinol is a potent free-radical trap that produced a lag phase in the chemiluminescence of cytochrome c, without affecting the slope and the maximal, chemiluminescence yield. At a concentration of 0.15 mM-2,5-di-t-butylquinol there was a lag phase of 7 min (Fig. 10) and at 0.5 mM a lag phase of 40 min (results not shown). The lag phase was augmented exponentially with increasing concentrations of 2,5-di-t-butylquinol. This effect indicates the involvement of free radicals in the decomposition of t-butyl hydroperoxide by cytochrome c.

Discussion

The requirements for chemiluminescence were oxidized cytochrome c, organic hydroperoxide and O2. The light-emission that arises from the reaction between a hydroperoxide and cytochrome c could be understood in terms of a process that includes (a) a cytochrome c-catalysed homolytic scission of the hydroperoxide, (b) a free-radical oxidation process and (c) the generation of chemiluminescent singlet oxygen, according to the following chemical reactions:

\[ \text{ROO}^+ + \text{ROO}^+ \rightarrow O_2 + \text{RO} + \text{ROH} \]  
\[ \text{Bu'OO}^+ + \text{Bu'OO}^+ \rightarrow O_2 + \text{Bu'OOBu'} \]  
\[ O_2 \rightarrow O_2 \]  
\[ O_2 + O_2 \rightarrow 2O_2 + h\nu \]

Reaction (1) is the cytochrome c-catalysed homolytic rupture of the oxygen–oxygen bond of the hydroperoxide molecule (Tappel, 1955; Desai & Tappel, 1963). The catalytic action of cytochrome c seems to involve the binding of the hydroperoxide to the sixth ligand position of the iron (Tsou, 1952; George & Tsou, 1952). This view is experimentally supported by the competitive inhibition exerted by cyanide on the process (Fig. 6). Reaction (1) seems to be applicable to primary (Fig. 1b; Misra & Fridovich, 1973), secondary (Tappel, 1955; Desai & Tappel, 1963; Banks et al., 1961) and tertiary (Fig. 1b; cumene and t-butyl hydroperoxides. Iron-bound hydroxyl radical may react with either t-butyl hydroperoxide [reaction (2)] or RH [reaction (3)] (RH meaning the primary carbons of t-butyl hydroperoxide molecule or any residue of the cytochrome c molecule), abstracting an hydrogen atom (Pryor, 1978) and yielding either t-butyl peroxy or alkyl radicals respectively. Alkyl radicals attached to the cytochrome c molecule are more able to undergo peroxydation reactions with subsequent cross-linking of cytochrome c molecules (Roubal & Tappel, 1966a,b) than to generate light-emission (Fig. 7).

Peroxidized cytochrome c would account for the 10% of cyanide-insensitive chemiluminescence (Fig. 6) as well as the cyanide-insensitive cytochrome c peroxidation (Fig. 7). Peroxidation of the cytochrome c molecule may not be regarded itself as the main source of light-emission. Iron-bound hydroxyl radical could explain the lack of effect of the hydroxyl-radical trap, mannitol (Halliwell, 1978), on light-emission. On the other hand, t-butyl alcohol, generated in reactions (6) and (7), could compete effectively with mannitol in scavenging hydroxyl radicals.

Incorporation of one molecule of oxygen to the alkyl radical [reaction (4)] is quantitatively important since the present system showed an O2 uptake of about 1 μmol/min per μmol of cytochrome c. The t-butylperoxy radical formed can react with available RH group, yielding again alkyl radicals [reaction (5)]. Reactions (4) and (5) are the propagation reactions of the autoxidation chain (Pryor, 1978). Reactions (8) and (9) are radical termination reactions that can be interpreted on the basis of a Russell's (1957) mechanism for the self-reaction of primary or secondary peroxy radicals and on the basis of the reaction proposed by Pryor (1978) respectively. The quadratic dependence of chemiluminescence on ethyl hydroperoxide
concentration (Fig. 1b) can also be taken as an experimental evidence for the occurrence of reaction (8) according to the proposition of Sugioka & Nakano (1976) for the hydroperoxide-induced chemiluminescence of a simplified microsomal system.

Experimentally support for reactions (10) and (11), i.e. that the observed photoemission is mainly due to the dimol emission of singlet oxygen (Khan & Kashka, 1963; Seliger, 1964; Kearns, 1971), is given by the effects of \( \beta \)-carotene and 1,4-diazabicyclo[2.2.2]octane (Figs. 8 and 9). Dimol emission of singlet oxygen apparently occurs at a rate of about \( 1 \times 10^6 \)–\( 2 \times 10^6 \) photons/s under the present conditions, which could correspond to the formation of one \([1^1O_2]^-\) pair per about \( 10^7 \) molecules of singlet oxygen generated and per about \( 10^9 \) oxygen molecules utilized in the radical chain (reaction (5)). The steady-state concentration of singlet oxygen would be about 10\(^{-11}\)M.

Participation of free-radical species on the chemiluminescence of hydroperoxide-supplemented cytochrome \( c \) seems supported by the inhibitory effect of the radical trap 2,5-di-t-butylquinoxal, which was also an effective inhibitor on the chemiluminescence of isolated microsomal fraction (Sugioka & Nakano, 1976).

It is unlikely that superoxide anion is involved in the chemiluminescence of cytochrome \( c \), as can be deduced by the lack of effect of superoxide dismutase. Moreover, the absence of superoxide anion generated discards the possibility of a Haber–Weiss-type reaction with the hydroperoxide as is possible with hydrogen peroxide (McCord & Day, 1978; Halliwell, 1978). The present system, in which no reductant such as NADH is included, unlike the one described by Misra & Fridovich (1973), does not involve a superoxide dismutase-inhibitable reduction of cytochrome \( c \), but there is a decrease in the absorption bands, which might be indicative of peroxidation of cytochrome \( c \).

The fact that cytochrome \( c \) (cyt. \( c \)) showed chemiluminescence when supplemented with t-butyl hydroperoxide was also extensible to other haemoproteins such as myoglobin (Mb), methaemoglobin (MetHb) and horseradish peroxidase (HRP). When the intensity of light-emission of the haemoproteins was compared on haem basis, the following order was found:

\[
\text{cyt. } c < \text{Mb} = \text{MetHb} < \text{HRP}
\]

Hawco et al. (1977) could not observe chemiluminescence during the decomposition of t-butyl hydroperoxide catalysed by haematin or methaemoglobin; the reasons of this disagreement escape our understanding.

Chemiluminescence required the oxidized forms of cytochrome \( c \), similarly to the necessity for haem compounds reported by Kashnitz & Hatefi (1975) on peroxidation reactions. Hydroperoxide-induced chemiluminescence of cytochrome \( c \) seems to account satisfactorily for the large increase of chemiluminescence of submitochondrial particles exerted by externally added cytochrome \( c \) (Cadenas et al., 1980).

The cellular generation of hydroperoxides, though at a low rate (Chance et al., 1979), under physiological or pathological conditions, might account for generation of singlet oxygen. At this point, we are able to state that generation of singlet oxygen, as responsible for light-emission, is identified by the effect of \( \beta \)-carotene and 1,4-diazabicyclo[2.2.2]-octane on submitochondrial particles, cytochrome \( c \)-supplemented submitochondrial particles and cytochrome \( c \) (the present work and Cadenas et al., 1980), and by \( \beta \)-carotene and spectral analysis on isolated microsomal fraction and simplified microsomal systems (Sugioka & Nakano, 1976; Nakano et al., 1975).

This research was supported by U.S. Public Health Service grants TW-02457 and HL-SCOR-15061. E. C. is a U.S. Fogarty International Research Fellow.

References


Desai, I. D. & Tappel, A. L. (1963) Lipid Res. 4, 204–207


O’Brien, P. J. (1968a) Biochem. J. 100, 12p
O’Brien, P. J. (1968b) Biochem. J. 100, 68p