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Chemiluminescence of Lipid Vesicles Supplemented with Cytochrome *c* and Hydroperoxide

Enrique CADENAS, Alberto BOVERIS and Britton CHANCE
Johnson Research Foundation, School of Medicine, G4, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

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The increase in light emission of hydroperoxide-supplemented cytochrome *c* observed on addition of lipid vesicles was related to the degree of unsaturation of the fatty acids of the phospholipids: dipalmitoyl phosphatidylcholine was without effect, whereas dioleoyl phosphatidylcholine and soya-bean phosphatidylcholine enhanced chemiluminescence 2- and 3-fold respectively. Effects on light-emission were similar to those on O_2 uptake. The chemiluminescence of the present system was sensitive to cyanide and to the radical trap 2,5-di-t-butylquinol, indicating a catalytic activity of cytochrome *c* and the presence of free-radical species respectively. Lipid-vesicle enhanced chemiluminescence showed different kinetic behaviours, apparently depending on unsaturation: three phases are described for soya-bean phosphatidylcholine, whereas only one phase was present in mixtures containing dipalmitoyl and dioleoyl phospholipids. Chemiluminescence of lipid vesicles supplemented with cytochrome *c* and hydroperoxide showed similar kinetic patterns with H_2O_2 and primary (ethyl) and tertiary (t-butyl and cumene) hydroperoxides. Participation of singlet molecular oxygen, mainly in the phase III of chemiluminescence, is suggested by the increase of light-emission by 1,4-diazabicyclo[2.2.2]-octane as well as by data from spectral analysis.

The ability of cytochrome *c* to induce lipid autoxidation was early demonstrated in linoleic acid suspensions (Tappel, 1955) and suggested for mitochondrial membranes (Tappel & Zalkin, 1959). By this process unsaturated lipids become peroxidized throughout the catalytic activity of haemoproteins (Kashnitz & Hatefi, 1975); the haemoprotein is generally damaged during the peroxidation process owing to reaction with free-radical intermediates generated in the system (Roubal & Tappel, 1966*a*,*b*). Participation of several oxygen intermediates and free-radical chain reactions are widely accepted as the mechanism of the process. Studies performed in either model systems or biological preparations have led to the proposal of the participation of O_2^-, $HO^\bullet$, H_2O_2 or singlet oxygen as initiators or as rate-limiting in the process of lipid peroxidation (Pederson & Aust, 1973; Bus *et al.*, 1974; King *et al.*, 1975; Kellog & Fridovich, 1975, 1977). However, details of the free-radical reaction and participation of defined oxygen intermediates or radicals may vary widely under different conditions. Chemiluminescence arising from peroxidized lipids and haemoproteins was also reported (Hawco *et al.*, 1977).

We have reported that organic-hydroperoxide-supplemented cytochrome *c* is an effective source of light-emission (Cadenas *et al.*, 1980*b*) as well as that cytochrome *c* has a conspicuous enhancing effect on the light-emission of submitochondrial particles (Cadenas *et al.*, 1980*a*). It is worth noting that, in the chemiluminescence of hydroperoxide-supplemented ferricytochrome *c*, damage to the cytochrome *c* molecule has been found to be un-associated with light-emission (Cadenas *et al.*, 1980*b*). In the present paper we report chemiluminescence from a model system composed of lipid vesicles, organic hydroperoxide and cytochrome *c* that offers a better extrapolation to biological membranes, and that apparently generates singlet molecular oxygen and other chemiluminescent species.

Materials and Methods

Preparation of lipid vesicles

Dipalmitoyl phosphatidylcholine and dioleoyl phosphatidylcholine were provided by Dr. J. C. Vidal (Department of Molecular Biology, Vanderbilt University, Nashville, TN, U.S.A.) and synthesized by the method of Cubero Robles & Van den

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Bergh (1969); these samples contained 99% palmitoyl and 99.6% oleoyl groups respectively, with traces of myristoyl and stearoyl groups or palmitoyl and stearoyl groups (less than 1%). Dipalmitoyl phosphatidylcholine, dioleoyl phosphatidylcholine and soya-bean phosphatidylcholine were kept in chloroform solution under N_2. The samples were evaporated under N_2 current, and the white powder adherent to the tube wall was suspended in 50mM-Tris/acetate/1mM-EDTA buffer, pH8.1, with a Vortex mixer. The aqueous suspensions of phosphatidylcholines were sonicated thrice for 5min periods with 5min intervals in the model W185 Sonifer Cell Disruptor (Heat System–Ultrasonics, New York, NY, U.S.A.). Sonication was carried out at 0°C and under N_2 for dioleoyl phosphatidylcholine and soya-bean phosphatidylcholine; dipalmitoyl phosphatidylcholine was kept above the transition temperature (42°C) for 1h before and during sonication. The vesicles were centrifuged at 50000*g* for 20min to remove debris from the sonicator probe and non-dispersed phospholipids; opalescent bands were discarded, thus leaving a single population of monolayer liposomes.

Photon counting and oxygen uptake

Chemiluminescence was measured in a photon counter as described by Boveris *et al.* (1978). Two photomultipliers were used alternatively, either an RCA 8850 photomultiplier, responsive in the range 300–650nm, with an applied potential of 1.8kV, or an EMI 9658 phototube, responsive in the range 300–900nm, with an applied potential of −1.2kV. Phototube output was connected to a Princeton Applied Research (Princeton, NJ, U.S.A.) model 1121 amplifier–discriminator adjusted for single photon counting. The output was connected to both a Heathkit IB1100 frequency counter and a recorder. Kodak Wratten filters (Eastman Kodak Co., Rochester, NY, U.S.A.) no. 29 (deep red) and no. 64 (blue–green) with spectral transmittance from 610nm into the infrared and from 390 to 610nm respectively were placed in front of the EMI phototube when indicated. Unless otherwise stated, the RCA 8850 phototube was used.

O_2 uptake was measured with a Clark-type oxygen electrode in a 2ml cuvette with constant stirring.

Assay conditions

All the reactions were carried out in 50mM-potassium phosphate buffer, pH7.2. The temperature was 37°C.

Chemicals

t-Butyl hydroperoxide, 1,4-diazabicyclo[2.2.2]-octane and 2,5-di-t-butylquinol were obtained from Aldrich Co. (Milwaukee, WI, U.S.A.). Cytochrome *c* (type VI) and L-α-phosphatidylcholine (type III-S) from soya bean were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). H_2O_2, cumene peroxide (αα′-dimethylbenzoyl hydroperoxide) and ethyl hydroperoxide were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.), Matheson, Coleman and Bell (Norwood, OH, U.S.A.) and Ferrosan (Malmo, Sweden) respectively. Other reagents were of analytical grade.

Results

Effect of lipid vesicles on the chemiluminescence of cytochrome c/hydroperoxide mixtures

Cytochrome *c* and organic hydroperoxide mixtures have been reported previously as effective sources of light-emission (Cadenas *et al.*, 1980*b*). Lipid vesicles, with a different degree of fatty acid unsaturation, increased chemiluminescence when

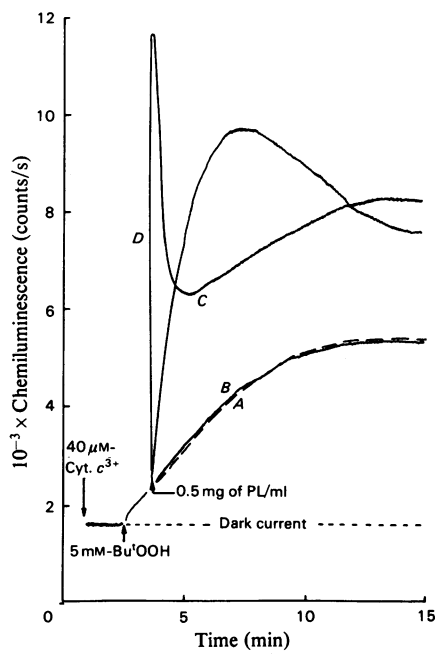


Fig. 1. *Effect of lipid vesicles on hydroperoxide-induced chemiluminescence of cytochrome c*

Assay conditions were as described in the Materials and Methods section. Curve *A* (----), light-emission of cytochrome *c* supplemented with t-butyl hydroperoxide without phospholipid vesicles added. For the remaining traces lipid vesicles (0.5mg of phospholipids/ml) were added as dipalmitoyl (curve *B*), dioleoyl (curve *C*) and soya-bean phosphatidylcholine vesicles (curve *D*). Abbreviations: Cyt. c^{3+}, cytochrome *c*; Bu^{t}OOH, t-butyl hydroperoxide; PL, phospholipids.

added to the cytochrome *c*/hydroperoxide system (Fig. 1). The extent of the stimulation depended on the degree of unsaturation of the fatty acids of the phosphatidylcholine vesicles: dipalmitoyl phosphatidylcholine ($C_{16:0}$) vesicles showed no effect, whereas dioleoyl phosphatidylcholine ($C_{18:1}$) vesicles produced a moderate increase in chemiluminescence. The more complex soya-bean phosphatidylcholine (predominantly $C_{18:2}$) vesicles were by far more effective at enhancing light-emission. Soya-bean phosphatidylcholine contains 13.8% palmitoyl, 3.6% stearoyl, 11% oleoyl, 65.3% linoleoyl and 6.3% linolenoyl groups (*Merck Index*, 1968); unsaturation is about 1.63 carbon double bonds per fatty acid chain; soya-bean phosphatidylcholine is referred to below as being '$C_{18:2}$' in comparisons with the other two phosphatidylcholines.

Unsaturated phospholipids also stimulated the O_2 uptake of the hydroperoxide-supplemented cytochrome *c* system from 0.5 to about 3-fold higher values by soya-bean phospholipids (1.7) and about 2-fold by dioleoyl phosphatidylcholine (0.95) Dipalmitoyl phosphatidylcholine vesicles had no effect (0.48). All values are expressed as μmol of O_2/min per μmol of cytochrome *c* and per 0.5 mg of phospholipid/ml.

Kinetics of light-emission in lipid vesicles/cytochrome c/hydroperoxide mixture

Lipid vesicles described above manifested different kinetic behaviour when added to cytochrome *c* and organic hydroperoxide; the different phases of light-emission are shown in Fig. 2. Only soya-bean phospholipids showed chemiluminescence on addition of ferricytochrome *c*: a small peak of light-emission was produced that returned to the background value within 1–2 min. This response is referred to as phase I. Subsequent addition of t-butyl hydroperoxide produced a rapid and larger enhancement of light-emission (phase II) that decayed to a value higher than the background, increased at a low rate until reaching a steady state (phase III) that lasted for about 2 h, and returned to the background value in about 6 h. Dipalmitoyl phosphatidylcholine and dioleoyl phosphatidylcholine vesicles showed neither phases I nor II but a long steady phase III.

The enhancing effect on chemiluminescence observed on addition of lipid vesicles to the hydroperoxide-supplemented cytochrome *c* was a linear function of the amount of lipid added (Fig. 3).

These experiments suggest that the presence of double bonds, mainly when an allylic carbon is

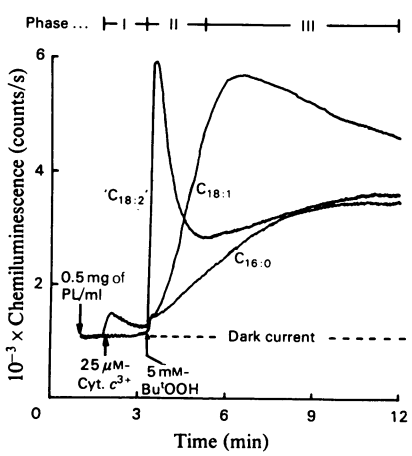


Fig. 2. *Kinetics of light-emission of lipid vesicles/cytochrome c/hydroperoxide mixtures*

Experimental conditions were as described in the Materials and Methods section. Dipalmitoyl ($C_{16:0}$), dioleoyl ($C_{18:1}$) or soya-bean ('$C_{18:2}$') phosphatidylcholine vesicles (each 0.5 mg of phospholipids/ml) were supplemented with cytochrome *c* and subsequently with t-butyl hydroperoxide. Abbreviations: Cyt. c^{3+}, cytochrome *c*; Bu^tOOH, t-butyl hydroperoxide; PL, phospholipids.

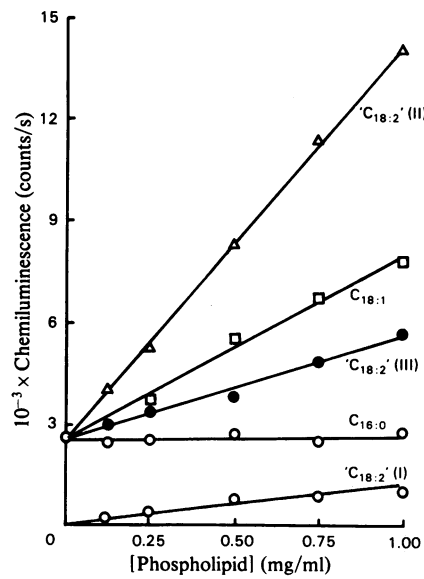


Fig. 3. *Dependence of hydroperoxide-induced chemiluminescence of cytochrome c on phospholipid vesicles concentration*

Experimental conditions were as described in Fig. 2 legend. The roman numbers in parentheses indicate the kinetic phases in the chemiluminescence of soya-bean phosphatidylcholine vesicles as shown in Fig. 2. Points plotted correspond to the maximum value of phase I or II and the plateau value of phase III. The chemiluminescence plotted at zero phospholipid concentration is the light-emission corresponding to hydroperoxide-supplemented cytochrome *c* devoid of lipid vesicles.

involved, as in soya-bean phosphatidylcholine, has a definitive effect on chemiluminescence of the cytochrome *c*/hydroperoxide system, as already reported on haemoprotein-catalysed lipid peroxidation (Kashnitz & Hatefi, 1975).

Chemiluminescence of lipid vesicles/cytochrome c on addition of different hydroperoxides

The system described in Fig. 2 was supplemented with different hydroperoxides replacing t-butyl hydroperoxide. Table 1 lists the effect on chemiluminescence of a mixture of cytochrome *c*, lipid vesicles and tertiary (cumene and t-butyl) or primary (ethyl) hydroperoxides as well as H_2O_2. H_2O_2 proved to be a quantitatively more active source of light-emission than the organic hydroperoxides when supplemented with cytochrome *c*, accounting for a very fast reaction with high chemiluminescence yields (experiments in Table 1 have a higher H_2O_2/cytochrome *c* ratio than the organic hydroperoxide/cytochrome *c* ratios) (Cadenas *et al.*, 1980*b*).

Addition of hydroperoxides to dipalmitoyl phosphatidylcholine vesicles showed no significant change of chemiluminescence emission because of its absence of double bonds (Table 1). When dioleoyl phosphatidylcholine vesicles were tested cumene hydroperoxide, t-butyl hydroperoxide and ethyl hydroperoxide were effective in that decreasing order, whereas H_2O_2, in lower concentration, was far more effective than the organic hydroperoxides. The same pattern could be seen when soya-bean phosphatidylcholine was the selected lipid, though the chemiluminescence yields were higher than those obtained with dioleoyl phosphatidylcholine.

Therefore the effect of t-butyl hydroperoxide on the chemiluminescence of vesicles with different fatty acid compositions supplemented with cytochrome *c* (Fig. 1) was paralleled by H_2O_2, ethyl hydroperoxide and cumene hydroperoxide.

Effect of cyanide and 2,5-di-t-butylquinol on chemiluminescence

Hydroperoxide-induced chemiluminescence of ferricytochrome *c* is inhibited by cyanide and sensitive to the radical trap 2,5-di-t-butylquinol (Cadenas *et al.*, 1980*b*). Fig. 4 shows the effect of cyanide on chemiluminescence of the lipid vesicles/cytochrome *c*/hydroperoxide system. When the inhibitor was present at the beginning of the reaction, 0.25 mM- and 1 mM-cyanide inhibited the maximal chemiluminescence (phase II) by about 33 and 66% respectively. The effect of cyanide was extended equally to all the three phases described for soya-bean phospholipid vesicles. On the other hand, when 1 mM-cyanide was added to the reaction before phase III started (Fig. 4*d*), an almost complete abolition of chemiluminescence of phase III was observed. Light-emission of dipalmitoyl phosphatidylcholine of dioleoyl phosphatidylcholine vesicles was inhibited by about 85% by 1 mM-cyanide. The concentration of cyanide necessary to inhibit the chemiluminescence of hydroperoxide-supplemented cytochrome *c* in the absence of lipid vesicles was slightly lower than the one required when lipid vesicles were present in the assay mixture.

The radical trap 2,5-di-t-butylquinol produced a lag of about 5–7 min on dipalmitoyl phosphatidylcholine and dioleoyl phosphatidylcholine chemiluminescence without affecting the total yield (results not shown). The concentration required to retard the chemiluminescence signal was higher than that necessary for hydroperoxide-supplemented cytochrome *c*, the simpler system (Cadenas *et al.*, 1980*b*).

Chemiluminescence and singlet oxygen

Involvement of singlet oxygen in the observed chemiluminescence was investigated with two different approaches: (*a*) by using the singlet-oxygen dimol-emission-enhancer 1,4-diazabicyclo[2.2.2]-

Table 1. *Chemiluminescence of lipid vesicles/cytochrome c on the addition of different hydroperoxides*

Assay conditions were as described in the Materials and Methods section and Fig. 2 legend. Lipid vesicles (0.5 mg/ml) containing fatty acids with different degree of unsaturation ($C_{16:0}$, $C_{18:1}$ or '$C_{18:2}$') were supplemented with cytochrome *c* and subsequently with H_2O_2 or t-butyl hydroperoxide or cumene hydroperoxide or ethyl hydroperoxide. For the H_2O_2-supplemented experiments the concentrations of reactants were 5 μM-cytochrome *c* and 1.3 mM-H_2O_2; for any other hydroperoxide they were 25 μM-cytochrome *c* and 5 mM-hydroperoxide. Values in the Table correspond to the maximal chemiluminescence yield observed in phase III. Chemiluminescence of these systems was a linear function of the phospholipid concentration, as shown in Fig. 3. Abbreviations: Bu^{t}OOH, t-butyl hydroperoxide; CuOOH, cumene hydroperoxide; EtOOH, ethyl hydroperoxide; cyt. c^{3+}, ferricytochrome *c*.

	Chemiluminescence (counts/s)			
Conditions	H_2O_2	EtOOH	Bu^{t}OOH	CuOOH
Cyt. c^{3+}	6900	1600	2500	4000
Cyt. c^{3+} + $C_{16:0}$	7100	1750	2500	4300
Cyt. c^{3+} + $C_{18:1}$	12000	2000	5500	13000
Cyt. c^{3+} + '$C_{18:2}$'	18000	2800	6800	15500

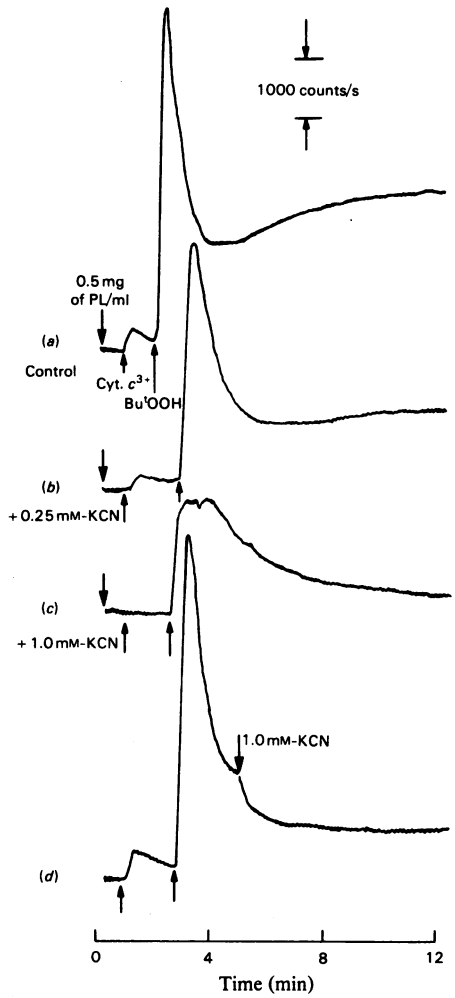


FIg. 4. *Inhibitory effect of cyanide on chemiluminescence of lipid vesicles/cytochrome c/hydroperoxide mixtures*

(*a*) Soya-bean phosphatidylcholine vesicles (0.5 mg of phospholipids/ml) were supplemented with 25 μM-cytochrome *c* and 5 mM-t-butyl hydroperoxide; (*b*) and (*c*) as in (*a*) plus 0.25 mM- and 1.0 mM-KCN respectively from the beginning; (*d*) as in (*a*) but 1.0 mM-KCN was added at the beginning of phase III. Abbreviations: Cyt. c^{3+}, cytochrome *c*; Bu^{t}OOH, t-butyl hydroperoxide; PL, phospholipids.

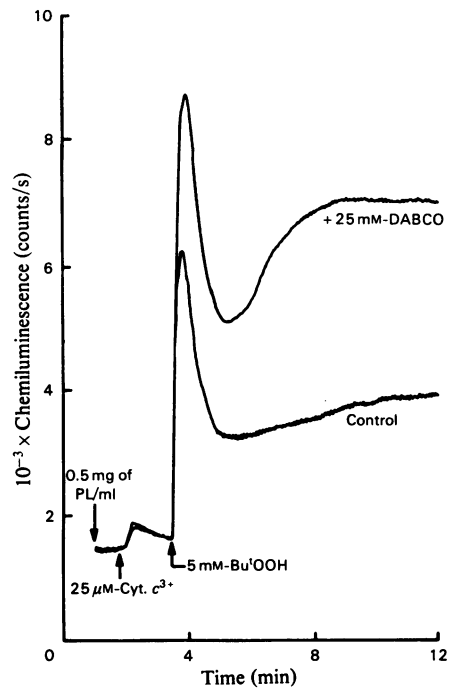


Fig. 5. *Effect of 1,4-diazabicyclo[2.2.2]-octane on chemiluminescence of soya-bean phospholipid vesicles/cytochrome c/hydroperoxide mixture*

Soya-bean phosphatidylcholine vesicles (0.5 mg of phospholipids/ml) were supplemented with cytochrome *c* and t-butyl hydroperoxide in the absence and presence of 1,4-diazabicyclo[2.2.2]-octane. Abbreviations: Cyt. c^{3+}, cytochrome *c*; Bu^{t}OOH, t-butyl hydroperoxide; PL, phospholipids; DABCO, 1,4-diazabicyclo[2.2.2]-octane.

octane (Deneke & Krinsky, 1977); (*b*) by a restricted spectral analysis.

The effect of 1,4-diazabicyclo[2.2.2]-octane was better observed on the so-called phase III of dipalmitoyl phosphatidylcholine or dioleoyl phosphatidylcholine or soya-bean phosphatidylcholine vesicles. Phase I, present only in the system containing soya-bean phosphatidylcholine vesicles, manifested no change with 1,4-diazabicyclo[2.2.2]-octane, whereas phases II and III were increased by about 53 and 100% respectively (Fig. 5); the increase observed in phase III was not only in total chemiluminescence yield but also in the rate in which maximal chemiluminescence was achieved. Maximal effect of 1,4-diazabicyclo[2.2.2]-octane was obtained at a concentration of about 25 mM; 10 mM-1,4-diazabicyclo[2.2.2]-octane was necessary to obtain the same stimulatory effect in a system of cytochrome *c* and hydroperoxide devoid of lipid vesicles.

Light-emission by the same system recorded with a red-sensitive phototube (EMI 9850) (Fig. 6) showed that the ratio of maximal chemiluminescence in phase II/phase III was lower (0.8) than that recorded with the green-sensitive tube (1.8). This was already indicative of a predominancy of red-light-emitting species in phase III that was

relatively undetected with the green-sensitive phototube (RCA 8850). Moreover, placing a red filter in front of the phototube showed that phase II was almost completely composed of emitted light of a wavelength lower than 610nm, and that phase III was predominantly composed of red light with a wavelength higher than 610nm. Similarly a blue–green filter showed phase II to be composed of 500–580nm light and phase III to contain little blue–green light.

Discussion

Hydroperoxide- and cytochrome *c*-supplemented lipid vesicles may be considered as a model system for reactions occurring in the membrane-bound electron-transport systems of the cell. Chemiluminescence from mitochondrial membranes and cytochrome *c* solutions supplemented with hydroperoxide (Cadenas *et al.*, 1980*a*,*b*) and from microsomal membranes (Nakano *et al.*, 1975) and microsomal simplified systems (Sugioka & Nakano, 1976) has been shown to be related to lipid peroxidation and to singlet-oxygen formation through spectral analysis and the effect of specific quenchers and enhancers of singlet-oxygen emission.

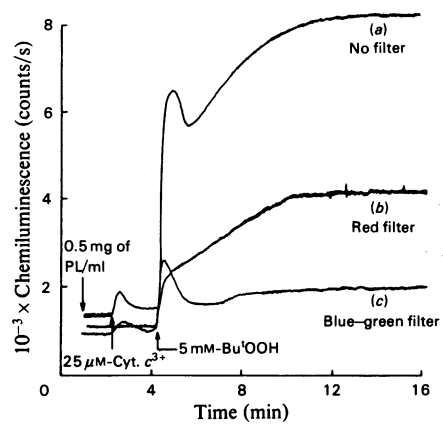


Fig. 6. *Partial spectral analysis of light-emission of a lipid vesicles/cytochrome c/hydroperoxide mixture*
Soya-bean phosphatidylcholine vesicles (0.5mg of phospholipids/ml) were used. Assay conditions were as described in the Materials and Methods section.

The kinetics of light-emission in polyunsaturated lipid vesicles supplemented with hydroperoxides and cytochrome *c* show a multiplicity of light-emitting reactions, depending on different modalities of the complex free-radical sequences of lipid peroxidation. One of the phases (the so-called phase III) that follows the initial burst of light-emission in the complete system is apparently constituted mainly by singlet-oxygen dimol emission. In this sense, the characteristic effect of 1,4-diazabicyclo[2.2.2]-octane, increasing light-emission from singlet oxygen in aqueous systems (Deneke & Krinsky, 1977), agrees with the principally red-light emission (Figs. 5 and 6). Moreover, the initial burst in light-emission that follows hydroperoxide addition seems to consist of blue–green light, and may be related to 1,2-dioxetane derivatives, which have emission maximal at about 350–480nm (Hasting & Wilson, 1976). However, it is possible that triplet–singlet energy transfer may activate another fluorophore emitting in the green region.

Fatty acid unsaturation is essential to chemiluminescence enhancement (Table 2). No chemiluminescence increase is produced by the saturated dipalmitoyl phosphatidylcholine ($C_{16:0}$) vesicles, whereas dioleoyl phosphatidylcholine ($C_{18:1}$) vesicles increased light-emission 2-fold and soya-bean phosphatidylcholine ('$C_{18:2}$') vesicles were even more effective (3-fold) in increasing light-emission. The rate of O_2 uptake showed effects that

Table 2. *Hydroperoxide-induced chemiluminescence and oxygen uptake by model systems and heart submitochondrial particles*
Assay conditions were as described in the Materials and Methods section. Additions were present at the following concentrations: 25μM-cytochrome *c*, lipid vesicles (0.5mg of phospholipids/ml) (as described in Fig. 2 legend), 1.2mg of protein/ml as bovine heart submitochondrial particles (approximately corresponding to 0.5mg of phospholipids/ml). The values are expressed per μmol of cytochrome *c* and were obtained on the addition of 5mM-t-butyl hydroperoxide.

Conditions	Chemiluminescence (counts/s)	O_2 uptake (μmol of O_2/min)
Cyt. c^{3+}	100	1.0
Cyt. c^{3+} + $C_{16:0}$ vesicles	110	1.0
Cyt. c^{3+} + $C_{18:1}$ vesicles	215	2.2
Cyt. c^{3+} + '$C_{18:2}$' vesicles	350	3.2
Submitochondrial particles	2000	7.2

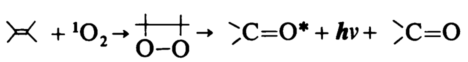
were parallel to that observed in chemiluminescence. Both parameters behaved in agreement with the well-known requirements of double bonds and O_2 for the lipid-peroxidation free-radical reactions (Tappel, 1973; Pryor, 1978). When lipid and cytochrome *c* were part of bovine heart submitochondrial particles, the chemiluminescence yield and the O_2 uptake were both about 3 times the corresponding chemiluminescence yield and O_2 uptake of phospholipids/cytochrome *c* mixtures. Considering that lipid unsaturation is about similar in both bovine heart mitochondrial (Keenan *et al.*, 1970) and soya-bean phospholipids, it seems that the close proximity of haem groups to these lipids assures a better efficiency of the reactions leading to lipid peroxidation (Demopoulos, 1973) and chemiluminescence.

Light-emission observed from isolated cells (Kakinuma *et al.*, 1979; Lloyd *et al.*, 1979), perfused organs (Boveris *et al.*, 1978) etc. appears to be related to side reactions of the complex free-radical process of lipid peroxidation. Tetroxides derived from peroxyl radicals seem to be the precursors of singlet oxygen (Russell, 1957; Sugioka & Nakano, 1976) [reactions (1) and (2)]:

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow {}^1O_2 + RO + ROH \quad (1)$$

$$2\,{}^1O_2 \rightarrow 2\,{}^3O_2 + h\nu \quad (2)$$

1,2-Dioxetane derivatives formed by addition of singlet oxygen to double bonds of unsaturated fatty acids may be the precursors of excited carbonyl groups (Hasting & Wilson, 1976; Foote, 1978; Faria-Oliveira *et al.*, 1978) [reaction (3)]:

 (3)

In the present paper we offer evidence (*a*) that the model system consisting of hydroperoxide, cytochrome *c* and unsaturated lipids generates light, (*b*) that the light-emission is related to unsaturation of fatty acids, (*c*) that more than one chemiluminescent species is involved, and (*d*) that singlet-oxygen dimol emission seems to contribute to light-emission under our experimental conditions.

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