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Low-Level Chemiluminescence of Bovine Heart Submitochondrial Particles

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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Submitochondrial particles from bovine heart mitochondria showed low-level chemiluminescence when supplemented with organic hydroperoxides. Chemiluminescence seems to measure integratively radical reactions involved in lipid peroxidation and related processes. Maximal light-emission was about 1500 counts/s and was reached 2–10 min after addition of hydroperoxides. Ethyl hydroperoxide, cumene hydroperoxide and t-butyl hydroperoxide were effective in that order. Antimycin and rotenone increased chemiluminescence by 50–60%; addition of substrates, NADH and succinate did not produce marked changes in the observed chemiluminescence. Cyanide inhibited chemiluminescence; half-maximal inhibitory effect was obtained with 0.03 mM-cyanide and the inhibition was competitive with respect to t-butyl hydroperoxide. Externally added cytochrome *c* (10–20 μM) had a marked stimulatory effect on chemiluminescence, namely a 12-fold increase in light-emission of antimycin-inhibited submitochondrial particles. Stimulation of hydroperoxide-induced chemiluminescence of submitochondrial particles by cytochrome *c* was matched by a burst of O_2 consumption. O_2 is believed to participate in the chain radical reactions that lead to lipid peroxidation. Superoxide anion seems to be involved in the chemiluminescence reactions as long as light-emission was 50–60% inhibitible by superoxide dismutase. Singlet-oxygen quenchers, e.g. β-carotene and 1,4-diazabicyclo[2,2,2]-octane, affected light-emission. β-Carotene was effective either when incorporated into the membranes or added to the cuvette. The present paper suggests that singlet molecular oxygen is mainly responsible for the light-emission in the hydroperoxide-supplemented submitochondrial particles.

The recent report on the chemiluminescence of perfused liver and isolated liver mitochondria (Boveris *et al.*, 1978) has directed attention to mitochondria as an important contributor to cellular chemiluminescence. Rat liver microsomal fraction has already been recognized as an effective source of light-emission (Nakano *et al.*, 1975; Sugioka & Nakano, 1976). Lloyd *et al.* (1979) have reported on the chemiluminescence of isolated *Acanthamoeba castellanii* cells and of subcellular fractions isolated therefrom, and Kakinuma *et al.* (1979) and Diaz *et al.* (1979) on the chemiluminescence of resting and phagocytosing polymorphonuclear leucocytes and macrophages respectively. The intensity of light-emission by rat liver mitochondria was found to be dependent on the metabolic state and to be markedly increased when the mitochondria were supplemented with organic hydroperoxides such as t-butyl hydroperoxide (Boveris *et al.*, 1978). The same workers also showed that the generation of superoxide anion and H_2O_2 and chemiluminescence were differently linked to mitochondrial metabolism. The apparent requirements for the chemiluminescence reaction in liver mitochondria were a hydroperoxide group, molecular O_2 and a membrane redox system.

In the present paper we summarize some observations on low-level chemiluminescence, so termed by Seliger (1975), of submitochondrial particles supplemented with hydroperoxides, and point out the probable nature of light-emission throughout the effect of relatively specific quenchers of singlet oxygen.

Materials and Methods

Biological preparations

Bovine heart mitochondria and bovine heart submitochondrial particles were isolated by the technique of Blair (1967) as already described (Boveris *et al.*, 1976), and suspended in 0.25 M-sucrose. Submitochondrial particles were obtained by sonicating

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bovine heart mitochondria (20mg of protein/ml) in 0.25M-sucrose/2mM-EDTA/KOH, pH 8.0, twice during 20s with 1min intervals in a model W185 Sonifier Cell Disruptor (Heat Systems–Ultrasonics, New York, NY, U.S.A.). Submitochondrial particles were washed twice with 0.25M-sucrose. Removal of cytochrome *c* from bovine heart mitochondria was carried out essentially by following the hypo-osmotic treatment described by Jacobs & Sanadi (1960), slightly modified for bovine heart mitochondria (Lenaz & MacLennan, 1967); incubation of mitochondria with 15mM-KCl at 0°C during 30min, with occasional stirring and two subsequent washings with 150mM-KCl, afforded over 70–90% decrease of cytochrome *c* estimated from reduced-minus-oxidized difference spectra.

Incorporation of β-carotene into submitochondrial particles was obtained by sonicating bovine heart mitochondria as indicated above in the presence of various concentrations of β-carotene (20–50nmol/mg of protein) dissolved in chloroform. Three or more successive washings, until the 105000*g* supernatant was colourless, were necessary to remove non-incorporated β-carotene. The amount of β-carotene present in the membranes was measured spectroscopically in a Johnson Research Foundation memory scanner (Chance *et al.*, 1977); three absorption bands (540, 494 and 463nm) were observed, and an ε_{494} value of $58\,mM^{-1}\cdot cm^{-1}$ (Grasselli, 1973) was utilized to calculate the amount of β-carotene incorporated into the membranes. Protein concentration was measured in β-carotene-supplemented membranes by the method of Lowry *et al.* (1951) in order to avoid the 540nm-band interference, and in the other membrane preparations by the biuret method (Gornall *et al.*, 1949).

Photon counting

Chemiluminescence was measured with a photon counter as described by Boveris *et al.* (1978) in a 35mm × 25mm × 5mm cuvette (12.25cm^2 surface) with constant bubbling with air to assure a constant O_2 concentration.

Oxygen uptake

O_2 consumption was measured in a 2ml-volume cell equipped with a Clark oxygen electrode.

Chemicals

β-Carotene, cytochrome *c* (type VI), antimycin and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Superoxide dismutase was also obtained from Truett Laboratories (Dallas, TX, U.S.A.). β-Carotene was dissolved in methanol and its concentration was calculated by using a ε_{450} value of $13.2\,mM^{-1}\cdot cm^{-1}$. Cytochrome *c* concentration was determined after reduction with $Na_2S_2O_4$ by its absorbance at 550nm ($\varepsilon = 27.6\,mM^{-1}\cdot cm^{-1}$) (Margoliash, 1954). 1,4-Diazabicyclo[2,2,2]-octane and t-butyl hydroperoxide were obtained from Aldrich Co. (Milwaukee, WI, U.S.A.); cumene hydroperoxide ($\alpha\alpha'$-dimethylbenzoyl hydroperoxide) and ethyl hydroperoxide were purchased from Matheson, Coleman and Bell (Norwood, OH, U.S.A.) and Ferrosan (Malmo, Sweden) respectively. Other reagents were of analytical grade.

Assay conditions

All assays were carried out either in 50mM-potassium phosphate buffer, pH 7.3–7.4, or in 0.23M-mannitol/0.07M-sucrose/20mM-Tris/HCl buffer, pH 7.3–7.4. The temperature was maintained at 37°C.

Results

Hydroperoxide-induced chemiluminescence of submitochondrial particles

Bovine heart submitochondrial particles showed low-level chemiluminescence when they were supplemented with organic hydroperoxide. Fig. 1(*a*) illustrates the light-emission of antimycin-inhibited submitochondrial particles. The reaction proceeded slowly, increasing its velocity at a rate of about 400 counts/s per min, reaching its maximal chemiluminescence yield of 1500counts/s about 8–10min after the addition of t-butyl hydroperoxide, and then remained constant for about 10min. It is worth noting that no abrupt increase of light-emission was detected on hydroperoxide addition, but a relatively slow progress in the process, indicating a reaction of the hydroperoxide with the biological material. The effects of NADH, succinate, rotenone and antimycin are shown in Table 1. NADH had no significant effect on the hydroperoxide-induced chemiluminescence of submitochondrial particles, whereas succinate showed a moderate inhibitory effect. Both antimycin and rotenone exhibited a moderate enhancing effect.

Chemiluminescence depended on the buffer system utilized in the assay: almost 2-fold-higher chemiluminescence yields were observed with phosphate buffer than with mannitol/sucrose/Tris buffer, presumably because of the reported inhibitory effect of Tris on chemiluminescence (Deneke & Krinsky, 1977) or the scavenging effect of mannitol on hydroxyl radicals (Halliwell, 1978). t-Butyl hydroperoxide and cumene hydroperoxide showed similar kinetic patterns of chemiluminescence when added to antimycin-inhibited submitochondrial particles. The primary hydroperoxide, ethyl hydroperoxide, showed faster response, achieving maximal light-emission after 2–3min of its addition to submito-

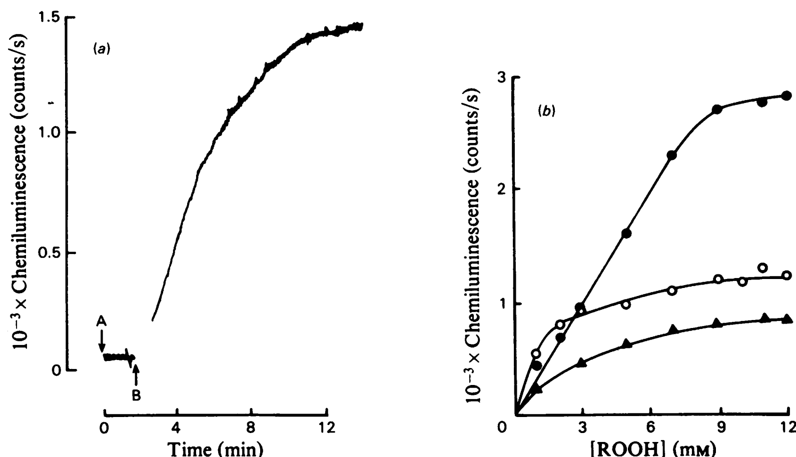

Fig. 1. *Chemiluminescence of hydroperoxide-supplemented submitochondrial particles*
(*a*) To submitochondrial particles (1.3 mg of protein/ml) and 10 μM-antimycin in 50 mM-phosphate buffer, pH 7.3 (arrow A), was added 5 mM-t-butyl hydroperoxide (arrow B). (*b*) The same conditions as in (*a*) (0.9 mg of protein/ml) with different amounts of added hydroperoxide (ROOH): ▲, t-butyl hydroperoxide; ○, cumene hydroperoxide; ●, ethyl hydroperoxide.

Table 1. *Hydroperoxide-induced chemiluminescence of submitochondrial particles*
Submitochondrial particles (1.65 mg of protein/ml) in 50 mM-potassium phosphate buffer, pH 7.3, were added to 5 mM-t-butyl hydroperoxide to start the reaction. Concentrations of different additions were: 10 μM-antimycin, 3 μM-rotenone, 5.5 mM-succinate or 0.4 mM-NADH. Abbreviation: SMP, submitochondrial particles.

Conditions	Maximal chemiluminescence (counts/s)	Rate of increase of chemiluminescence (counts/s per min)
SMP	1750	400
SMP + antimycin	3300	1100
SMP + rotenone	3050	900
SMP + NADH	1700	350
SMP + succinate	1000	—*
SMP + antimycin + NADH	3200	1050
SMP + antimycin + succinate	1700	—*

* A rapid burst of the chemiluminescence that returned rapidly to the initial value (1–2 min) was observed.

chondrial particles, and a higher chemiluminescence yield than those of the two tertiary hydroperoxides described above (Fig. 1*b*).

Characteristics of the hydroperoxide-induced chemiluminescence of submitochondrial particles

Submitochondrial particles showed a 50–60% enhancement of light-emission when they were inhibited by either antimycin (about 2 nmol/mg of protein) or rotenone (about 1 nmol/mg of protein). The concentrations of the inhibitors utilized are slightly higher than those usually required to inhibit electron transfer (Fig. 2). Both inhibitors, antimycin and rotenone, were tested on particles with an endogenous substrate concentration approaching zero; these assays were always performed in the absence of externally added substrate unless otherwise stated.

Cyanide and azide manifested marked inhibitory effects at a concentration of about 0.03 mM, and azide needed 30-fold this concentration to show a half-maximal inhibitory effect (Fig. 2). The inhibitory effect of azide on chemiluminescence cannot be regarded as an unique effect on cytochrome oxidase, since azide has been reported to decrease chemiluminescence in organic systems devoid of enzymic activity (Kearns, 1971; Deneke & Krinsky, 1977). The inhibitory effect of 0.2 mM-cyanide was tested at different hydroperoxide concentrations in

bovine heart submitochondrial particles (Fig. 3*a*). The reciprocal plot (Fig. 3*b*) shows cyanide inhibition to be competitive with the tertiary hydroperoxide. This effect identifies light-emission as a process dependent on a component of mitochondrial membranes that is able to bind either cyanide or hydroperoxide.

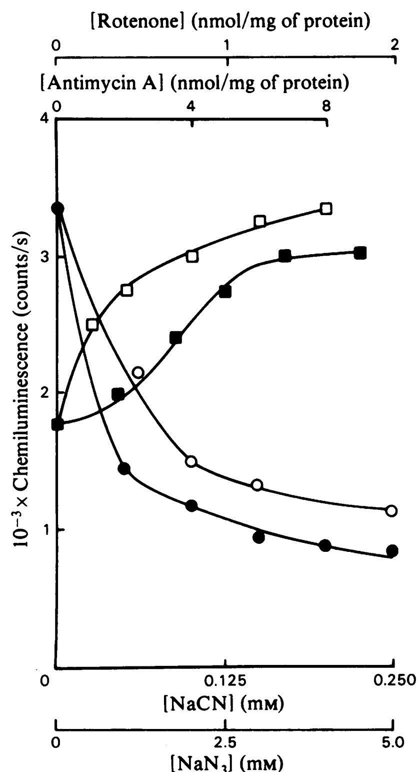

Fig. 2. *Effect of different inhibitors on chemiluminesence of hydroperoxide-supplemented submitochondrial particles*

The reaction mixture contained submitochondrial particles [1.82 (□, ■) or 3.2 (○, ●) mg of protein/ml] in 50mM-phosphate buffer, pH 7.3; the reaction was initiated by the addition of 5mM-t-butyl hydroperoxide. Inhibitors: □, antimycin A; ■, rotenone; ○, NaN_3; ●, NaCN.

Cytochrome c and chemiluminescence of hydroperoxide-supplemented submitochondrial particles

According to the results shown in Figs. 2 and 3, it seems that light-emission of mitochondrial membranes strongly depends on the participation of the oxidized form of a member of the respiratory chain located on the O_2 side of the rotenone-sensitive site. We have partially focused our attention on the possible role of cytochrome *c* on chemiluminescence.

Table 2 shows that depletion of cytochrome *c* from bovine heart mitochondria throughout the saline-extraction method resulted in a decrease of the total chemiluminescence yield and that cytochrome *c*-containing supernatant showed noticeable degrees of light-emission. Depletion of cytochrome *c* from mitochondria was not complete, accounting for 78% of the total cytochrome *c* present in the membranes. On the other hand, a total abolition of chemiluminescence in the hydroperoxide-supplemented and cytochrome *c*-depleted mitochondria was not expected, since phospholipids and other haemoproteins could also be contributing to light-emission.

Fig. 4 shows that light-emission was substantially increased when external ferricytochrome *c* was added to NADH-supplemented submitochondrial particles. The effect was more marked when the membranes were inhibited with antimycin (12-fold increase with 20μM-cytochrome *c*), whereas in the absence of antimycin the maximal increase observed (in the same conditions) was about 4-fold. These results strengthen the idea that oxidized cytochrome *c* is a major contributor to light-emission of antimycin-inhibited and hydroperoxide-supplemented mitochondrial membranes.

Effects of superoxide dismutase on chemiluminescence

The participation of superoxide anion in chemiluminescence of hydroperoxide-supplemented submitochondrial particles is indicated by the inhibitory effect (50–60%) of superoxide dismutase (McCord & Fridovich, 1969) when the assay was performed

Table 2. *Relationship between cytochrome c content of mitochondria membranes and chemiluminescence*

Extraction of cytochrome *c* from mitochondrial membranes was developed as explained in the Materials and Methods section. The assay mixture contained 1.04mg of protein/ml as mitochondrial membranes (intact or cytochrome *c*-depleted) or supernatant and 5mM-t-butyl hydroperoxide in 50mM-phosphate buffer, pH 7.3.

	Mitochondria	Cytochrome *c*-depleted mitochondria	Supernatant
Cytochrome *c* content (nmol of cytochrome *c*/mg of protein)	0.42	0.09	1.48
Chemiluminescence (counts/s)	3800	1120	1100

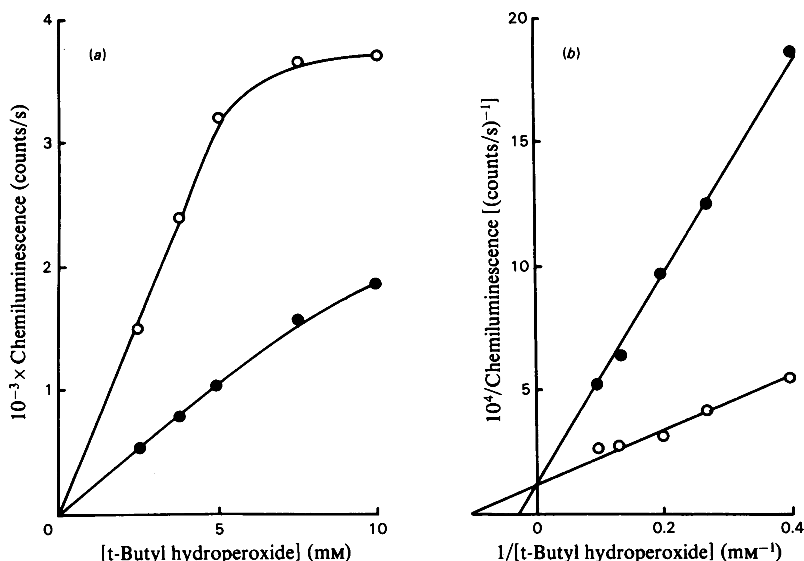

Fig. 3. *Effect of cyanide on chemiluminescence of hydroperoxide-supplemented submitochondrial particles*
(*a*) Submitochondrial particles (3.5 mg of protein/ml) and 10 µM-antimycin in 50 mM-phosphate buffer, pH 7.35, were supplemented with various amounts of t-butyl hydroperoxide in the absence (○) and presence (●) of 0.2 mM-cyanide. (*b*) Reciprocal plot obtained from data of (*a*).

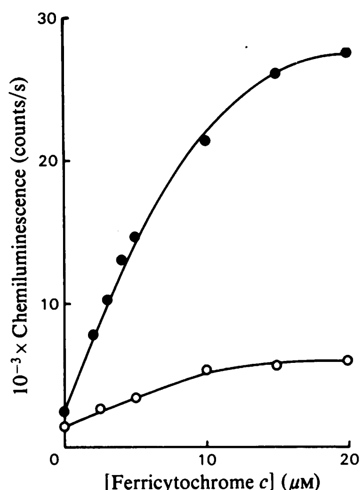

Fig. 4. *Effect of externally added cytochrome c on chemiluminescence of hydroperoxide-supplemented submitochondrial particles*
The reaction mixture contained submitochondrial particles (2.6 mg of protein/ml) supplemented with 0.34 mM-NADH in the presence (●) or absence (○) of 10 µM-antimycin in 50 mM-phosphate buffer, pH 7.3; the reaction was started by the addition of 5 mM-t-butyl hydroperoxide.

in the presence of antimycin and supporting substrate (Fig. 5), conditions in which superoxide anion generation by submitochondrial particles is markedly increased (Boveris & Cadenas, 1975; Boveris *et al.*, 1976). Reciprocal plots show that superoxide dismutase inhibites chemiluminescence competitively with the hydroperoxide (results not shown). Superoxide dismutases produce a half-maximal inhibitory effect at a concentration of about 0.8–2.1 µg/ml (Fig. 5); this concentration is similar to that reported by Kellog & Fridovich (1975) to inhibit lipid peroxidation.

It is unlikely that the observed inhibitory effect of superoxide dismutase on chemiluminescence is due to a direct molecular collision of the enzyme with molecular singlet oxygen, taking into account the lifetime of singlet oxygen and concentration of dismutase (Fridovich, 1975).

Nature of the light-emission

The effects of known quenchers of singlet oxygen were tested on the chemiluminescence of hydroperoxide-supplemented submitochondrial particles. β-Carotene, a physical quencher of singlet molecular oxygen (Foote & Denny, 1968), proved to be a potent inhibitor of light-emission of the antimycin-inhibited particles, both when it was previously incorporated into the submitochondrial particles by sonication (Fig. 6) and when it was added to the cuvette (Fig. 7). β-Carotene was much more effective (about 30-fold) on a molar basis when it was incorporated into the membranes, pointing to a hydrophobic domain as the medium in which it quenches singlet oxygen. Externally added β-caro-

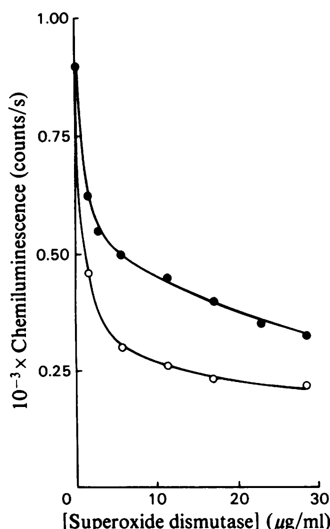

Fig. 5. *Effect of superoxide dismutase on chemiluminescence of hydroperoxide-supplemented submitochondrial particles*

The reaction mixture contained submitochondrial particles (0.8 mg of protein/ml), 10 μM-antimycin and 5.5 mM-succinate in 50 mM-phosphate buffer, pH 7.3. Reaction was started by the addition of 5 mM-t-butyl hydroperoxide. ●, Sigma superoxide dismutase; ○, Truett superoxide dismutase.

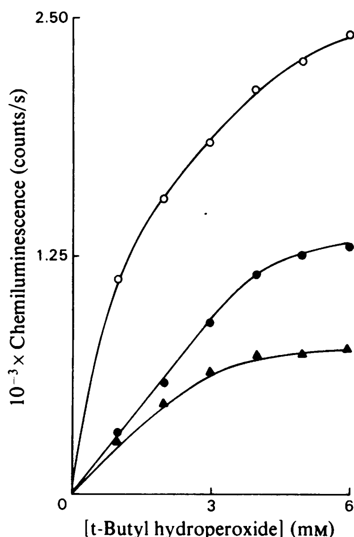

Fig. 6. *Effect of membrane-incorporated β-carotene on hydroperoxide-induced chemiluminescence of submitochondrial particles*

The reaction mixture contained submitochondrial particles (2.2 mg of protein/ml) [without (○) or with membrane-incorporated β-carotene (●, 2.6 nmol/mg of protein; ▲, 5 nmol/mg of protein)] and 10 μM-antimycin in 50 mM-phosphate buffer, pH 7.35. t-Butyl hydroperoxide was added to start the reaction.

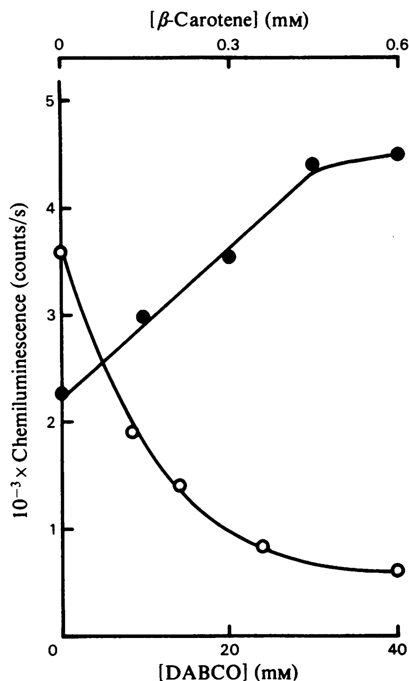

Fig. 7. *Effect of singlet-oxygen quenchers on hydroperoxide-induced chemiluminescence of submitochondrial particles*

The reaction mixture contained submitochondrial particles (●, 2.0 mg of protein/ml; ○, 3.5 mg of protein/ml) and 10 μM-antimycin in 50 mM-potassium phosphate buffer, pH 7.3, and also 1,4-diazabicyclo[2,2,2]-octane (DABCO) (●) or β-carotene (○).

tene was capable of decreasing by 83% the total chemiluminescence at a concentration of about 0.6 mM (Fig. 7); this fact seems to indicate that singlet-oxygen light-emission is responsible for at least 83% of the chemiluminescence, since it is possible that part of the total singlet oxygen produced is not accessible to the quencher, owing to the membrane permeability barrier. Furthermore, 1,4-diazabicyclo[2,2,2]-octane, which enhances singlet-oxygen dimol emission in aqueous media (Deneke & Krinsky, 1977), showed a 2-fold increase in light-emission when present at a concentration of 30 mM (Fig. 7), thus identifying singlet oxygen as responsible for chemiluminescence.

Oxygen uptake by hydroperoxide-supplemented submitochondrial particles

Submitochondrial particles, devoid of endogenous substrates, and in the absence of exogenous substrates, consume O_2 at a rate of 2–3 nmol/min per mg of protein when supplemented with t-butyl hydroperoxide (Fig. 8*a*); there were no significant

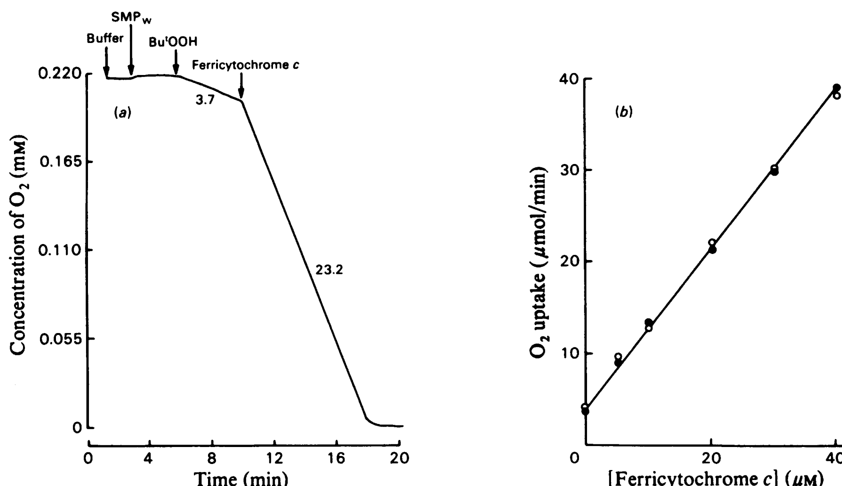

Fig. 8. ***Effect of externally added cytochrome c on oxygen uptake by hydroperoxide-supplemented submitochondrial particles***

(*a*) Submitochondrial particles (1.2 mg of protein/ml) in 50 mM-phosphate buffer, pH 7.3, were added after 5 mM-t-butyl hydroperoxide (Bu^{t}OOH) and 20 μM-cytochrome *c*; numbers close to the traces indicate nmol of O_2 consumed/min per mg of protein. (*b*) The same conditions as in (*a*) in the presence (●) and absence (○) of 10 μM-antimycin. SMP$_w$, Submitochondrial particles washed twice.

changes in the rate of O_2 uptake when antimycin was present in or absent from the assay mixture. Externally added ferricytochrome *c* markedly enhanced O_2 consumption by hydroperoxide-supplemented submitochondrial particles; the extent of this increase depended on the amount of cytochrome *c* present in the reaction mixture (Fig. 8*b*). No O_2 uptake was recorded when cytochrome *c* was added to submitochondrial particles in the absence of t-butyl hydroperoxide. Apparently, cytochrome *c*, like other haemoproteins or heavy-metal haem groups already described, catalyses the free-radical reaction of lipid peroxidation by which O_2 is incorporated into membranes. This process is favoured by the presence of hydroperoxide and the high content of polyunsaturated fatty acids in mitochondrial membranes.

Discussion

Chemiluminescence seems to integrate radical reactions that are involved in lipid peroxidation and related processes. It might be worth recalling the involvement of free-radical chains within the reactions detected by chemiluminescence that leads to measurements of non-stoicheiometric reactions. This fact raises a difficulty in estimating the absolute participation of different species. Moreover, the observed light-emission (about 10^3 counts/s) corresponds to a rate of 10^{-17} M/s (considering a detection efficiency of about 0.1%), which implies that the chemiluminescent species are produced in minor side reactions.

Components of the respiratory chain participate in reactions that lead to chemiluminescence; this view is supported by the effect of respiratory inhibitors on light-emission of submitochondrial particles. It appears that one of these components is more effective in the oxidized state, since (*a*) the submitochondrial particles used in these experiments are devoid of endogenous substrates and very small O_2 uptake rates were recorded in the absence of added substrate, (*b*) exogenous substrates did not significantly affect light-emission in antimycin-inhibited particles, (*c*) rotenone and antimycin exert a stimulatory effect, and finally (*d*) cyanide shows an inhibitory effect.

The identity of the component of the respiratory chain that participates in light-emission is indicated by the effect of external cytochrome *c* on the chemiluminescence of antimycin-inhibited and hydroperoxide-supplemented submitochondrial particles. The cytochrome *c* effect seems to be a result of molecular interactions with components other than the respiratory chain, since in the presence of antimycin effective electron transfer to cytochrome *c* is unlikely. It is known that heavy-metal complexes such as haem act as catalysts or initiators of lipid peroxidation (Demopoulos, 1973). Moreover, mitochondrial phospholipids are rich in polyunsaturated fatty acids, which make the organelle particularly susceptible to peroxidation reactions. This fact is helped by the close molecular proximity of haemoproteins to the unsaturated lipids (Tappel, 1973). The role of ferricytochrome *c* as an initiator of lipid

peroxidation in biological membranes has been demonstrated by Kashnitz & Hatefi (1975). On the other hand, Banks *et al.* (1961) and Roubal & Tappel (1966) have shown the susceptibility of cytochrome *c* to peroxidation and subsequently destruction when exposed to hydroperoxides. Moreover, systems undergoing lipid peroxidation or lipid peroxide breakdown are known to produce chemiluminescence (Sugioka & Nakano, 1976). We have been able to detect light-emission from a model system composed of oxidized cytochrome *c* and organic hydroperoxide (Cadenas *et al.*, 1979).

The present results indicate that ferricytochrome *c* participates in light-emission by submitochondrial particles, but it is not the only source, since the percentage of depletion of cytochrome *c* did not correlate with the percentage decrease of chemiluminescence.

The involvement of superoxide anion in light-emission is likely, considering the effect of superoxide dismutase observed in the succinate- and antimycin-supplemented submitochondrial particles. Antimycin and rotenone effects, increasing light-emission (Fig. 2), might also be interpreted as indicating a superoxide anion-mediated process, since it has been reported that antimycin (Boveris & Cadenas, 1975) and rotenone (Boveris *et al.*, 1979) enhance superoxide anion production. Superoxide anion has been claimed to participate in lipoperoxidation reactions either by a self-reaction with polyunsaturated lipids (Lynch & Fridovich, 1978) or through a metal-catalysed Haber–Weiss-type reaction (Halliwell, 1978; McCord & Day, 1978; Kellog & Fridovich, 1977) leading to the production of more effective oxidant species. We believe that the participation of superoxide anion in light-emission could rest on the same basis, though its relative contribution is not yet established. Misra & Fridovich (1973) have reported an ethyl hydroperoxide decomposition catalysed by cytochrome *c* that involved a superoxide dismutase-inhibitible reduction of cytochrome *c* in the presence of NADH.

It is worth noting that cyanide exerts its effects according to the individual metabolic conditions of each system, and that chemiluminescence assays can discriminate among them, excluding cyanide as a specific effector on light-emission. We have been able to observe both an enhancing effect of cyanide in the chemiluminescence of leucocytes (Kakinuma *et al.*, 1979) and *Acanthamoeba castellanii* cells (Lloyd *et al.*, 1979), and an inhibitory effect on submitochondrial particles (reported in the present paper).

With regard to the quality of light-emission, the effects of β-carotene and 1,4-diazabicyclo[2,2,2]-octane suggest an involvement of molecular singlet oxygen in the chemiluminescence of mitochondrial membranes. Cytochrome *c*-supplemented submitochondrial particles showed the same response to β-carotene and 1,4-diazabicyclo[2,2,2]-octane (results not shown) as that described in Fig. 7, thus pointing out that the nature of the increased light-emission was not altered on addition of ferricytochrome *c*. In this sense, bovine heart submitochondrial particles and rat liver microsomal fraction (Sugioka & Nakano, 1976) seem to behave similarly with respect to light-emission: in both cases singlet molecular oxygen appears to be mainly responsible for the observed chemiluminescence.

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