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Oxygen- or organic hydroperoxide-induced chemiluminescence of brain and liver homogenates

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Oxygenation of anaerobically isolated brain and liver homogenates is associated with chemiluminescence and formation of lipid hydroperoxides, the latter determined by the thiobarbituric acid assay. Light emission and formation of malonaldehyde are 20-fold higher in brain than in liver; chemiluminescence of both decays when accumulation of malonaldehyde ceases. Exogenous organic peroxides, such as t-butyl hydroperoxide, inhibit the light-emission response to oxygenation by brain homogenate, whereas they enhance that of liver homogenate. t-Butyl hydroperoxide-induced photoemission of liver homogenate shows a polyphasic kinetic pattern that is O_2-dependent. The spectral analysis of chemiluminescence arising from brain and liver homogenates on oxygenation shows a spectrum with five emission bands at 420–450, 475–485, 510–540, 560–580 and 625–640nm. These bands are subjected to intensity changes or shifts of the wavelength whenever t-butyl hydroperoxide is present, either inhibiting or stimulating light emission. The blue-band chemiluminescence, around 435nm, is possibly due to the weak light emission arising from excited carbonyl compounds [Lloyd (1965) *J. Chem. Soc. Faraday Trans.* **61**, 2182–2193; Vassil'ev (1965) *Opt. Spectrosc.* (*USSR*) **18**, 131–135], whereas the presence of other bands suggests generation of singlet molecular oxygen either in the process triggered on oxygenation (lipid peroxidation) or after supplementation with organic hydroperoxides. We offer several explanations for the spectral analysis presented here.

Low-intensity- or ultraweak-chemiluminescence of systems undergoing lipid peroxidation reflects oxidation reactions involving molecular O_2, especially generation of short lived free radicals or excited states derived from the termination reactions of the free-radical process of lipid peroxidation (Vassil'ev & Vichutinskii, 1962; Stauff *et al.*, 1963; Barenboim *et al.*, 1969; Cadenas *et al.*, 1980*c*,*d*). The generation of excited species formed during the free-radical decomposition of lipid peroxides, as reflected by chemiluminescence, represents only side reactions of the overall lipid peroxidation; therefore, the effective quantum yield of these chemiluminescence reactions is extremely low, in the range 10^{-9}–10^{-12} (Stauff *et al.*, 1963; Vassil'ev, 1965; Cadenas *et al.*, 1980*c*,*d*), in terms of photon emitted/hydroperoxide decomposed or photon emitted/O_2 consumed.

Chemiluminescence accompanying lipid peroxidation reactions has been demonstrated in intact and perfused organs (Boveris *et al.*, 1980; Cadenas *et al.*, 1980*a*), tissue homogenates (Di Luzio & Stege, 1977), and isolated mitochondrial (Vladimirov *et al.*, 1971; Cadenas *et al.*, 1980*b*) and microsomal (Howes & Steele, 1971; Nakano *et al.*, 1975; Sugioka & Nakano, 1976) subcellular fractions.

The chemiluminescence technique permits continuous monitoring of lipid-peroxidation reactions occurring in tissue homogenates and, therefore, the detection of complex kinetic patterns that are difficult to follow with other techniques; this establishes an advantage over the detection of malonaldehyde by the thiobarbituric acid assay.

The aim of the present study was to support the hypothesis that singlet oxygen is yielded in the lipid peroxidation process of tissue homogenates as a response to oxygenation; furthermore, we have extended our research to a hypothetical hyperoxic condition created by supplementation with organic hydroperoxides. We describe here the kinetics and spectral analysis of the chemiluminescence of brain and liver homogenates in conditions that involve lipid peroxidation. We have chosen spectral analysis

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to identify the photoemissive species involved in light emission of tissue homogenates, since their identification by chemical analysis is not reliable due to their high unspecificity (Foote, 1979).

On the other hand, this report emphasizes the use of homogenates as compared with intact tissue, cell preparations, or cell-free preparations because of (1) our observations of maximal chemiluminescent signals from the whole homogenate; (2) the increasing lipid peroxidation of membranes on subsequent fractionation procedures and (3) the suitability of homogenates, compared with intact organs, to the experimental models carried out in this research.

Materials and methods

Preparation of tissue homogenates

The liver (or brain) of Charles River male rats (250–300 g) was perfused *in situ* with an O_2-free, ice-cold 0.9% NaCl solution, driven into either the portal vein or the inferior vena cava with a peristaltic pump (Harvard Apparatus, Millis, MA, U.S.A.) at a flow rate of 32 ± 2 ml/min. Liver or brain was then removed and homogenized with N_2-gassed, ice-cold 140 mM-KCl/5 mM-potassium phosphate solution. Homogenates were centrifuged at 500 ***g*** for 10 min to eliminate cellular debris. The 500 ***g*** supernatant was kept at 0°C under anaerobic conditions until it was used for the different assays.

Photon counting

Chemiluminescence of tissue homogenates was measured with a photon counter as described by Boveris *et al.* (1980). A red sensitive EMI (9658A) photomultiplier responsive in the range 300–900 nm and cooled to −40°C by a thermoelectric cooler (EMI-Gencom, Plainview, NY, U.S.A.) was used. The phototube output was connected to an amplifier-discriminator (model 1121; Princeton Applied Research, Princeton, NJ, U.S.A.) adjusted for single photon counting and connected to both a frequency counter (Heathkit IB 1100; Heath, Benton Harbor, MI, U.S.A.) and a recorder. Efficient light collection and isolation of the sample were established by using a Lucite rod as optical coupler. Dark current (recorded with the shutter in the 'closed' position) was about 50 ± 7 counts/s and background value (shutter in 'open' position) was about 150 ± 25 counts/s. Chemiluminescence was expressed in counts/s; according to the efficiency of our photon counter, 1 count/s corresponds approximately to 10 photons/s.

Chemiluminescence reactions were carried out in 0.1 M-potassium phosphate buffer, pH 7.2–7.3, in a 34 mm × 35 mm × 5 mm (11.9 cm^2 surface) cuvette thermostatically controlled at 37°C. Oxygenation was carried out with 100% pure O_2 flushing constantly into the sample through thin polyethylene tubing; when anaerobic conditions were required, 100% N_2 was used instead of O_2.

Spectral distribution of the homogenate chemiluminescence was carried out with different Kodak Wratten filters (Eastman-Kodak Co., Rochester, NY, U.S.A.) of the cut-off type attached to a rotating disc that moved within a 2 mm-gap between the light guide and the sample; each rotating disc or wheel contained seven Wratten gelatin filters and a filter-free space that served as control; the rotating disc was operated manually to the position desired from the outside of the light-tight box. The counts obtained through successive filters proceeding from red to blue were subtracted to give the corresponding incremental counts plotted in Figs. 6 and 7. The counts were corrected for the sensitivity characteristic of the photomultiplier, according to the manufacturer's data (EMI–Gencom, Plainview, NY, U.S.A.); thus, the precaution of the measurements in the red region was greater than in the blue region. Kodak Wratten filters selected were: 2B, 2A, 3, 4, 8, 9, 12, 16, 21, 22, 23A, 24, 25, 26, 29, 92, 70 and 89B.

Chemical assays

The thiobarbituric acid assay (Bernheim *et al.*, 1948) was carried out as follows. Homogenate (0.1 ml) with a protein concentration of 1.0–1.5 mg/ml was diluted to 1.5 ml with water and precipitated with trichloroacetic acid; the supernatant was incubated with thiobarbituric acid for 10 min at 100°C; the amount of malonaldehyde formed at different incubation periods was expressed in nmol/mg of protein by using $\varepsilon_{535} = 156\,\text{litre}\cdot\text{mmol}^{-1}\cdot\text{cm}^{-1}$ (Sinnhuber & Lu, 1958).

Glutathione peroxidase (EC 1.11.1.9) activity in tissue homogenates was determined by following spectrophotometrically the rate of NADPH oxidation coupled to glutathione peroxidase activity in an assay mixture containing 0.15 mM-β-NADPH, excess glutathione reductase (1.6 m-units/ml), 0.2 mM-reduced glutathione, 5 mM-t-butyl hydroperoxide and 0.04–0.08 mg of protein/ml of tissue homogenate. The activity was expressed in nmol of NADPH oxidized/min per mg of protein by using $\varepsilon_{340} = 6.22\,\text{litre}\cdot\text{mmol}^{-1}\cdot\text{cm}^{-1}$ (Pinto & Bartley, 1969; Günzler *et al.*, 1974).

Protein concentration was assayed as described by Lowry *et al.* (1951).

Chemicals

t-Butyl hydroperoxide was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 2-Thiobarbituric acid, glutathione reductase (type III), reduced glutathione and β-NADPH were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other reagents were of analytical grade.

Results

Chemiluminescence of liver and brain homogenates

Brain homogenate, maintained under anaerobic conditions, showed chemiluminescence immediately after oxygenation; the rate of increase of chemiluminescence was approx. 150 counts/s per min (Fig. 1); maximal light-emission intensity was obtained in about 50–60 min, and after a brief plateau, decayed slowly to the background value. Chemiluminescence of liver homogenates showed a lag phase that varied between 20 and 40 min and a maximal chemiluminescence intensity in about 1.5–2.0 h.

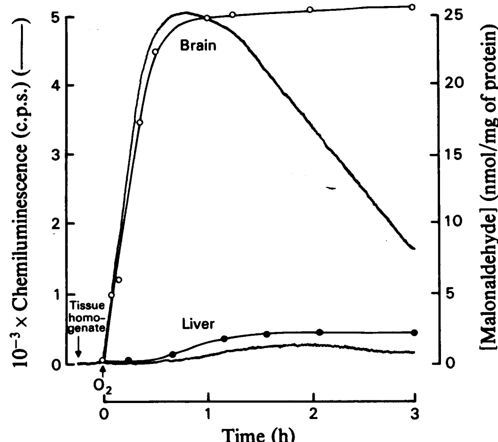


Fig. 1. *Chemiluminescence and malonaldehyde formation of brain and liver homogenates as a response to oxygenation*

Brain and liver homogenate (both 4.3 mg of protein/ml) were kept under anaerobic conditions in 0.1 M-potassium phosphate buffer, pH 7.2–7.3. The chemiluminescence reaction was started on oxygenation of the reaction mixture. Malonaldehyde formation by brain (O) and liver (●) homogenates was determined as described in the Materials and methods section.

Total chemiluminescence yield of brain homogenate was about 20-times higher than that of liver homogenate when compared on the basis of protein concentration. This is also reflected in the mounts of lipid hydroperoxide formed (measured as malonaldehyde by the thiobarbituric acid assay). Formation of malonaldehyde and chemiluminescence showed similar increases after oxygenation started; however, when the accumulation of malonaldehyde ceased, with a maximum of 2.3 (liver) and 25 (brain) nmol of malonaldehyde/mg of protein, the chemiluminescence intensity started a slow decay (Fig. 1).

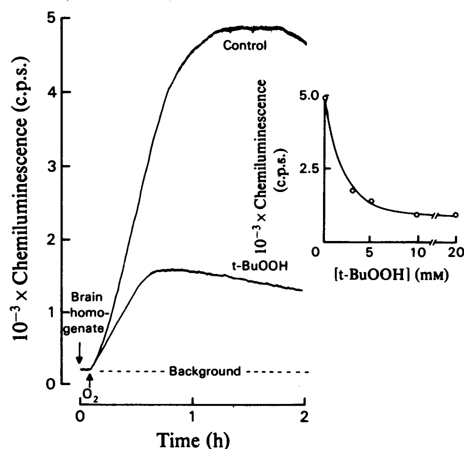


Fig. 2. *Inhibitory effect of t-butyl hydroperoxide on the O_2-induced chemiluminescence of brain homogenate*

The assay mixture consisted of brain homogenate (4.5 mg of protein/ml) in 0.1 M-potassium phosphate buffer, pH 7.2–7.3; the reaction was started on oxygenation in the absence (control) or presence of 5 mM-t-butyl hydroperoxide (t-BuOOH). The inset shows the effect of increasing concentrations of t-butyl hydroperoxide on the O_2-induced chemiluminescence of brain homogenate (assay conditions were as in the main Figure).

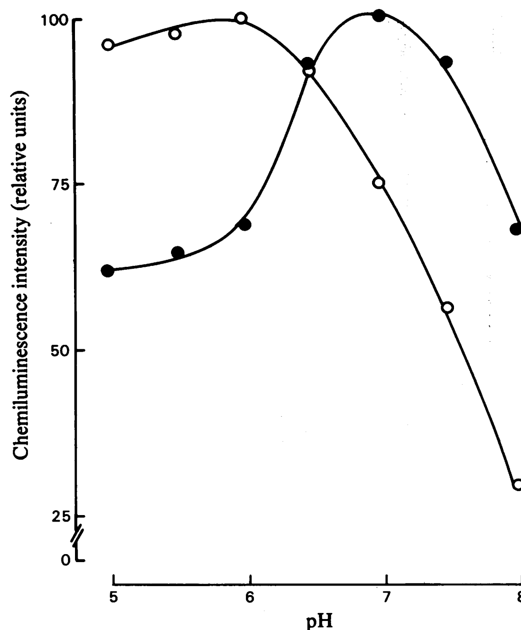


Fig. 3. *pH-dependence of the O_2-induced chemiluminescence of brain homogenate in the absence* (O) *and presence* (●) *of t-butyl hydroperoxide*

Assay conditions consisted of brain homogenate (4.2 mg of protein/ml) in 0.1 M-potassium phosphate buffer, at different pH values, with and without t-butyl hydroperoxide (5 mM). Brain homogenate was kept under anaerobic conditions and, to start the reaction, O_2 was flushed into the reaction mixture.

This kinetic difference between malonaldehyde formed and chemiluminescence was also observed by Vladimirov *et al.* (1971) in the Fe^{2+}-induced light emission of mitochondria.

Effect of t-butyl hydroperoxide on chemiluminescence of brain homogenate

The free-radical decomposition of organic peroxides, such as t-butyl hydroperoxide, is accompanied by low-intensity chemiluminescence; ferricytochrome *c* (Cadenas *et al.*, 1980*c*) and mitochondrial membranes (Wang & Kimura, 1976; Cadenas *et al.*, 1980*b*) catalyse effectively the decomposition of organic hydroperoxide. When supplemented with t-butyl hydroperoxide, however, brain homogenate did not yield light emission, and, moreover, its response to oxygenation was inhibited (Fig. 2). This is indicated by the slower rate of change of chemiluminescence in the t-butyl hydroperoxide-added brain homogenate, compared with the unsupplemented homogenate; the inset of Fig. 2 shows a half-maximal inhibition at 1.3mM-t-butyl hydroperoxide. It is worth noting that the rate of increase of chemiluminescence of hydroperoxide-supplemented brain homogenate is more in the order of its response to oxygenation than in the range of a t-butyl hydroperoxide-induced light emision, which, in general, reached its maximum within 3–5 min (Boveris *et al.*, 1980; Cadenas *et al.*, 1980*b,c,d,e*).

The O_2-induced light emission and the t-butyl hydroperoxide-inhibited light emission of brain homogenate showed a different pH dependence (Fig. 3). In the former condition, chemiluminescence intensity is maximal between the pH range 5.0–6.0, and diminishes beyond pH 6.0, whereas light emission of the t-butyl hydroperoxide-supplemented brain homogenate shifted the optimal chemiluminescence intensity to pH 6.5–7.5, diminishing before and after these values.

t-Butyl hydroperoxide-induced chemiluminescence of liver homogenate

Kinetics. Unlike brain homogenate, liver homogenate supplemented with t-butyl hydroperoxide immediately yielded light emission (Fig. 4). O_2-induced and t-butyl hydroperoxide induced chemiluminescence of liver homogenate differ in their kinetic pattern, in addition to total chemilumi-

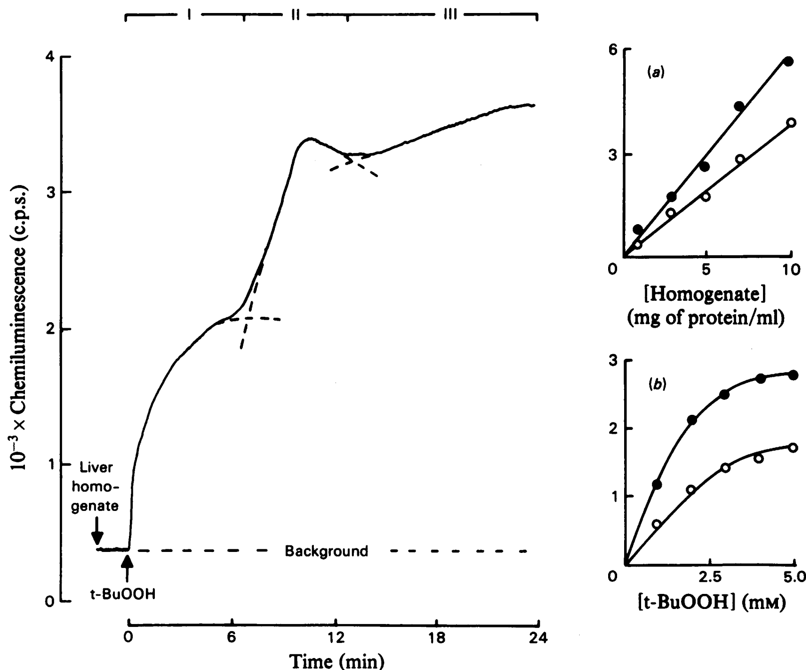


Fig. 4. *Kinetics of the t-butyl hydroperoxide-induced chemiluminescence of liver homogenate*
Liver homogenate (5.1 mg of protein/ml) in 0.1 M-potassium phosphate buffer, pH 7.2–7.3, was supplemented with 5 mM-t-butyl hydroperoxide. The insets show (*a*) dependence of phases I (○) and II (●) (as marked in the main Figure) on protein concentration and (*b*) dependence of phases I (○) and II (●) on t-butyl hydroperoxide concentration. The experiments were carried out under aerobic conditions. Abbreviation used: t-BuOOH, t-butyl hydroperoxide.

nescence intensity values and rate of chemiluminescence, which are significantly higher with t-butyl hydroperoxide. On the other hand, the O_2-induced and t-butyl hydroperoxide-induced light emission of liver differ kinetically; the former shows a simple kinetic response (Fig. 1), whereas the latter shows a complex kinetic response in three phases (Fig. 4). The first phase (I) rises rapidly and reaches its maximum in about 6 min; the second phase (II) reaches its maximum about 5–6 min later, and continues with a mildly-increasing third phase (III) that becomes stable within 12–15 min. Phases I and II depend linearly on protein concentration and show saturation values in their dependence on t-butyl hydroperoxide concentration (insets in Fig. 4) (half-maximal chemiluminescence yields for phases I and II were obtained with about 1.9 and 1.3 mM-t-butyl hydroperoxide respectively). The transition from phase I to phase II is more distinctive at high t-butyl hydroperoxide concentrations, whereas at low concentrations the phases merge.

Oxygen dependence. The polyphasic chemiluminescence response of liver homogenate to t-butyl hydroperoxide is completely aborted under anaerobic conditions (Fig. 5*a*). Presence of O_2 in the medium is also necessary for complete development of the second phase; Fig. 5(*b*) shows a somewhat decreased phase II when oxygenation is halted after completion of phase I and replaced by N_2-flushing in the incubation medium. When the reaction is started under anaerobic conditions no chemiluminescence is detected (Fig. 5*c*); however, it seems that not all free-radical processes are arrested, since subsequent oxygenation leads to a rapid burst in chemiluminescence, the rate of which shows a quadratic dependence on the time of incubation under anaerobic conditions (Fig. 5*d*). Thus it is probable that under these conditions only the formation of O_2-containing free radicals (ROO$^•$) is arrested, but that of O_2-devoid free radicals (R$^•$) will continue; the longer the incubation under anaerobic conditions, the higher the accumulation of R$^•$, and consequently the higher the rate of the chemiluminescence burst when oxygenation is restored.

Glutathione peroxidase activity of brain and liver homogenates

Glutathione peroxidase activity of brain homogenate (0.4 ± 0.05 nmol/min per mg of protein) was found to be negligible when compared with that of

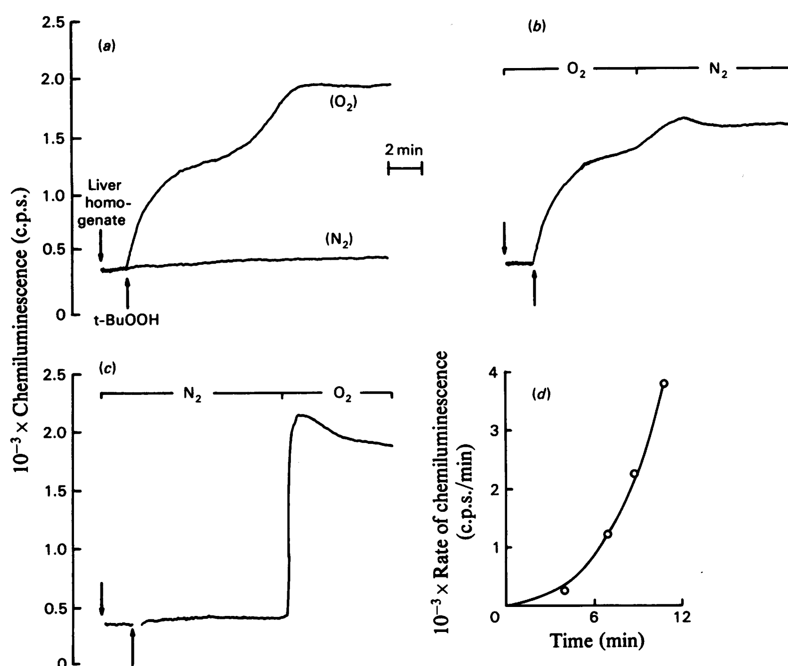


Fig. 5. *O_2-dependence of the chemiluminescence of liver homogenate supplemented with t-butyl hydroperoxide*
Liver homogenate (4.1 mg of protein/ml) in 0.1 M-potassium phosphate buffer, pH 7.2–7.3, was supplemented with 5 mM-t-butyl hydroperoxide. (*a*) Under aerobic (O_2) and anaerobic (N_2) conditions; (*b*) and (*c*), the changes from aerobic to anaerobic (and vice versa) conditions are marked in the Figure; (*d*) dependence of the rate of the burst of chemiluminescence on the incubation time under anaerobic conditions (Fig. 5*c* as a model). Abbreviation used: t-BuOOH, t-butyl hydroperoxide.

liver homogenate (182 ± 12 nmol/min per mg of protein); these values agree with previous reports of glutathione peroxidase activity in brain and liver (Prohaska & Ganther, 1976; Sies *et al.*, 1979).

Spectral analysis of the chemiluminescence of brain homogenate

Fig. 6(*a*) illustrates spectral analysis of the chemiluminescence response of brain homogenate to oxygenation. Five emission bands were found at around 420–450, 475–485, 510–540, 560–590 and 625–650 nm, with a shoulder at 650–710 nm, with relative intensities of 0.06, 0.29, 0.51, 0.58 and 1.0, for the five bands respectively, and 0.52 for the shoulder. The red-light band (625–650 nm), with its shoulder (650–710 nm), is the largest contribution to the total photoemission. Since the O_2-induced chemiluminescence kinetic pattern of brain homogenate shows three phases (cf. Fig. 1; an increasing chemiluminescence phase, a plateau and a decaying phase), spectral analysis of each of these phases was performed; no substantial difference in the spectral distribution was found, and the bands remained unshifted.

Spectra analysis of the chemiluminescence response of liver to oxygenation was similar to that of brain shown in Fig. 6.

The spectral distribution of t-butyl hydroperoxide-inhibited chemiluminescence of brain homogenate remains basically the same as the O_2-induced chemiluminescence, except for a small shift in the wavelengths now at 410–420, 460–480, 515–530, 555–585 and 629–645 nm, with relative intensities of 0.1, 0.31, 0.46, 0.68 and 1.0 respectively (Fig. 6*b*).

Spectral analysis of chemiluminescence of liver homogenate supplemented with t-butyl hydroperoxide

Because of the complex kinetics of t-butyl hydroperoxide-induced light emission of liver homogenate (Fig. 4), spectral analysis might afford different chemiluminescence species, or at least different contributions of the same species in each kinetic phase. Phases I and II were found to differ quite significantly, not in the distribution, but in the relative contribution of each band (Fig. 7); emission bands in phase I were found at 410–425, 460–480, 510–530, 555–575 and 590–655 nm, with relative intensities of 0.32, 0.71, 0.88, 0.85 and 1.0 respectively (Fig. 7*a*). On the other hand, phase II showed emission bands at 420–450, 460–485, 505–525, 560–575 and 590–650 nm, with relative intensities of 0.43, 0.66, 0.48, 0.32 and 1.0 respectively (Fig. 7*b*). This implies an increase in the bands at about 420 nm, and decreases in the bands at about 470, 520 and 570 nm, and a small red-shift in the 420 and 610–630 nm bands.

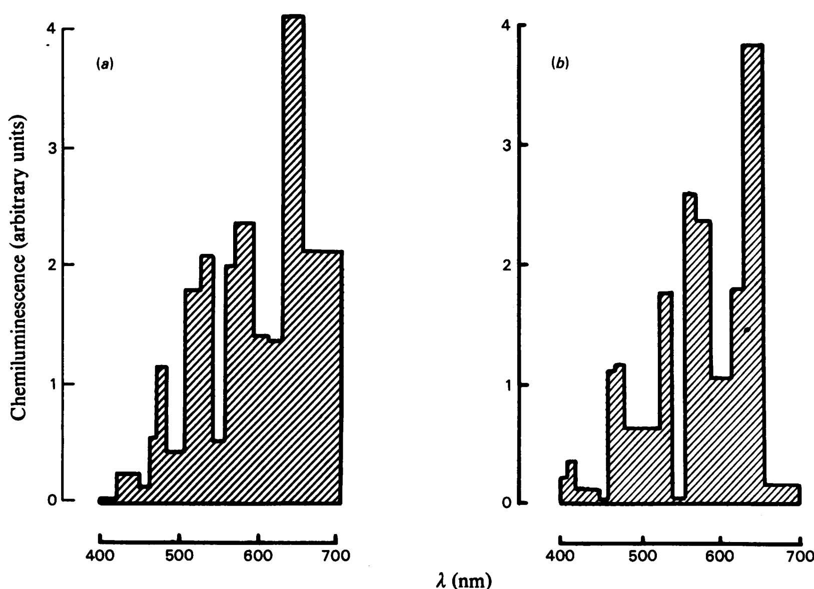


Fig. 6. *Spectral analysis of the chemiluminescence response of brain homogenate to oxygenation in the absence* (*a*) *and presence* (*b*) *of t-butyl hydroperoxide*
Assay conditions were as described in the legend to Fig. 2.

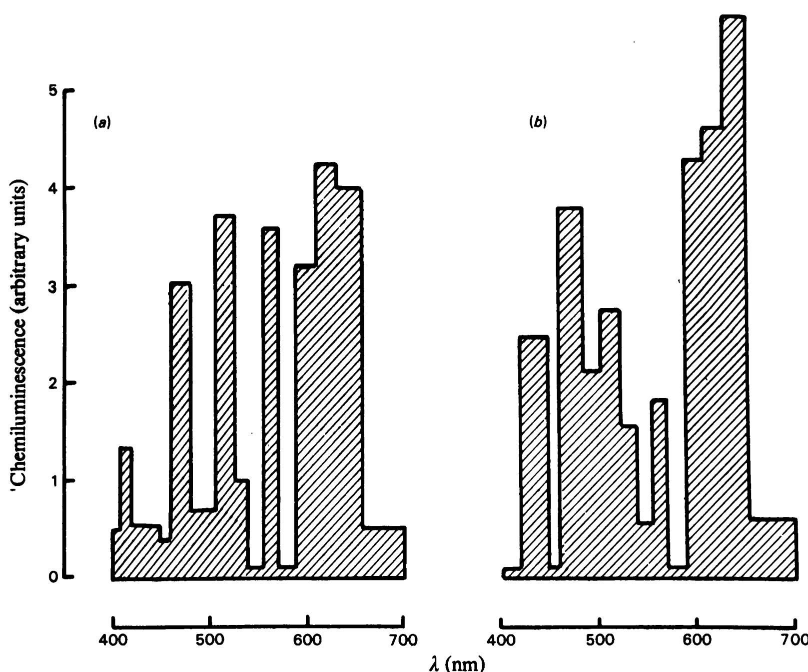


Fig. 7. *Spectral analysis of the t-butyl hydroperoxide-induced chemiluminescence of liver homogenate*
Liver homogenate (5.1 mg of protein/ml) in 0.1 M-potassium phosphate buffer, pH 7.2–7.3, was supplemented with 5 mM-t-butyl hydroperoxide, under aerobic conditions. Spectral distribution was carried out as described in the Materials and methods section. Spectral distributions corresponding to phase I (*a*) and phase II (*b*) are shown.

Discussion

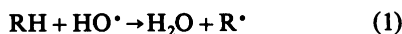
Chemiluminescence and thiobarbituric acid assay point to an outstanding difference between lipid peroxidation in brain and liver homogenates on oxygenation. This seems to reflect a series of factors, which can be examined in terms of lipid composition, molecular organization and defence mechanisms particular to each tissue, along with the molecular mechanisms operating in each case.

The lipid composition of rat brain (1.85 mg/g wet wt.) and liver (1.27 mg/g wet wt.) (White, 1973) can hardly explain the 20-times difference in both chemiluminescence intensity and malonaldehyde formation. The unsaturated/saturated lipids ratio is close to 1.0 for both tissues; the only major difference is the three-times higher content of oleic acid in the brain, but even comparing chemiluminescence of both tissues on this basis, the brain remains seven-times more effective.

The defence mechanisms of each tissue against lipid peroxidation are represented by several lipid-soluble vitamins in addition to the glutathione peroxidase activity. The tissue content of lipid-soluble antioxidants, such as vitamin E, acting as a free-radical scavenger (Tappel, 1979), and vitamin A, acting as a singlet-oxygen quencher (Foote, 1976), could certainly differentiate between liver and brain chemiluminescence. Liver is known to accumulate vitamin A at concentrations that are 10–20-times higher than those of other tissues (Olson, 1968). In addition, the O_2-induced lipid peroxidation of tissue homogenates is a rather slow process; malonaldehyde formation (46 and 4 nmol/h per mg of protein in brain and liver respectively; Fig. 1) would correspond to about 460 and 40 nmol of lipid peroxide/h per mg of protein in brain and liver, since malonaldehyde formation accounts for about 10% of the O_2 uptake during lipid peroxidation (Hochstein *et al.*, 1964; Chance *et al.*, 1979). Then, this slow formation of lipid hydroperoxide could be scavenged partially by glutathione peroxidase, negligible in brain but high in liver, although this activity would be restricted by lack of reduced cofactors (reduced glutathione and NADPH, active through glutathione reductase).

The molecular mechanisms that on oxygenation, and eventually on lipid peroxidation, lead to light emission in brain and liver homogenates might be expressed through the classical free-radical reactions involving unsaturated lipids (RH) with further formation of lipid hydroperoxides (ROOH) (Pryor, 1976) [reactions (1), (2), and (3)]. It is beyond the scope of the present paper to establish the oxidizing

species (O_2^-, H_2O_2, $HO^\bullet$ and $^1O_2$) triggering lipid peroxidation, and moreover, it is apparent that all these species participate in the process of lipid peroxidation (Chance *et al.*, 1979). However, we have selected $HO^\bullet$ as the most probable triggering species [reaction (1)] (Chance *et al.*, 1979), and we consider that singlet oxygen is a product rather than an initiator of lipid peroxidation.



$$RH + HO^\bullet \rightarrow H_2O + R^\bullet \quad (1)$$

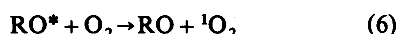
$$R^\bullet + O_2 \rightarrow ROO^\bullet \quad (2)$$

$$ROO^\bullet + RH \rightarrow ROOH + R^\bullet \quad (3)$$

Chemiluminescence is associated with the fraction of lipid hydroperoxides that undergoes free-radical decomposition, one of its products being lipid peroxy radicals ($ROO^\bullet$) (Evans *et al.*, 1967). The self-reaction of primary and secondary peroxy radicals (Russell, 1957) generates either singlet oxygen [reaction (4)] or an excited triplet carbonyl group (RO^*) [reaction (5)], which may yield singlet oxygen after quenching by molecular oxygen [reaction (6)].

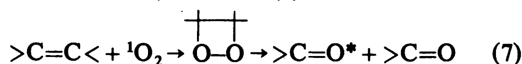
$$ROO^\bullet + ROO^\bullet \rightarrow ROH + RO + {}^1O_2 \quad (4)$$

$$ROO^\bullet + ROO^\bullet \rightarrow ROH + RO^* + O_2 \quad (5)$$



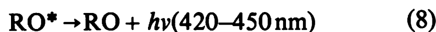
$$RO^* + O_2 \rightarrow RO + {}^1O_2 \quad (6)$$

Generated singlet oxygen might react with unsaturated and substituted lipids (>C=C<) leading to the formation of unstable dioxetan intermediates (Foote, 1976; Hastings & Wilson, 1976) that on decomposition yield excited carbonyl compounds (>C=O* or RO*) [reaction (7)].

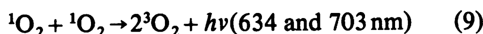


$$>C{=}C< + {}^1O_2 \rightarrow O{-}O \rightarrow >C{=}O^* + >C{=}O \quad (7)$$

The sources of visible-range photons are the spontaneous triplet–singlet transitions of the excited carbonyl groups and the dimol emission of singlet oxygen [reactions (8) and (9)].

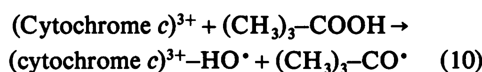


$$RO^* \rightarrow RO + h\nu(420\text{–}450\,nm) \quad (8)$$



$${}^1O_2 + {}^1O_2 \rightarrow 2{}^3O_2 + h\nu(634 \text{ and } 703\,nm) \quad (9)$$

Brain and liver homogenates supplemented with t-butyl hydroperoxide show a different chemiluminescence response; liver homogenate yields photoemission but brain homogenate does not, which is opposite to their chemiluminescence responses on oxygenation. Light emission from the free-radical decomposition requires: (*a*) a homolytic scission of the hydroperoxide; (*b*) a free-radical oxidation process; and (*c*) the generation of chemiluminescent species (Cadenas *et al.*, 1980*c*). Reaction (10) accounts for the haemoprotein (cytochrome *c*)-catalysed rupture of the hydroperoxide molecule (Tappel, 1955; Cadenas *et al.*, 1980*c*).



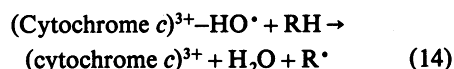
$$(\text{Cytochrome } c)^{3+} + (CH_3)_3\text{–}COOH \rightarrow (\text{cytochrome } c)^{3+}\text{–}HO^\bullet + (CH_3)_3\text{–}CO^\bullet \quad (10)$$

The tertiary t-butoxy radicals give a primary methyl peroxy radical via rearrangement and β-scission and subsequent O_2-addition [reactions (11) and (12)] (Ingold, 1969). Other free-radical propagating reactions would include hydrogen atom abstraction by the alkoxy radical or the haem-bound hydroxyl radical [reactions (13) and (14)], yielding the primary and secondary peroxy radicals, which are the precursors of the chemiluminescent species [reactions (4) and (5)].

$$(CH_3)_3\text{–}CO^\bullet \rightarrow CH_3\text{–}CO\text{–}CH_3 + CH_3^\bullet \quad (11)$$

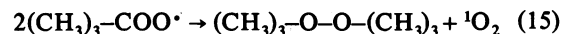
$$CH_3^\bullet + O_2 \rightarrow CH_3OO^\bullet \quad (12)$$

$$(CH_3)_3\text{–}CO^\bullet + RH \rightarrow (CH_3)_3\text{–}COH + R^\bullet \quad (13)$$



$$(\text{Cytochrome } c)^{3+}\text{–}HO^\bullet + RH \rightarrow (\text{cytochrome } c)^{3+} + H_2O + R^\bullet \quad (14)$$

An additional source of chemiluminescent species could be expected by the self-reaction of t-butyl peroxy radicals (Pryor, 1978) [reaction (15)].



$$2(CH_3)_3\text{–}COO^\bullet \rightarrow (CH_3)_3\text{–}O\text{–}O\text{–}(CH_3)_3 + {}^1O_2 \quad (15)$$

Experimental evidence for the involvement of peroxy radicals is given by the O_2 requirement of photoemission (Fig. 5) along with the inhibitory effect of the radical trap 2,5-di-t-butylquinol (results not shown). An accumulation of $R^\bullet$ seems possible because of the chemiluminescence burst on restoring oxygenation, as well as the quadratic dependence of maximal photoemission on the time under anaerobiosis (Fig. 5).

The lack of efficiency of brain homogenate in yielding light emission when supplemented with t-butyl hydroperoxide is understood as an inability to catalyse reaction (10) effectively (ferricytochrome *c* and cytochrome *P*-450 are likely to be the more important catalysts for this reaction), as well as to the presence of an endogenous trapping agent capable of reacting with t-butoxy radicals. Alkoxy radicals are trapped effectively by =NH and –COH groups (Ingold, 1969).

Spectral analysis of the chemiluminescence of brain and liver homogenates on oxygenation and/or supplementation with organic hydroperoxide shows a five-band emission spectrum (Figs. 6 and 7). The bands were found at 420–450, 475–485, 510–540, 560–580 and 625–650 nm. Small shifts, usually in the range 10–15 nm, were found on supplementation with t-butyl hydroperoxide.

Four of these bands are located around 480, 520, 580 and 635 nm, wavelengths that correspond to the

dimol emission of singlet molecular oxygen in several electronic excited and vibrational states (Seliger, 1960; Khan & Kasha, 1970; Inaba *et al.*, 1979). A better correspondence of emission bands and the wavelengths corresponding to the various electronic and vibrational transitions cannot be expected for the low-intensity chemiluminescence of biological samples; shifts of ± 15 nm are to be expected due to the complex composition of the system (Andersen *et al.*, 1978). A Wedge interference filter (Cadenas *et al.*, 1980*e*) might allow a better resolution but it is not applicable at the present time due to the low counts rate.

Emission bands at about 480, 520, 580 and 640 nm were reported for the chemiluminescence of a reconstituted microsomal system undergoing lipid peroxidation (Sugioka & Nakano, 1976) and for the reaction of linoleic acid hydroperoxide and Ce^{4+}, which yields $ROO^{\bullet}$ (Nakano *et al.*, 1976). It is then apparent that these four emission bands identify singlet oxygen as a product of lipid peroxidation. The differences between these four-band spectra, attributable to singlet oxygen dimol emission, and the two-band spectra of the chemiluminescence of H_2O_2/NaOCl mixtures, also attributable to singlet oxygen (Khan & Kasha, 1963; Seliger, 1964) are: (*a*) lack of a 703 nm band, clearly seen in the latter case, and (*b*) high intensity of the 480, 520 and 580 nm bands in the former case. The 580 nm band is almost negligible in the photoemission of H_2O_2/NaOCl mixtures (Seliger, 1964). The 480, 520 and 580 nm emission bands of singlet oxygen seem to appear in heterogeneous biological systems undergoing lipid peroxidation. Since singlet oxygen is sensitive to quenching by water (Merkel *et al.*, 1972), it seems that hydrophobic domains would favour emission at 480, 520 and 580 nm. Evidence for the 703 nm band was not obtainable in our case due to the lack of suitable filters.

The spectra in Figs. 6 and 7 show a small blue band of chemiluminescence between 410 and 440 nm that does not correspond to any known transition of singlet oxygen. This emission wavelength, though contributing very little to the total chemiluminescence spectrum, might be due to excited carbonyl compounds formed through the dioxetan mechanism [reaction (7)] or the self-reaction of peroxy radicals [reaction (5)]. Excited carbonyl compounds are believed to be generated in the chain-termination process of hydrocarbon oxidation and to emit light with a maximum in the 420–450 nm range (Lloyd, 1965; Vassil'ev, 1965).

The malonaldehyde accumulated in the tissue homogenates due to the lipid-peroxidation process is ruled out as an additional source of photoemission, since it does not emit radiation in the 300–700 nm range (Sugioka & Nakano, 1976). On the other hand, the possible generation of singlet oxygen through a peroxidase-catalysed decomposition of malonaldehyde (Vidigal-Martinelli *et al.*, 1979) seems very unlikely to occur in the present conditions; although liver homogenate might provide a peroxidase activity necessary to these reactions, its strict requirement of Mn^{2+} would halt the reaction. Moreover, we have reported on the inhibitory effect of Mn^{2+} on chemiluminescence (Cadenas *et al.*, 1980*f*).

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