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Organ chemiluminescence: Noninvasive assay for oxidative radical reactions

(light emission/nondestructive biochemistry/free radical reactions/liver *in situ*/perfused liver)

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ABSTRACT ***In situ*** **and perfused rat livers showed a spontaneous chemiluminescence of 7–12 counts/sec·cm^2 (corresponding to 7–12 × 10^3 photons/sec·cm^2); chemiluminescence was increased up to 30 times by infusion of exogenous hydroperoxides. The chemiluminescence of the perfused liver was oxygen dependent. Ethyl, *t*-butyl, and cumene hydroperoxides were almost equally effective in inducing light emission in the perfused liver. Glutathione release and chemiluminescence showed a parallel increase upon hydroperoxide supply to the perfused liver. A partial spectral analysis of the chemiluminescence of the perfused liver showed a predominance of red-light-emitting species, presumably arising from the singlet oxygen dimol-emission peaks. Many side reactions derived from the complex free radical sequence of lipid peroxidation could afford the chemistry leading to light emission, which represents only about 10^{-14} of the utilization of peroxide.**

Luminescence of organisms has fascinated scientists [see E. N. Harvey's classic volume, *Living Light* (1)], and the phenomenon generally can be related to peroxide and free radical metabolism, a topic of current interest in disease processes (2). The fact that luminescence might not be restricted to those forms of life having special organs containing enzyme systems such as luciferins/luciferases was underlined by the remarkable findings of Tarusov *et al.* in 1961. Those experimenters used photon counting to identify a weak light emission from mouse liver *in situ* (3); the observation was extended to brain, muscle, intestine, tissue homogenates, and lipid extracts (3, 4). (For a review, see ref. 5.) The existence of such light emission, which should be termed "low-level chemiluminescence" to differentiate it from the more effective photoemission of the luciferin/luciferase systems (5, 6), was soon related to oxygen-dependent chain reactions involving biological lipids (3–5). This early work lay fallow for years, notwithstanding the reports by Stauff and Ostrowski on the chemiluminescence of mitochondria (7) and Howes and Steele on the chemiluminescence of microsomes (8, 9), both isolated from rat liver. The more recent reports by Nakano *et al.* (10) and Sugioka and Nakano (11) of light emission during lipid peroxidation and other oxidative reactions (12) in microsomes revived interest in the phenomenon and suggested chemiluminescence as a tool for the investigation of the radical reactions of lipid peroxidation under physiological conditions.

We have recently reported that maximal light emission in isolated mitochondria and microsomes (13) and in submitochondrial particles (14) requires an electron transfer system, hydroperoxide, and oxygen, and that hydroperoxide-supplemented cytochrome *c* provides a chemiluminescent model system suitable for the elucidation of some of the molecular mechanisms responsible for light emission (15). On the other hand, isolated cells such as amoebae (16) and phagocytizing leukocytes (17) also have been found to be effective chemiluminescent sources.

The most important aspect of the organ chemiluminescence is that it gives readily detectable, continuously monitorable, noninvasive signals of oxidative metabolism. This article explores the possibility of continuously monitoring the metabolism of exposed or fiberoptic probed organs *in vivo* by the chemiluminescent technique. In this paper we report the spontaneous and hydroperoxide-induced chemiluminescence of the *in situ* and perfused rat liver, as well as a partial spectral analysis of the chemiluminescence of the perfused liver. Light emission seems to indicate the generation of short-lived free radicals and excited states derived from the side reactions of the free radical process of lipid peroxidation. A preliminary report on light emission has been published elsewhere (13).

MATERIALS AND METHODS

Photon Counting. A single-photon-counting apparatus was used (Fig. 1). Both an EMI 9658 photomultiplier, responsive in the range 300–900 nm, with an applied potential of −1.2 kV (dark current: 20–30 counts per second), and an RCA 8850 photomultiplier, responsive in the range 300–650 nm, with an applied potential of −1.8 kV (dark current: 300–400 counts per second) were used. Phototube output was connected to an amplifier–discriminator (model 1121; Princeton Applied Res., Princeton, NJ) adjusted for single photon counting and connected to both a frequency counter (Heathkit IB 1100, Heath, Benton Harbor, MI) and a recorder. The EMI phototube, cooled down to −40°C by a thermoelectric cooler (EMI-Gencom, Plainview, NY) and the RCA phototube were placed in an Ortec housing, sealed and surrounded by dry ice with a tube temperature of about −30°C. Efficient light collection and isolation from the sample were established by using a Lucite rod (22 cm long, 5-cm diameter, and 9.6-cm^2 surface) as optical coupler. A silicone oil-filled shutter allowed continuous operation of the photomultiplier. Either the animal for the liver measurements *in situ* or the perfused liver was placed in a special lighttight box, as close as possible to the end of the light guide (Fig. 1). Partial spectral distribution of chemiluminescence of the perfused liver was determined by placing Kodak Wratten filters (Eastman Kodak, Rochester, NY) in front of the EMI phototube. Filters used for this purpose were 2A (pale yellow), 29 (deep red), 70 (dark red), with spectral transmittance from 400 nm, 600 nm, and 650 nm, toward the infrared, respectively, and 64 (light blue-green) with spectral transmittance from 400 to 600 nm, which also provides some transmission beyond 710 nm. With studies *in vitro* a wedge interference filter (Schott Glass, Germany) was used as a monochromator. Erythrocyte measurements were performed in a

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Abbreviations: cps, counts per second; *t*BuOOH and CumOOH, *t*-butyl and cumene hydroperoxides.

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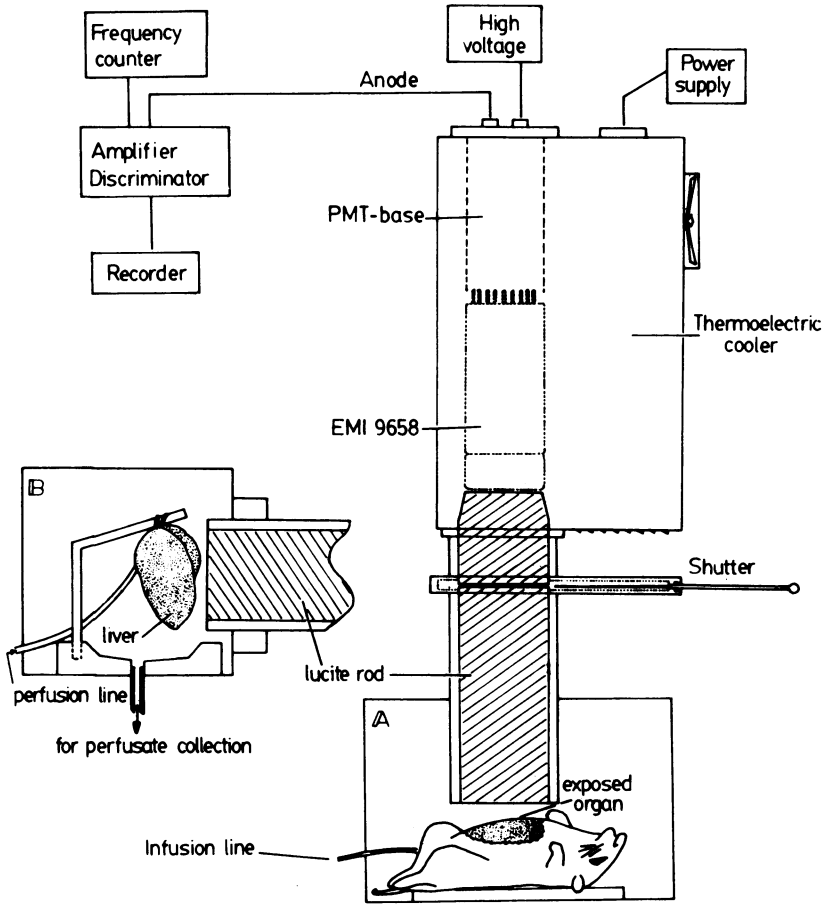

FIG. 1. Single-photon-counting apparatus for the measurement of organ chemiluminescence. The Lucite rod used as optical coupler is placed in front of the exposed liver *in situ* (*A*) or the perfused liver (*B*). PMT, photomultiplier tube.

10-ml Plexiglas cuvette equipped with a Clark oxygen electrode and a magnetic stirrer. All chemiluminescence measurements are expressed in arbitrary units, counts per second (cps; 1 cps corresponds to about 10^3 photons per sec).

Animal Preparations. Male Sprague–Dawley rats (200–260 g) maintained on a commercial diet ad lib and anesthetized with pentobarbital (10 mg/100 g of rat weight) were used. The liver was exposed *in situ* by opening the abdominal wall. Hydroperoxide was infused by a pump-driven microsyringe connected to a microcannula inserted in an intestinal branch of the portal vein. Isolated livers were perfused according to the conditions of a hemoglobin-free noncirculating system, as described (18). The perfusion medium consisted of 115 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$, and 25 mM $NaHCO_3$ equilibrated with an O_2/CO_2 (19:1) gaseous mixture. The perfusion fluid, kept at room temperature (22–25°C), was driven by a pump (Harvard Apparatus, Millis, MA) adjusted to a flow of 30 ± 2 ml/min (3.5 ± 0.3 ml/min per g of liver). Hydroperoxides were pumped into the perfusion fluid via a three-way valve by a syringe pump (model 241; Sage Instruments, Orion Research, Cambridge, MA). Erythrocytes from decapitated rats were obtained in the presence of heparin; the cells were sedimented by centrifugation and washed three times with 0.15 M NaCl.

Chemical Assays. Heme content of erythrocyte suspensions was determined by the pyridine/hematochrome method (19). Total glutathione in the perfusate was measured by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay (20) as described (18). The results are expressed as $\mu mol \cdot min^{-1} \cdot g$ of $liver^{-1}$ and the assay interval was every 1 min (Fig. 6).

Chemicals. Hydroperoxides were used as 0.2–0.4 M solutions in water (ethyl and *t*-butyl hydroperoxides) and in 10–20% dimethyl sulfoxide/water mixtures (*t*-butyl and cumene hydroperoxides). Ethyl hydroperoxide was obtained from Ferrosan (Malmo, Sweden), *t*-butyl hydroperoxide (*t*BuOOH) from Aldrich (Milwaukee, WI), and cumene hydroperoxide (CumOOH; α,α′-dimethylbenzyl hydroperoxide) from Matheson, Coleman, and Bell (Norwood, OH). All other chemicals were of analytical grade.

RESULTS

Chemiluminescence of the Liver *In Situ*: Effect of Hydroperoxides. The *in situ* liver of anesthetized rats showed an endogenous light emission of about 67 ± 10 cps (mean ± SEM) measured with an EMI 9658 red-sensitive phototube (Fig. 2) and of about 120 ± 25 cps when measured with an RCA 8850

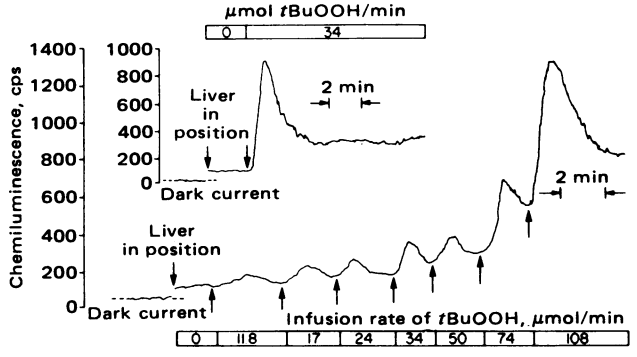

FIG. 2. Chemiluminescence of the rat liver *in situ*. *t*BuOOH was infused in an intestinal branch of the portal vein at the rates indicated by the numbers in the blocks. (*Inset*) Detail of the light emission overshoot observed upon changing hydroperoxide infusion rates. An EMI 9658 phototube was used.

green-sensitive phototube for a surface of about 10 cm^2 of the exposed liver. This light emission from liver tissue was about 25 times greater than the one previously recorded by Tarusov *et al.* (4); technical improvements may account for the difference. Infusion of *t*BuOOH into the portal vein at rates of 12–17 μmol/min caused only a moderate increase in chemiluminescence, but higher rates, 24–108 μmol/min, produced a marked augmentation of light emission (Fig. 2). During the first minute after the hydroperoxide infusion rate was changed, light emission fluctuated, but soon thereafter it reached a constant intensity. Indeed, light emission showed an overshoot every time that hydroperoxide levels were increased, returning afterward to a lower steady-state level. A detail of this initial burst of photoemission, characteristic of the *in situ* organ chemiluminescence, is illustrated in the *Inset* of Fig. 2. When hydroperoxide infusion was stopped, liver chemiluminescence decreased to a level corresponding to 20–30% of the maximal induced light emission within 10–15 min (data omitted). At hydroperoxide infusion rates higher than 100 μmol/min, a constant level of light emission was reached, and a rapid and severe damage of the liver was observed. Change in blood color due to methemoglobin formation, appearance of white spots on the liver surface, and rapid mortality of the animal were the most significant indications of tissue damage. In several experiments, replacing blood with saline solution lowered the erythrocyte content of the blood about 20–30% without significant change in the hydroperoxide-induced liver chemiluminescence, as compared with normal animals. Control experiments on isolated erythrocytes confirmed this conclusion.

Chemiluminescence of the Perfused Liver: Effect of Hydroperoxides. The perfused liver showed a spontaneous chemiluminescence of about 50 ± 12 cps (mean ± SEM) as measured with an EMI 9658 red-sensitive phototube, a level to similar to the one observed in the organ *in situ* (Fig. 3). Chemiluminescence reached steady states about 1 min after the hydroperoxide level was changed, and no overshoots were observed. Withdrawal of hydroperoxide reduced light emission to the initial levels in about 2–3 min (Fig. 3). Hydroperoxide infusion at high rates, over 120 μmol/min, especially when sustained over periods of 5–10 min, gave light emissions that lacked proportionality with hydroperoxide infusion rates and that did not return to background levels when hydroperoxide was removed from the perfusion fluid. These effects were accompanied by biochemical and morphological indications of severe and irreversible tissue damage, such as an abrupt decrease in glutathione release and the appearance of white spots on the liver surface.

Nitrogen-saturated perfusion fluid decreased the light

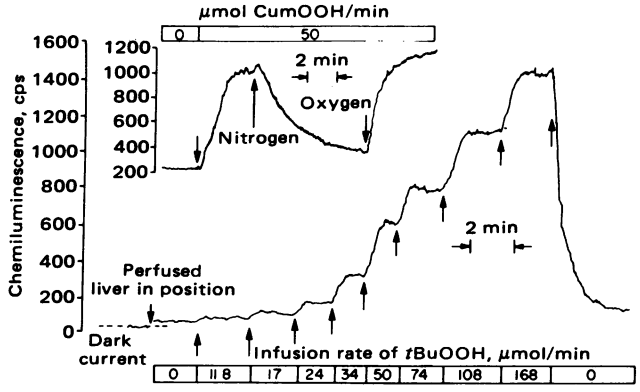

FIG. 3. Chemiluminescence of the perfused rat liver. *t*BuOOH and CumOOH were infused at the rates indicated by the numbers in the blocks. For the traces in the main figure and in the *Inset*, red-sensitive EMI 9658 and green-sensitive RCA 8850 phototubes were used, respectively.

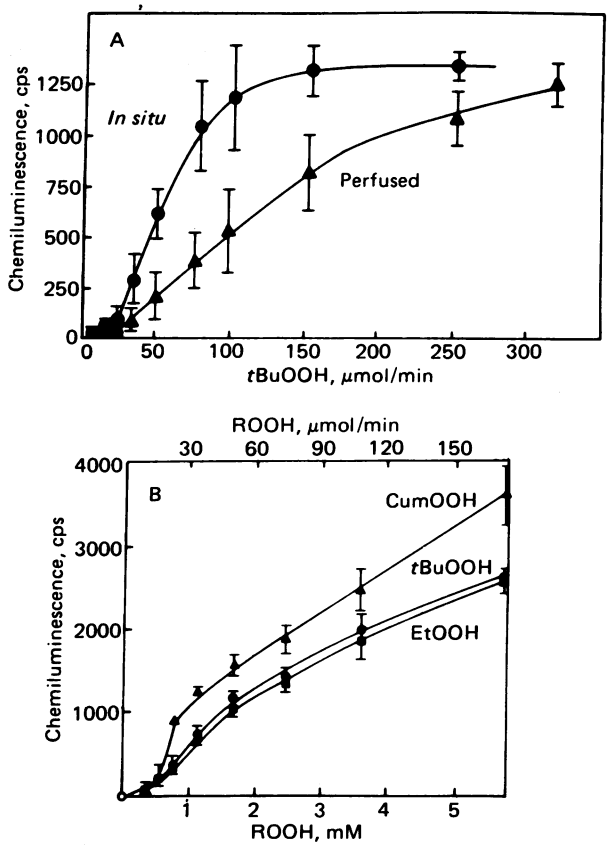

FIG. 4. Titrations of hydroperoxide-induced chemiluminescence in rat liver. (*A*) *In situ* and perfused liver chemiluminescence produced by *t*BuOOH infusion. An EMI 9658 phototube was used. (*B*) Effect of ethyl hydroperoxide (EtOOH), *t*BuOOH, and CumOOH on the chemiluminescence of perfused rat liver. The hydroperoxide concentration in the perfusion fluid is indicated on the bottom; the top scale indicates the hydroperoxide infusion rate. Symbols and bars indicate mean values ± SEM.

emission by 70–80%, and the emission returned to the aerobic level in about 1 min upon reoxygenation (Fig. 3 *Inset*). The incomplete inhibition of light emission produced by nitrogen-saturated perfusion fluid could be accounted for by an incomplete exclusion of oxygen due to gas diffusion into the perfusion system, or by a persistence of the radical reactions in the absence of oxygen.

Dependence of the Induced Chemiluminescence on Hydroperoxide Levels. The sigmoidal concentration–effect curves for hydroperoxide action on the chemiluminescence of both the *in situ* and the perfused liver indicate the existence of effective cellular protection systems against the accumulation of reactive intermediates (Fig. 4*A*). On the basis of similar hydroperoxide infusion rates, chemiluminescence in the *in situ* liver was found to be about twice as great as in the perfused liver up to a hydroperoxide infusion rate of about 150 μmol/min; higher infusions seemed to approach a similar maximal light emission. The difference is probably greater than a factor of 2, because light absorption by hemoglobin and consumption of *t*BuOOH by the glutathione peroxidase system of the erythrocytes during its transport to the liver and consumption by hemoglobin-catalyzed hydroperoxide decomposition (21, 22) could occur *in vivo*.

Comparison of the concentration–effect curves for different hydroperoxides in the perfused liver (Fig. 4*B*) shows that ethyl hydroperoxide and *t*BuOOH were similarly active, whereas CumOOH was about 35% more effective in inducing chemiluminescence. The shape of the curves and the relationship between the hydroperoxides were similar to the ones observed

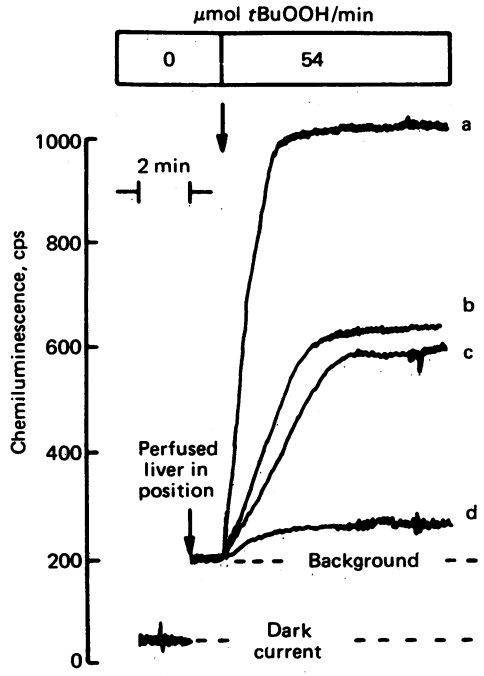

FIG. 5. Partial spectral distribution of the hydroperoxide-induced chemiluminescence of the perfused liver. Experimental details are given in *Materials and Methods* and in the text. *t*BuOOH was infused at the rate indicated by the numbers in the blocks. Wratten filters 2A (trace a), 29 (trace b), 70 (trace c), and 64 (trace d) were placed in front of the EMI (red-sensitive) phototube.

with isolated mitochondria and different from the ones observed with isolated microsomes (13). Consequently, it seems reasonable to assume that mitochondrial membranes are mainly responsible for the photoemission of the liver.

Partial Spectral Analysis of Chemiluminescence of the Perfused Liver. In a model system composed of ferricytochrome *c* and *t*BuOOH, we have found a major emission peak at 662 nm and a minor shoulder at 580 nm (unpublished work). Because the signals from the tissue are too small for narrow band spectral analyisis, cutoff filters were placed in front of the EMI (red-sensitive) phototube, as described in *Materials and Methods*. No difference in chemiluminescence was found in the presence or absence of a pale yellow filter (2A) (Fig. 5, trace a), thus implying that the light emission observed was above 400 nm with no components between 300 and 400 nm.

Red-light-emitting species in the perfused liver are suggested by traces b and c of Fig. 5. Trace b was obtained with a filter with spectral transmittance from 600 nm toward the infrared (filter 29), whereas trace c was obtained with a filter with spectral transmittance from 650 nm toward the infrared (filter 70). The contribution of green-emitting species (400–600 nm) as well as those with a wavelength beyond 710 nm seems quite limited, as is shown on trace d of Fig. 5, obtained with a blue-green filter (filter 64).

As a whole, Fig. 5 is indicative of a main involvement of red-emitting species in the hydroperoxide-induced chemiluminescence of the perfused liver. This partial evidence supports the presence of emission bands between 600 and 710 nm.

Relationship of Chemiluminescence and the Rate of Formation of Oxidized Glutathione. The release of oxidized glutathione constitutes a sensitive indicator of oxidative stress in liver tissue. The effect has been documented in liver cells, the perfused organ, and the liver *in situ* (2, 8, 23, 24). In our experiments, total glutathione (GSSG + GSH) rather than oxidized glutathione (GSSG) was measured in the perfusate, because reduced glutathione (GSH) release is not modified upon *t*BuOOH perfusion to the liver (25); moreover, remaining hydroperoxide in the perfusion fluid could lead to the nonenzymatic oxidation of reduced glutathione. Fig. 6 shows the simultaneous measurement of chemiluminescence and the rate of glutathione release in the perfused rat liver upon hydroperoxide infusion. There was a parallel increase in both parameters, although glutathione release seemed a more sensitive indicator at low hydroperoxide levels. Moreover, chemiluminescence and the rate of glutathione release were found, in a series of four perfused livers, to be statistically correlated ($r = 0.90$; $P < 0.1$).

DISCUSSION

Chemiluminescence, glutathione release (2), and evolution of hydrocarbons (26, 27) are indirect assays of lipid peroxidation. We have emphasized chemiluminescence because it is a truly noninvasive method, that gives precise information related to the early stages of peroxidative breakdown of phospholipids.

Perfused and *in situ* livers exhibit a chemiluminescence of 7–12 cps/cm^2; this value may be increased up to 30-fold upon infusion of exogenous hydroperoxides. The latter chemiluminescence of the perfused liver is due mainly to a red-light-emitting species (600–710 nm), presumably arising from the decay of singlet oxygen [$O_2(^1\Delta g)$] to the ground, triplet state

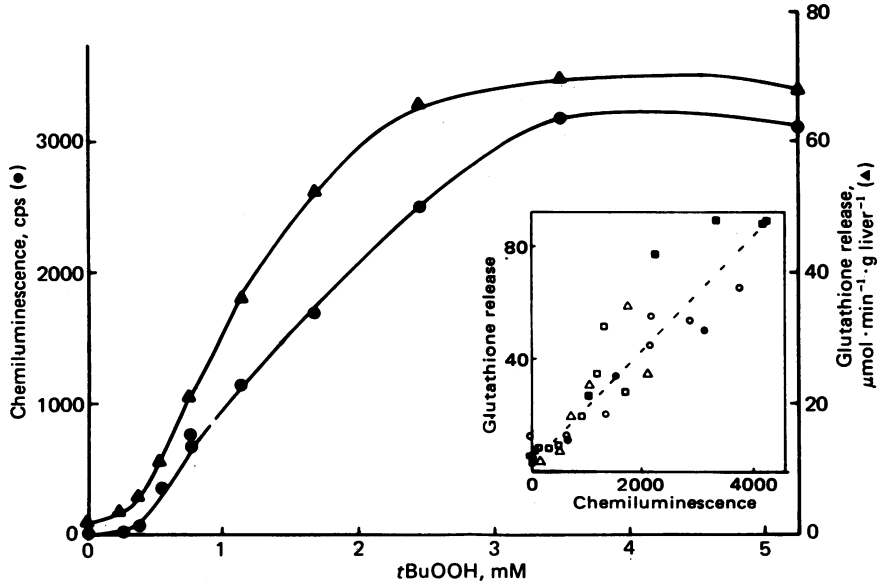

FIG. 6. Chemiluminescence (●) and glutathione release (▲) from the perfused rat liver upon infusion of *t*BuOOH at the indicated concentration in the perfusion fluid. (*Inset*) Statistical correlation between chemiluminescence and glutathione release into the perfusate in four rat livers. Units are the same as for the main figure. Different symbols indicate different livers. An RCA phototube was used.

$[O_2(^3\Sigma\bar{g})]$ (28, 29). The origin of singlet oxygen can be manifold, and its identification becomes difficult because of the small concentration and rate of reaction of the light-emitting species, for example: 10 cps/cm^2 corresponds to approximately 20 cps/cm^3·sec if the light originates through a 5-mm thickness of the liver lobe. This value represents 3×10^{-20} mol/liter· sec, which reflects only 10^{-14} of the rate of release of glutathione.

Chemiluminescence of the intact organ may involve several photoemissive reactions: (*i*) the singlet oxygen dimol emission, in which singlet oxygen can be generated by the reaction of primary and secondary or tertiary peroxy radicals, according to Russell's (30) or Pryor's (31) mechanism, and (*ii*) the formation of 1,2-dioxetane derivatives either from the lipid peroxidation free radical process or from the reaction of singlet oxygen with double bonds that makes likely the generation of excited carbonyl groups after rupture of the carbon–carbon and oxygen–oxygen linkages (32–35). Experimental evidence for the generation of singlet oxygen has been obtained mainly through the effect of specific quenchers (14, 15, 36) or spectral analysis (10) in biological or model systems.

Optimization of light emission in mitochondria and microsomes (13) and submitochondrial particles (14) apparently requires (*i*) a membrane-bound electron transfer system, (*ii*) added hydroperoxide, and (*iii*) the presence of O_2. Oxygen-containing species, O_2^-, H_2O_2, HO·, RO·, ROO·, singlet oxygen, generated either by an interaction of oxygen with the components of the respiratory chain or by the homolytic scission of hydroperoxides by hemoproteins (14, 15, 21, 37, 38), are able to initiate free radical reactions, mainly through increased HO· and RO· formation, leading to lipid peroxidation (2, 39–41). For example, the direct reaction of O_2^- with organic hydroperoxides (ROOH) may also yield HO· and RO· radicals (42).

The future application of these methods to the noninvasive assay of organ redox and hyperoxic states may be carried out through the fiberoptic probes, for example, to accessible internal organs such as portions of the lung, or by catheterization of portions of the heart or liver. In each case, the low levels of endogenous light emission may require enhancement by hyperoxic stress. In addition, two-dimensional display of chemiluminescence from the surface of an exposed organ may be possible, for example, in the margin of the liver where portions of the acinus (43) that are most reactive in lipid peroxidation may be identified. In this case, the experimental difficulties are challenging: endogenous rates of 10 cps/cm^2 correspond to 1 cps from a tissue area of 300×300 μm. If, however, peroxide-stimulated emission is studied, the corresponding tissue area is 60×60 μm, approaching an appropriate value for localized study.

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