Spontaneous Chemiluminescence of Human Breath

SPECTRUM, LIFETIME, TEMPORAL DISTRIBUTION, AND CORRELATION WITH PEROXIDE*

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Human breath spontaneously emits photons at a rate of approximately 7,000/liter-s. The emission has a peak in the red part of the spectrum and an ultraviolet contribution. The emission count rate correlates with peroxide concentration in a saturating manner under normal breathing conditions. When trapped in a balloon, the breath luminescence count rate has a half-decay time of approximately 20 min and exhibits more than one mode of decay. The photomultiplier pulses generated by breath luminescence arrive in bursts. The chemiluminescence process appears by these criteria to include chain reactions, long-lived emitters, or both.

CL of phagocytosing cells (1), the large populations of these cells in lungs (2), the association of peroxides with the CL of these cells (3), and the membrane-damaging properties of peroxides and peroxy radicals (4) have been combined in recent studies of pulmonary pathology and oxygen toxicity (5). To put these results in the context of the lung, studies of perfused rat lung and the roles of peroxides and edema in CL count rates have also been made (6). Concomitant studies of the production of malondialdehyde as a function of CL intensity, and of the CL-optical spectrum have suggested roles of lipid peroxidation and singlet oxygen in the CL of these reactions. Products of lipid peroxidation, such as pentane, are found in breath after chronic alcohol administration in rats (7) and lipid peroxide radicals as well as other possible emitters, including singlet oxygen and hydroxyl radical, are sufficiently volatile to be found in breath (8).

Breath is a voluminous and readily accessible waste product. Though not as complex as urine or blood, breath is known to contain at least 100 complex molecules present at nanogram/liter quantities (9). Most components of breath originate in the atmosphere and the CL of the atmosphere is well known (10). Human breath also emits CL spontaneously, without addition of any substance or perturbation. The possibility of determining pulmonary condition by measurement of breath luminescence (SBL) is being investigated by this laboratory. Initial experiments have been done to determine aspects of subjects' daily lives which produce physiological conditions detectable by these measurements. A preliminary spectral study has also been reported (11). The presence of peroxide and the effects of breathing pure oxygen have been observed, as have the effects of cigarette smoking (12). In the present study, results of optical spectral, radiative lifetime, peroxide concentration correlation with luminescence intensity, and temporal distribution measurements are reported.

MATERIALS AND METHODS

The photon counter employed as detector an EMI-Gencom (Plainview, NY) 9638 A/R photomultiplier with cathode sensitivity of 322 UA/L operated at −952 kV with a dynode-resistor chain wired for photon counting. The tube was selected at the factory for low dark count and high red response. This unit was maintained at −25 °C by a thermostatic cooler type F 50 Mark II (EMI-Gencom). A fused silica window with an evacuated air space provided a thermal barrier between the photomultiplier/cooler combination and the breath collector. A polyvinyl chloride cylinder was attached by means of a bayonet lock to the cooler to serve as an optical parabolic polished aluminum light collector (13). The photomultiplier was bored to provide two inlet/outlet ports, fitted with 3/8-inch pipe nipples, to exhaust exhaled breath. A mask consisting of double-thick rubberized darkroom drapery cloth fitted with Velcro ties was employed to maintain darkness around the subject's mouth and cheeks. This was secured to the adapter by a wire "drawstring" seated in a channel milled in the annulus of the adapter. The room was also darkened.

High voltage was provided to the photomultiplier by a Hewlett-Packard (Santa Clara, CA) 1600 A supply. The pulse train from the photomultiplier was amplified and passed through a window discriminator (−2 to −7 mV) (Princeton Applied Research, Model 1121) to a frequency counter (Hewlett Packard Model 5300/5308-A) and to a Digital Equipment Corporation PDP 11/10 computer. A histogram depicting the frequency distribution of photomultiplier counts was constructed for each experiment. The frequency of occurrence of numbers of counts, ranging from 0 to 256, recorded in fixed counting intervals ranging from 7 to 25 ms, was recorded. The peroxide correlation, intensity decay, and optical spectrum experiments each employed 25-ms intervals and the temporal distribution experiment employed 10-ms intervals. The temporal distribution experiment required recording of 1,024 intervals and display of every fourth interval. The other experiments employed 10,240 intervals. The temporal distribution experiment employed an EMI-9788 QB photomultiplier. The combination of this tube and associated apparatus had a quantum efficiency of 2.32 × 10⁻⁴ by luminol calibration (14) when the high voltage was −1100 V and the discriminator passed pulses between −0.21 and −7 mV. The count rates thus obtained in breathing experiments were approximately equal to those obtained with the 9658 tube as were dark counts. The dispersion in count rate (noise) was 4 times greater with the 9788 (probably due to its 10-year age).

The subject inhaled ambient air nasally and exhaled orally into the breath/light collector. A typical experiment lasted 5 min and consisted of approximately 20 full (inhale and exhale) breaths. Counting proceeded continuously during the experiment so that both inhalation and exhalation count rates were recorded except in the temporal distribution experiment which consisted of a single exhalation.

The correlation of breath luminescence intensity and peroxide concentration was determined from the results of four series of simultaneous measurements of these parameters and several control experiments described under "Results." Dr. Takehashi Yonatani kindly provided cytochrome c peroxidase for use in a combined spectrophotometric assay with cytochrome c (15). The results of a different test for peroxides employing scopoletin have been presented (12).

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‡ The abbreviations used are: CL, chemiluminescence; SBL, spontaneous breath luminescence.
The optical spectrum measurements were performed using a combination of the apparatus employed in the other experiments and the apparatus described by Cadenas et al. (1). This consisted of the addition of a Wratten filter wheel in a light-tight box mounted between the breath/light collector and the detector. Position of 633-nm band and transmittance of 633-nm light outside the band were determined using a helium-neon laser.

RESULTS

When breath peroxide concentrations are measured from the same exhale as SBL count rates the correlation of these two variables is described by a curve indicating a saturation effect as is shown in Fig. 1. The point at which the count rate ceases to increase as a function of peroxide concentration increases is at $2 \times 10^{-3}$ M. The curve shown in Fig. 1 gives results that would be obtained if the reaction were bimolecular in peroxide as in the $\text{H}_2\text{O}_2$ disproportionation reaction described by Smith and Kulig (16). The result for the unimolecular case is essentially the same but more gently sloping. The concentration values are those measured in the peroxide assay cuvette. To correct them to moles/liter of breath, the square root must be found and this value multiplied by 3.33 x $10^{-4}$ (to account for the volume difference between cuvette and 20 exhalations). This produces results in the range of $10^{-3}$ M. Various control experiments were performed such as measurement of the concentration of peroxide in laboratory air ($\sim 10^{-8}$ M on the day measured), determining the possibility of contamination of the assay reaction with ambient peroxide by pumping argon through the breath luminescence collector and through the assay mixture in the same way as breath (none found), determining the trapping efficiency of the assay by running it in tandem, determining breath volume using both a gas flow valve with timed exhalations and a manometric technique to determine exhalation size explicitly. The amount of peroxide lost in the apparatus was determined by breathing single breaths directly into separate aliquots of assay mixture through a short piece of "Tygon" tubing.

In Fig. 2 is shown an optical spectrum of SBL CL. The red region of the spectrum is especially represented in this and a replicate experiment not shown. This red peak is transmitted by a Wratten filter 26 but not by filters 25 or 29. The bandpass thus defined is centered at 610 nm. Experiments employing Wratten 2B filters, which cut off the near UV at approximately

![Fig. 1. Correlation of peroxide concentration and SBL count rate. Ovals indicate the error in both count rate and peroxide measurement. Filled squares and circles represent two different types of measurement. Count rates have dark count subtracted. Concentrations are those found in the cuvette and are not corrected to breath volume. The peroxide assay mixture described in the methods section was prepared in 10-ml aliquots in 50-ml polyethylene graduated cylinders and breath exhausted from the photon counter bubbled through them during the respective photon-counting measurements. Corrections based on control experiments described under "Results" yield breath peroxide concentration in the $10^{-3}$ M range.](http://www.jbc.org/)

![Fig. 2. The red region optical spectrum of SBL obtained using a Wratten filter wheel. Each bandwidth represents a 5-min count of SBL through the respective filter. Position of the 633-nm transmittance and transmittance of 633-nm light out of band were determined with a helium-neon laser. Results indicated here were not averaged with very similar results obtained in a replicate experiment because of the probable real variations in breath spectra. In both cases, peak wavelength was the same and relative peak intensity varied less than a factor of 2.](http://www.jbc.org/)

![Fig. 3. Decay of SBL intensity. Breath trapped in an aluminized Mylar balloon generates photomultiplier counts for at least 30 min. The decrease of count rate with time is at least biphasic. Filled circles indicate the decay rate obtained when the balloon is first filled. Open circles indicate two intervening experiments. The decrease of intensity after vigorous pulmonary exercise is a standard feature of SBL. Closed squares represent SBL count rate decay obtained when the balloon is filled 5 min after smoking a cigarette.](http://www.jbc.org/)
macrophages and other phagocytes as a function of the respiratory burst accompanying CL and phagocytosis in many cases (3). Lung microsomes also express hydrogen peroxide when subject to hyperoxia (21). Hydrogen peroxide is also liberated by mitochondria under a variety of conditions besides hyperoxic stress (22). If these hydrogen peroxide molecules attack lipids as is suggested by the measurements of malondialdehyde referred to in the introduction, the resulting alkylation peroxides and their known atmospheric reactions (8) and CL emission (4) require consideration of their possible roles in SBL. Furthermore, many reactions of peroxides produce hydroxyl radicals and/or singlet oxygen (8, 17). The saturation characteristics of the correlation of peroxide concentration and count rate may indicate the operation of a branched chain reaction with a dark pathway competing with the radiative process under concentration control. It is also possible that the emitter has a dark mode of reaction as in the physical quenching of singlet oxygen (19).

The known sources producing atmospheric luminescence must be considered as possible sources of SBL since the atmosphere constitutes a major portion of exhaled breath.

The atmospheric sources all have a red emission band except for nitrogen which emits yellow. These red sources include singlet oxygen (6324 A) (23), hydroxyl radical (6329 A) (24), atomic oxygen (5577 A) (22), and the water cation H2O+ (6140 and 6190 A) (26). The reaction of NO + O or NO + O yields several bands in the red region (27). The presence of a red peak in the SBL optical emission spectrum thus has several possible interpretations. The multitude of possible energy transfer acceptors and re-emitters (9) must also be taken into account. The presence of a near-UV component is equally nonspecific. Various forms and reactions of CO₂ emit in this region (28, 29). There are also singlet oxygen (23) and hydroxyl radical bands (24) in the near UV.

The alteration of the atmosphere’s luminescence count rate by breathing requires consideration of the atmospheric components which are known to react with biological molecules at substantial rates. Singlet oxygen is present at 10⁻¹⁴ M (30). Ozone is present in air at 10⁻⁷ to 10⁻⁵ M concentrations, H₂O₂ at 10⁻⁷ to 10⁻⁵ M, OH⁻ at 10⁻²⁰ to 10⁻¹⁸ M and HO₂⁻ at 10⁻¹⁵ to 10⁻¹⁷ M (31). Singlet oxygen and hydroxyl radical are formed in the excited state and may therefore luminesce spontaneously whereas the other bioreactive atmospheric species serve as substrates in reactions leading to excited state products. Ozone reacts with linoleate and other unsaturated molecules yielding trioxides which decompose to aldehydes and peroxo radicals (32, 33). Hydrogen peroxide (or HOO⁻) oxidizes lipids to their hydroperoxy products of cleavage at unsaturated bonds and the reaction is propagated by a chain mechanism. This reaction and the physiological role of hydrogen peroxide have recently been reviewed (34). Hydroxyl radical participation in lipid peroxidation appears to proceed via a Fenton reaction, the exact mechanism of which is currently debated (35, 36). In sum, any of these bioactive atmospheric molecules could be substrates in the reactions leading to SBL. Singlet oxygen and hydroxyl radical give up their role as emitters if they serve as substrates.

Bovens and Lloyd (37) have found CL accompanying the thermal decomposition of several hydroperoxy radicals. Russell has found that the self-reaction of secondary peroxo radicals yields excited state ketones and ground state triplet O₂ or ground state ketones and excited state singlet oxygen (38). Kellogg (39) also has found that secondary peroxo radicals yield CL on decomposition. Several workers have found that cerium oxidation of secondary peroxides yields CL which has been attributed to singlet oxygen by various tests including optical spectra and deuterium oxide enhancement (40, 41). In their study of tertiary butyl peroxide perfused rat lung

**Fig. 4. Temporal distribution of SBL photomultiplier counts.** The counts generated by a single breath were collected in 1024 10-ms wide counting intervals. Every fourth interval was used in the representation here. This permits visualization of the fact that fewer than 29 of the 256 bins represented had any counts. The total count rate was 199/s versus 33 for dark count (not subtracted).

400 nm, resulted in definite reductions of luminescence intensity. Three measurements of each case yielded 147 ± 2.2 counts/s for the unfiltered count rate versus 125 ± 2.3 for the filtered case. When a sheet of cheesecloth is loosely wadded into a ball and stuffed into the entrance of the light/breath collector the count rate is increased. In one set of three experiments each with and without gauze, the average count rates were 27 ± 1.7 without gauze and 147 ± 2.2 with gauze. Pumping air through the gauze produced no signal in excess of dark count rate.

The decay of SBL count rate when breath is trapped in an aluminized Mylar balloon is shown in Fig. 3. It should be noted that the lifetime is very long before as well as after cigarette smoking. The open circles represent intervening experiments each with and without gauze, the average count rates were 20. The decrease of luminescence after filling the balloon the first time is a typical response of healthy subjects to vigorous pulmonary exercise. A control experiment employing USP oxygen to fill the balloon produced no signal in excess of dark count rate.

When SBL counts are collected from a single breath and plotted versus time, as shown in Fig. 4, it may be seen that the luminescence is produced in bursts. Counts were collected continuously for a single exhalation. The data string was divided into 1024 10-ms intervals and every fourth segment employed in the result. Use of every fourth interval instead of all intervals facilitates visualization of the result. Fewer than 20 of the 256 illustrated segments have any counts in them. The maximum counts/interval was 20. The total count rate/s was 120 versus 33 for the dark count.

**DISCUSSION**

Peroxides are known to be present in air at 10⁻⁸ M concentrations even in the absence of industrial or urban waste gases (17). Blowing ambient air into the breath collector of the apparatus employed here, however, resulted in no signal distinguishable from the dark count of the detector. The peroxide content of breath is altered from that of air with respect to its luminescence properties. This is apparent from the peroxide-correlated count rates of SBL. The intensification of this signal by filtering breath through cheesecloth may be due to the removal of part of the liquid phase of breath. Protilic environments quench free radicals and singlet oxygen, two of the possible emitters in this system (18, 19). There are many different peroxide reactions (20) and they are considered to be among the most complex, kinetically, of all chemical reactions (8). Hydrogen peroxide is known to be excreted by lung mitochondria under conditions of hyperoxic stress (21) and by macrophages and other phagocytes as a function of the respiratory burst.
phagocytosing opsonized zymosan granules is inhibited by form dioxetanes.

The chemiluminescence of polymorphonuclear leukocytes present lack of evidence for ozone generation by tissue. The appearance of counts in bursts is consistent with such a radical chain reaction since a chain reaction provides multiple opportunities for emission from a limited number of reactants. The appearance of a plateau in that plot and the multimodal decay of the luminescence intensity are both indicative of the operation of more than one process leading to photon emission. The very slow decay of SBL-count rates may be indicative of the operation of more than one process, including the generation of a long-lived species.

The correlation of peroxide concentration and photocounts may have an overall character of being bimolecular in peroxide since $10^{-3}$ M peroxide/liter yields $10^7$ photons. The appearance of a plateau in that plot and the multimodal decay of the luminescence intensity are both indicative of the operation of more than one process leading to photon emission. The very slow decay of SBL-count rates may be indicative of the operation of more than one process leading to photon emission. The very slow decay of SBL-count rates may be indicative of the operation of more than one process leading to photon emission. The very slow decay of SBL-count rates may be indicative of the operation of more than one process leading to photon emission.

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