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GENERALIZED CHEMILUMINESCENCE SPRAY CELL FOR LIQUID CHROMATOGRAPHY DETECTION: SELECTIVE DETECTION USING O_2 ($^1\Delta_g$)

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SUMMARY

We have introduced a series of improvements to the chemiluminescence spray cell described earlier. The new detector displays dead-volume behavior dependent on both gas and liquid flow-rates; its best dead volumes rival those of standard ultraviolet-absorption liquid chromatography detectors. A simple, modular assembly, the spray cell is capable of accepting a wide range of chemiluminescent reaction systems; we present data here on luminescence induced by ozone and by singlet oxygen produced chemically *in situ*. By combining chemical with spectroscopic selectivity, the spray cell becomes a powerful tool for high-performance liquid chromatography detection.

INTRODUCTION

Chemiluminescence (CL) is no new phenomenon; fireflies and luminous bacteria have fascinated humans practically since the dawn of our race. Even as an area of chemical investigation, light from chemical reactions has been a subject of study for some time: the NaOCl + H_2O_2 reaction, for example, has been recognized since early this century[1].

The bulk of chemiluminescence research has until recently involved explaining what happens: reaction mechanisms, structures of intermediates, singlet *vs.* triplet product yields and substituent effects, to name a few areas. Although Seitz and Hercules[2] demonstrated metal-catalyzed luminol detection combined with ion-exchange chromatography in 1973, only in the last few years has CL found widespread analytical application. Today, chemiluminescent analysis of liquid-phase samples is becoming so commonplace that a recent review of luminescence methods includes the term explicitly in its title[3].

A variety of analytical CL systems has now surfaced. In addition to the classic luminol–metal ion–H_2O_2 system, capable of species or even oxidation-state specificity[4], and gallic acid–metal ion–H_2O_2 emission[5], organic detection is possible using 1,2-dioxetanedione decomposition and sensitized luminescence[6].

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We have developed a liquid-spray technique for chemiluminescence detection which can utilize a wide range of gas–liquid or liquid–liquid reactions. In expanding the capabilities of our ozone-based detector, we have devised a cell which can in principle serve as a detector using any reasonably rapid chemiluminescent reaction system, and which has a sufficiently small dead volume to operate in conjunction with high-performance liquid chromatography (HPLC).

EXPERIMENTAL

The work described here began as an attempt to extend the abilities of the chemiluminescent aerosol spray (CLAS) detector described earlier[7] from ozone-induced to singlet oxygen-stimulated luminescence. The setup which resulted, however, has proved versatile far beyond its original design.

Detector cell

The spray detector in its current form, illustrated in Fig. 1, works according to the same confined-spray principle discussed before. It consists of an opaque PTFE base; a Swagelok 1/4 in. tube to 1/8 in. pipe-thread adapter screwed into the base to allow attaching a gas hose; a stainless steel "sheath tube" (No. 14 needle gauge) press fit into the base; three stainless steel capillary tubes (No. 22 needle gauge) glued in place with black RTV silicone sealer (General Electric, Waterford, NY, U.S.A.) and ending 2 mm below the tip of the sheath tube; a Pyrex cover, built from an Ace-Thred No. 11 adapter (Ace Glass, Vineland, NJ, U.S.A.) sealed to appropriate diameter Pyrex tubing and closed off at the top end as shown, screwed onto the threaded top of the base; and a stainless steel drain tube, bent in a double curve and press fit into place.

Gas entering via the Swagelok fitting travels up the sheath tube, since sealant blocks the lower end of the base. As gas passes the capillary tube tips, the liquid in the capillaries nebulizes and rapidly impacts against the inner walls of the glass cover. If different solutions are traveling up the three capillaries, they mix thoroughly during spray and impaction steps.

The sheath and capillary sizes provide a tight fit of three tubes inside a fourth, with the smaller three approximately the size of standard atomic absorption aspirator tubes (Micro-Line No. 6492-02; Cole-Parmer, Chicago, IL, U.S.A.). Relative tube sizes and positions were chosen experimentally to maximize the cell's spray pattern and to minimize its dead volume.

A small triangular PTFE centering collar slid onto the sheath tube, fitting snugly into the 9-mm cell, aids in keeping the sheath tube centered and thereby ensures effective rinsing of the cell walls.

The outside of the glass cover is spray painted black to eliminate unwanted light, with the uppermost 3/8 in. left unpainted to provide the luminescence region inside a reflector-photomultiplier housing. A rubber gasket between glass cover and base prevents liquid leakage from around the threads.

The spray cell in its current form, due to all-internal mating of gas and liquid flows, is considerably simpler and more rugged than its predecessor. Disassembly and cleaning are more straightforward; breakage is much less of a problem since the cell has no glass sidearm. The elimination of external fittings means fewer places for liquid to collect and foul or corrode the assembly.

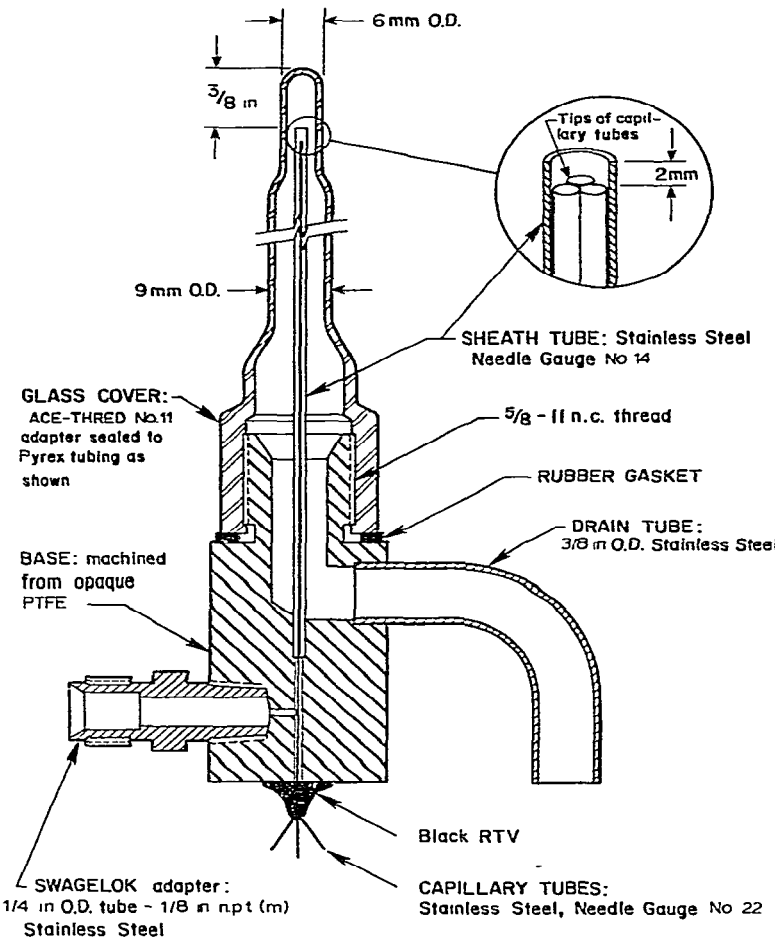


Fig. 1. Diagram of the chemiluminescence spray cell. 5/8–11 n.c. thread = 5/8 in., 11 threads per inch, National Coarse; 1/8 in n.p.t. (m) = 1/8 in., National Pipe Thread (male). These units are defined according to the United States standards for thread diameter and pitch.

The three-spray design was originally intended to allow *in situ* formation of excited metastable oxygen, O_2 ($^1\Delta_g$), but is not limited to this chemical system, nor to this number of spray tubes.

With the present geometry, eliminating stray light is much easier than before since the exhaust is a doubly curved tube rather than a straight vertical opening.

Light detection

By design, the 9-mm outside diameter of the main portion of the glass cover fits directly into the light collection housing illustrated earlier[7]. The unpainted tip of this cell, as before, is located (by empirical adjustment) at one focus of a rhodium-coated ellipsoidal reflector (Melles Griot 02-REM-005), with the photocathode of a red-sensitive photomultiplier located at the other focus. Early work employed the same

photomultiplier tube, cooling, power supply and photon counting apparatus as described previously.

For more recent experiments, we have constructed a two-chamber housing to hold (1) the spray cell and ellipsoidal mirror in its lower portion, (2) a light filter or glass plate, as appropriate, between the cavities and (3) a red-sensitive side-on photomultiplier tube (R928P; Hamamatsu Corporation, San Jose, CA, U.S.A.), with its photocathode at the ellipsoid's second focus, in the upper section. Both regions have air inlets and outlets to allow passing heated or cooled air in order to prevent cell-tip frosting or reduce photomultiplier dark current. Fig. 2 depicts this newer assembly.

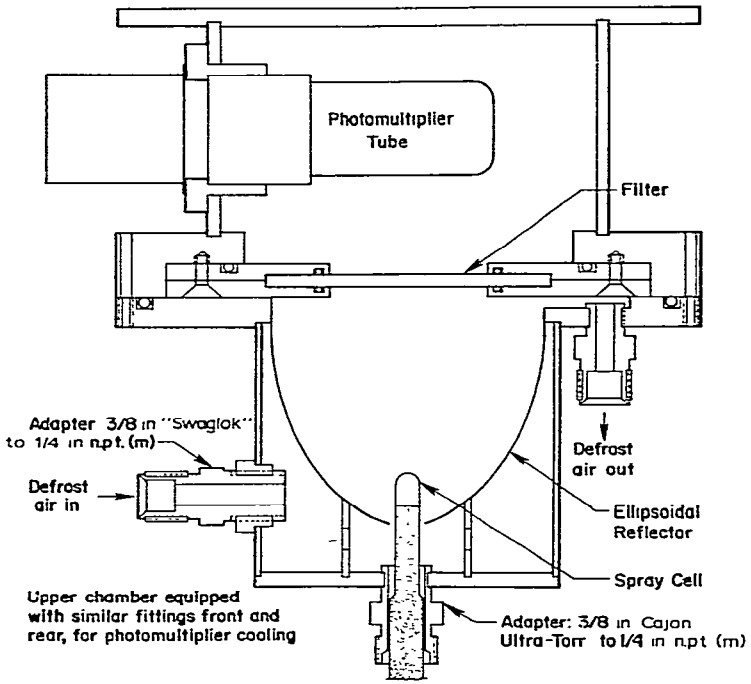


Fig. 2. Cross-section of the cell housing used for the chemiluminescence–HPLC experiments.

In order to obtain spectra, the cell was removed from the reflector housing and mounted with its unpainted tip directly in front of the entrance slit to the polychromator of an intensified diode-array rapid scan spectrometer (IDARSS, TN-1710; Tracor-Northern, Middleton, WI, U.S.A.). Spectra described below are digitized versions from oscilloscope photographs of the IDARSS multichannel analyzer output.

Routine HPLC runs employed either of two filters (obtained from Klinger Scientific Corporation, Richmond Hill, NY, U.S.A.) for wavelength selection: one a bandpass interference filter with its maximum at 671 nm [full width at half maximum (FWHM) 12 nm] to transmit radiation between the O_2 ($^1\Delta_g$) dimol peaks, and the other a long-wavelength cutoff interference filter transmitting between roughly 390 nm and a sharp edge at 600 nm.

Reagents

The principal reagents were chosen for low cost and ready availability. Gases used included industrial grade oxygen and USP nitrogen (obtained from General Air Service and Supply, Denver, CO, U.S.A.). Helium proved far too light a choice of aspirating gas to nebulize sufficient liquid at reasonable flow-rates.

Sodium hypochlorite, needed for O_2 ($^1\Delta_g$) production, was tried both in the form of a 4–6% reagent solution (Fisher Scientific, Pittsburgh, PA, U.S.A.) and as standard commercial bleach (5.25% NaOCl). The bleach gave more satisfactory results in that it did not clog the spray assembly. Both were used undiluted.

Hydrogen peroxide solutions were prepared by dilution of a small volume (usually 1 ml) of nominal 90% H_2O_2 (FMC, Philadelphia, PA, U.S.A.) in an appropriate volume of methanol. Aqueous 3% or 30% H_2O_2 solutions gave unacceptably high background-emission levels; the best signal-to-noise values resulted from methanol–H_2O_2 solutions of less than 1% H_2O_2.

Use of technical rather than reagent grade methanol as carrier solvent did not appear to affect the results of chemiluminescence response tests. Reagent methanol (Fisher) served for all dead-volume determinations. Except for a private sample of Celanthrene Fast Blue FFS [a dye mixture labeled 1-methylamino-4-(β-hydroxyethylamino)anthraquinone], all other emitting substances were ACS certified reagents.

Apparatus

Early experiments with this spray cell employed the same setup as diagrammed previously[7], except for removal of the ozonizer for O_2 ($^1\Delta_g$)-induced emission.

For later experiments, in particular the dead-volume measurements, an Altex Model 110A liquid chromatograph pump replaced the Spectra-Physics liquid chromatograph. The associated injector (20-μl sample loop), column and UV absorption detector also served where needed; a dual-pen recorder (Model 285/MM; Linear Instruments Corporation, Irvine, CA, U.S.A.) allowed simultaneous tracing of UV absorption and luminescence intensity. For chromatography, peristaltic pumps (Masterflex Model 7013, Cole-Parmer) delivered luminescence reagents to the spray cell. Luminescence signals detected at the R928P photomultiplier passed as current to a Keithley Model 480 picoammeter, and thence as voltage to one input of the dual-trace recorder. Fig. 3 illustrates this setup.

Because the spray cell will accept virtually any liquid introduced *via* its capillaries, it should be compatible with almost any pump, injector and column combination.

Chemical systems

The ozone-induced mechanism was discussed before; it appears to consist, in many cases, of energy transfer from an excited oxidation product of the solvent molecule to the emitting substance.

In the singlet oxygen case, the excited O_2 molecule can form *in situ* using the classic reaction of sodium hypochlorite with hydrogen peroxide[1,3–10]. The overall mechanism has been characterized[11] as a reaction to form the chloroperoxide ion, followed by the ion's unimolecular decomposition:

$$OCl^- + HOOH \rightarrow\rightarrow H_2O + ClOO^- \quad (1)$$

$$ClOO^- \rightarrow Cl^- + O_2\,(^1\Delta_g) \quad (2)$$

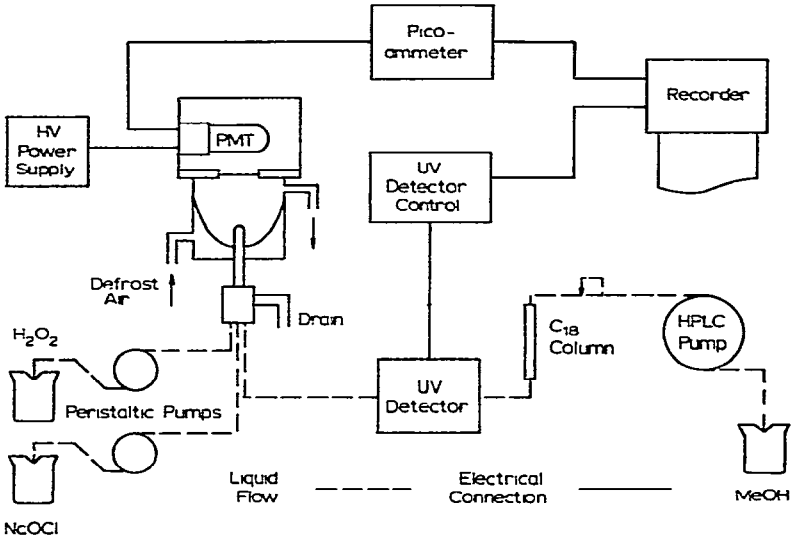


Fig. 3. Block diagram of the dual-detector HPLC system.

Cahill and Taube[12] showed, via ^{18}O labeling, that the oxygen formed in this reaction derives entirely from the hydrogen peroxide; the chloroperoxide formation is therefore likely to consist of several steps. Decomposition of the chloroperoxide leads to O_2 ($^1\Delta_g$) formation due to spin conservation[11].

In our detector, it is sufficient to spray commercial bleach and a dilute H_2O_2 solution (usually 1 % or less in methanol) together into the luminescence region without premixing. Both reagents feed by combined gravity and aspiration from syringes suspended higher than the spray cell, or can be pumped to the cell. The remaining cell inlet may receive the output from a liquid chromatograph. The O_2 ($^1\Delta_g$) forms and either emits, reacts or transfers energy during its residence time in the luminescence region.

Dead volume analysis

In order to demonstrate our cell's usefulness as a practical detector, we produced a series of time-resolved traces of the decaying luminescence signal using two different chemical systems. Subsequent data analysis yielded exponential time constants of these decays; dead volumes calculated were products of these time constants and the liquid flow-rates.

An "inserted volume" approach to introducing a sample plug yielded the fastest decay constants by eliminating all other devices which contribute to dead volume. In this method, a volume of appropriate dye (Rhodamine B for ozone experiments, Celanthrene Fast Blue for O_2 ($^1\Delta_g$) experiments), filling a length of No. 22 needle-gauge stainless steel tube, became a part of the liquid flow on physical insertion of the metal tube into lengths of flexible tubing leading from the pump and into the detector. During insertion, the pump remained off and the flexible tube leading to the detector was pinched to prevent drawing the dye sample into the detector prematurely. With the inserted volume in place, the pump was turned on to a preset flow-rate. A sharp

dividing line between blue or red solution and following methanol gave visual evidence of sample plug flow.

Once the signal reached a high level, monitored using the photon counting system, data recording began at the signal analyzer (Tracor Northern TN-1505), operating in multichannel scaling mode. A subsequent trace of background signal level at the same time scale provided a zero reference line. Dwell times ranged from 20 to 0.8 msec per channel, and were adjusted so that no significant time constant resulted from the data-acquisition process.

Decay curves, traced out on an x–y recorder (Esterline Angus XY-575) and digitized at approximately twelve-channel intervals into data files on a PDP 11/70 computer, served as input into an exponential least-squares analysis restricted to the first two 1/e lives of the signal. A sample digitized data plot and its corresponding curve fit appear in Fig. 4. Each point on Fig. 5 results from at least two replicate data-curve fits; the points in Fig. 6 result from single observations.

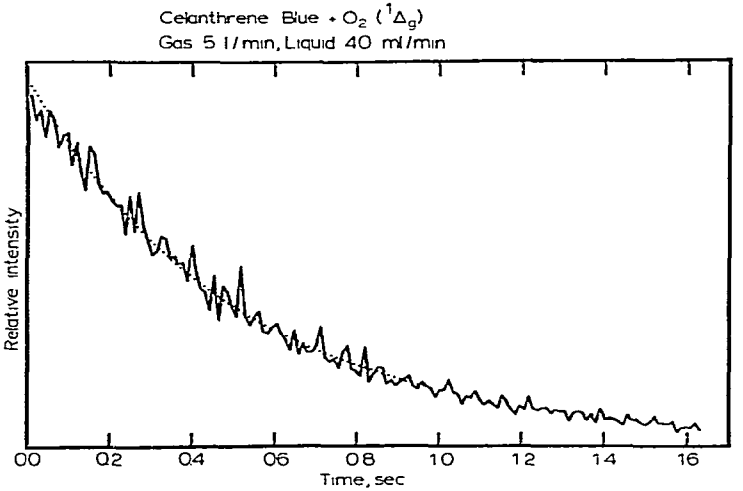


Fig. 4. Sample decay curve (———) with exponential least-squares fit (·····) for comparison. Time = 0 when data acquisition began; dwell set at 0.9 msec per channel. Recorder plot digitized at twelve-channel intervals.

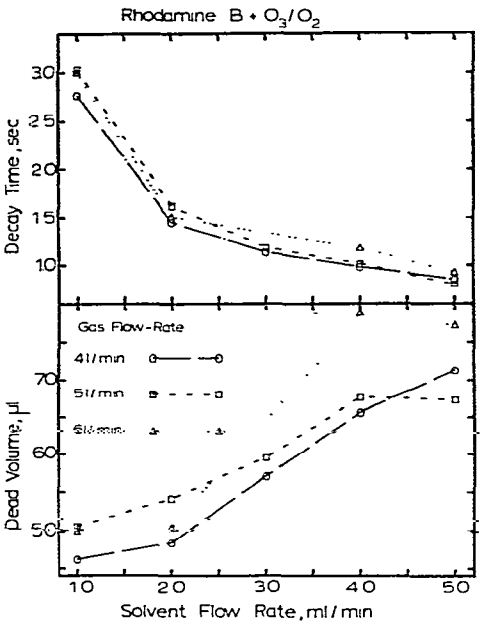
RESULTS AND DISCUSSION

Work to date has demonstrated the practicality, versatility and multiple selectivities of the chemiluminescence spray cell detector.

Dead volumes

Figs. 5 and 6 illustrate the decay time and dead volume behavior of the spray cell under different conditions: only one liquid flow (O_3 + Rhodamine B system), and three liquid flows [O_2 ($^1\Delta_g$) + Celanthrene Blue system].

Although the measured decay constants decline with increasing flow-rate in the single-flow case (Fig. 5), their decrease is clearly less than proportional to the increase in liquid flow-rate. From the three curves, it is difficult to note any clear trend of decay time with gas flow-rate.



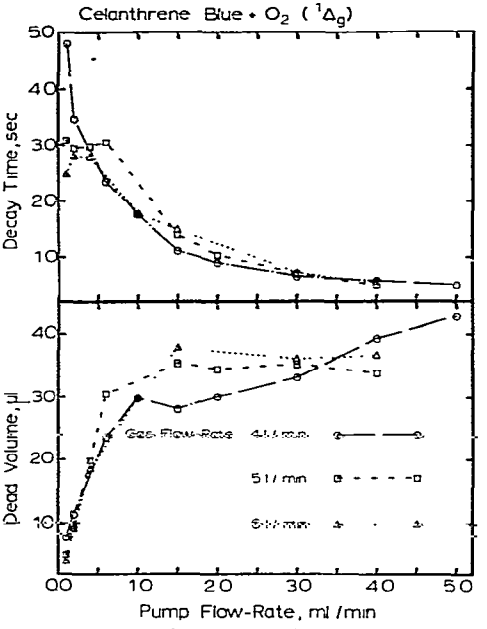


Fig. 5. Decay time and dead volume, at several liquid and gas flow-rates, for a single liquid spray.
Fig. 6. Decay time and dead volume for three liquid sprays *vs.* flow-rate from pump only.

The larger dead volume at higher liquid flow-rates apparently results from an increase in the mass of liquid residing in the observation region with increasing liquid flow-rate. This effect is due in part to a smaller fraction of solvent evaporating at higher liquid flow-rate, and in part to the gas–liquid flow dynamics. Most important for chromatography considerations is the fact that the decay constant decreases with greater liquid flow-rate.

In the three-liquid case, dead volumes calculated from total flow-rate range from 50 to 180 µl, and would appear unacceptable. For liquid chromatography, however, we are concerned with the detector's behavior as we change flow-rate through a column; regarding reagent flows as a downstream "black box" and considering only the HPLC pump flow yields the values plotted in Fig. 6. Here, the trend of increased dead volume with flow-rate is again clear, with the dead volumes at 1–5 ml min^{-1} shifted downward by *ca.* 20 µl relative to the single-flow case. In addition, it was possible to measure dead volumes at flows less than 1 ml min^{-1} due to the extra rinsing effect of the reagents, whereas attempts to measure decay times in the single-flow system resulted in an extremely slow return to background signal level. With three flows, the lowest measured dead volumes fall in the 5–10 µl range.

Spectroscopy

Fig. 7 gives spectra taken with the IDARSS apparatus, using the O_2 ($^1\Delta_g$) system with and without an energy acceptor.

In all three spectra, peaks at 634 and 703 nm result from the well known oxygen "dimol" emission[13]:

$$O_2\,(^1\Delta_g) + O_2\,(^1\Delta_g) \rightarrow 2O_2\,(^3\Sigma_g^-) + h\nu\,(634\text{ nm}) \quad (3)$$

$$O_2\,(^1\Delta_g) + O_2\,(^1\Delta_g) \rightarrow O_2\,(^3\Sigma_g^-, v=0) + O_2\,(^3\Sigma_g^-, v=1) + h\nu\,(703\text{ nm}) \quad (4)$$

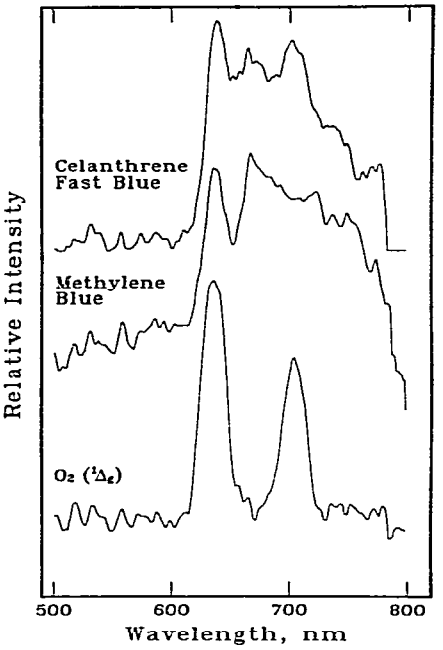


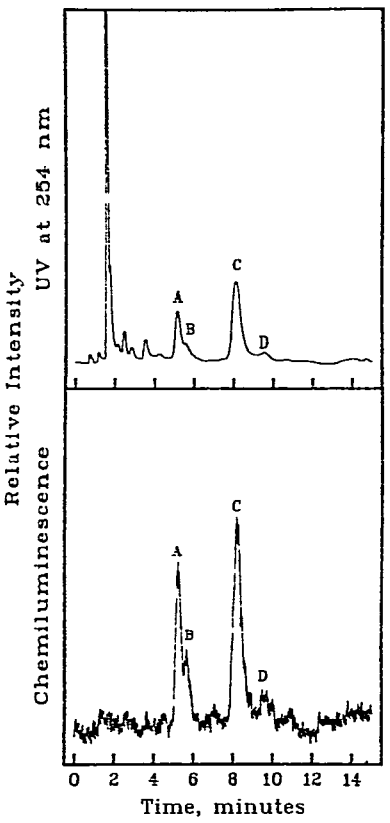
Fig. 7. Uncorrected diode-array spectra from O_2 ($^1\Delta_g$) chemiluminescence in spray cell. Concentrations: H_2O_2, *ca.* 10%; NaOCl, 5.25%; methylene blue, 100 ppm; Celanthrene Fast Blue > 100 ppm. Dispersion 1.16 nm per channel. Spectra produced by accumulating four times at 20 sec each, then subtracting blank (produced same way, spraying only methanol and bleach) and smoothing. Vertical scales shifted for clarity.

Further emission from the acceptors is clearly due to energy transfer, since it occurs at wavelengths greater than or equal to the dimol emission.

Other reactions known for singlet oxygen do not simply transfer electronic energy, but actually yield excited product molecules[14,15]. We can simultaneously distinguish between luminescence mechanisms and eliminate the unwanted "dimol" background emission by our selection of filters. To detect a likely energy-transfer acceptor, we employ a bandpass filter transmitting in the wavelength region between the two dimol peaks. If a luminescent reaction occurs, of course, the emission may still occur in the region between 634 and 703 nm, but is also likely to include shorter wavelengths. To select for compounds which react with singlet O_2 to form excited products, we choose a filter blocking all wavelengths longer than, say, 600 nm. In either case, we have selectivity due to both chemistry and spectroscopy.

Chromatography

Figs. 8 and 9 illustrate the spray cell's potential as a selective HPLC chemiluminescence detector. The setup diagrammed in Fig. 3 served in producing these chromatograms, with UV absorbance at 254 nm and luminescence intensity tracing simultaneously at the dual-pen recorder. For both chromatograms, the sample was an extract from frozen spinach, prepared according to literature methods[16,17], with peaks identified by eluting a portion of the sample on a standard chromatography tube packed with powdered sugar[17]. Visible spectroscopy and HPLC of the fractions thus obtained confirmed the peak identifications reported in a comparable HPLC analysis[18].



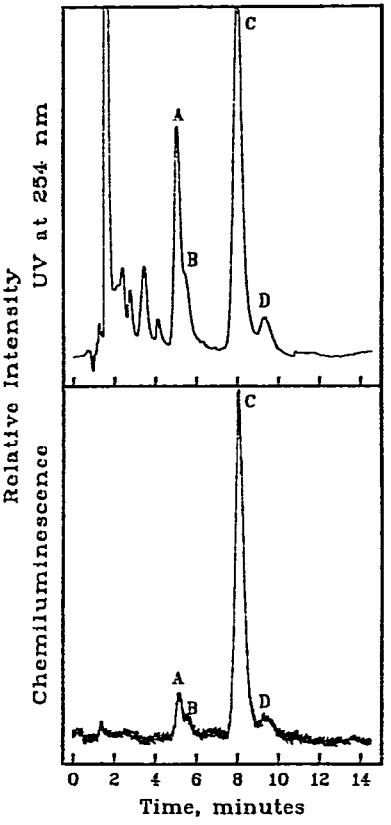


Fig. 8. UV–CL chromatogram of spinach extract using 671-nm filter for wavelength selection. Peaks: A = chlorophyll *b*; B = chlorophyll *a'*; C = chlorophyll *a*; D = pheophytin *a*. Flow-rate 2.0 ml min^{-1}; eluent methanol; column Altex LiChrosorb C_{18}, 10 μm; pump Altex Model 110A.

Fig. 9. Spinach extract chromatogram using 600-nm cutoff filter. Peak identifications and conditions as in Fig. 8.

Chlorophyll *a* is known to undergo a chemical reaction with singlet oxygen, producing luminescence with immediate[19] and long-lived components[20]. Using the 671-nm filter, as Fig. 8 demonstrates, produces CL responses for both chlorophylls *a* and *b* that are in roughly the same ratio as their 254-nm UV absorption peaks. If we select for shorter wavelengths as in Fig. 9, however, we obtain a significant enhancement of the chlorophyll *a* signal over those of the other emitting compounds in the plant extract. Apparently the chlorophyll *a* CL spectrum includes more emission below 600 nm than that of chlorophyll *b*; the CL filter spectrum reported earlier[19] indicates that at least a portion of the chlorophyll *a* emission occurs in this region.

In either chromatogram, the variety of carotene and xanthophyll components present produces no detectable response in O_2 ($^1\Delta_g$) chemiluminescence. We therefore have both chemical selectivity (for the chlorophylls) and wavelength selectivity (for chlorophyll *a* over chlorophyll *b*).

This work is still preliminary in that we have not yet explored all parameters which might affect signal intensity or signal-to-noise ratio. A rough optimization

resulted in the choice of 0.036% H_2O_2 in methanol for singlet oxygen formation, but more detailed work remains on this factor and on cell heating, aspiration-gas heating, pump damping and photomultiplier-tube cooling.

CONCLUSIONS

Our experience to date has shown the chemiluminescent spray cell to be a versatile and practical detector due to its inherent simplicity: other devices can couple with the assembly of Fig. 1 in virtually unlimited ways.

Even where the spray feature is not specifically required for a gas–liquid reaction, it is nonetheless convenient to spray reagents simultaneously into a confined zone and thereby ensure complete, rapid mixing of all components in a small dead volume.

Luminescence chemistry and detection wavelength range, both externally selected, can vary widely without requiring any alterations within the cell itself. In addition, proper choice both of chemical system and of filter gives the possibility of a dual selectivity.

Perhaps most importantly, a wide variety of chemiluminescence schemes can function in the spray cell, including gas–liquid, liquid–liquid and photoinduced mechanisms.

Although gas–liquid reaction luminescence provided the original impetus to build a spray cell, ozone is perhaps the only practical example of this system. Venting toxic exhaust no longer requires a hood, since the drain tube can connect to an appropriately vented liquid reservoir. To obtain lower dead volumes and allow lower flow-rates, the detector would operate best with carrier solvent spraying from the two otherwise unused capillaries.

In the case of liquid–liquid reaction schemes, both energy-transfer and chemical reaction systems are possible. Reagents formed *in situ*, such as O_2 ($^1\Delta_g$), or prior to entering the cell, such as tetramethyl-1,2-dioxetane[21] or 1,2-dioxetanedione[22], can serve as sources of electronic energy (see Figs. 10 and 11). Where the emission results from energy transfer, detection can require some cleverness to eliminate background luminescence (although 1,2-dioxetanedione does not have this problem)[23]. Chemical reaction mechanisms, or true chemiluminescence, should provide the greatest selectivity on both the chemical and the spectroscopic fronts.

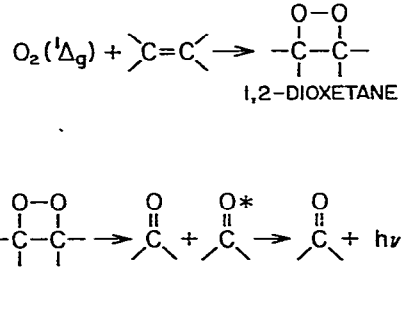
Yet another dimension is the area of photoinduced luminescence; here again, a kind of energy-transfer mechanism stands alongside a series of chemical reaction schemes.

In the 1930's, Kautsky[24,25] first demonstrated the transfer of energy from an absorbing substance (designated S or sensitizer) to molecular oxygen and thence to a third substance (designated A or acceptor):

$$S + h\nu \rightarrow {}^1S \rightarrow {}^3S \text{ (intersystem crossing)} \tag{5}$$

$${}^3S + O_2\,({}^3\Sigma_g^-) \rightarrow S + O_2\,({}^1\Delta_g) \tag{6}$$

$$O_2\,({}^1\Delta_g) + A \rightarrow O_2\,({}^3\Sigma_g^-) + A^* \rightarrow A + h\nu \tag{7a}$$



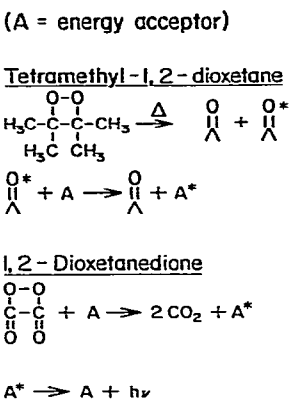


Fig. 10. General mechanism of the O_2 ($^1\Delta_g$)–olefin chemiluminescence. From Lee and Wilson[14].

Fig. 11. Chemical mechanism of tetramethyl-1,2-dioxetane and 1,2-dioxetanedione sensitized luminescence. From Lechtken and Turro[21] and Rauhut[23].

or

$$O_2\,(^1\Delta_g) + A \rightarrow \text{product}^* \rightarrow \text{product} + h\nu \qquad (7b)$$

In our detector, the analyte can serve as either the sensitizer or the acceptor in a Kautsky mechanism. Where the analyte serves as Kautsky sensitizer, we can irradiate the column output and add an efficient acceptor in the cell to produce greatly enhanced emission. Such a scheme might be appropriate for detecting humic substances, whose O_2 sensitizing ability is currently a subject of study[26,27].

With the analyte serving as acceptor, we can place some appropriate sensitizer in a secondary cell-input stream and thereby provide O_2 ($^1\Delta_g$) without the oxidizing reagents. Since some compounds, such as methylene blue, act both as sensitizers[28,29] and as energy acceptors[30], it will be important to select wavelengths carefully or to immobilize the sensitizer in an irradiation cell.

Work is currently under way on a photochemical scheme in which a quinone sensitizer, on irradiation, undergoes a sequence of reactions in the presence of alcohols[31] and other oxygen-containing compounds to produce hydrogen peroxide. The H_2O_2 thus formed, detected in our cell by its 425-nm luminol-reaction luminescence[32], is then a marker for either the sensitizer or the oxygen-containing compound, depending on which we choose to detect[33]. If we use photoproduced radicals to bring about a chemical reaction sequence leading to luminescence, the only limitation in coupling to the spray cell is the experimenter's imagination.

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