

SINGLE PHOTON COUNTING AND SPECTROSCOPY OF  
LOW INTENSITY CHEMILUMINESCENT REACTIONS<sup>1</sup>

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*With electron multiplier phototubes selected for low dark noise and with direct light coupling, chemiluminescent reactions in solution emitting as little as 200 photons s<sup>-1</sup> will produce noise-equivalent counting rates. With this extremely sensitive system it is possible to observe the adventitious chemical production of excited states during the course of oxidase reactions in biological systems and therefore to use this low intensity chemiluminescence as a tracer for specific metabolic reaction rates. The microsomal metabolism of carcinogenic polycyclic aromatic hydrocarbons and the bactericidal action of phagocytic cells are accompanied by significant chemiluminescence. In the former, the chemiluminescence emission spectrum has been measured and the mechanism appears to proceed through a dioxetane intermediate to a diradical exciplex. The Luminol chemiluminescent reaction is proposed for the absolute calibration of single photon detectors and for specifying the intrinsic sensitivity of spectrometers. Procedures for these calibrations will be described.*

I. INTRODUCTION

The contrast between light and darkness is so striking to the human being that in almost all religions the origin of

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light and the creation of the world are intimately related. This intuitive association of light with "order" and darkness with "chaos" is the interpretation that the large neural bundle, the brain, evolved to rationalize the myriad of electrical signals transmitted to it by the retinal light receptor. The free energy of the photons of sunlight is the driving force for life on earth. Aside from the heating of the earth's crust by the decay of naturally-occurring radioactive elements, volcanism and cosmic rays, all of the energy for the "creation" and maintenance of life on earth derives from a narrow spectral region of electromagnetic radiation from the sun, incident on the atmosphere and the surface of the earth. Whether by direct pre-biological photochemical synthesis or by chlorophyll-sensitized photochemistry, an irradiance of  $0.09 \text{ W cm}^{-2}$  has produced "order" from "chaos" and the evolution of a signal processing system that requires outside stimuli for its existence. A major selection process for the structure of all molecules involved in the efficient capture of the free energy of photons, pigment molecules, pigment-protein complexes and electron-transport molecules, has been directed to the production of excited states and the coupling of these electronically excited states to chemical reactions. The advantage of photochemistry over the so-called dark chemical reactions is in its selectivity. Specific pigment molecules at ambient temperatures can be raised to stable energy levels corresponding to 20,000-50,000 °K, providing activation energies for specific chemical reactions that are not accessible otherwise. Evolution, whether on a molecular level of the structure and function of macromolecules such as amino acids, proteins or nucleic acids or as exhibited in the most complex life forms is the expression of the availability of a source of free energy and the Third Law of Thermodynamics. The biochemical apparatus of the lowly bacterium or virus parallels the biochemistry of the trilobite, the dinosaur, the alga and man. The chemical reactions and sequences of chemical reactions that provided the energy and the information for the first self-replicating life forms were the most efficient on a thermodynamic basis. When environmental stress over and over again selected for those most efficient mutant combinations for structure and function these same sets of chemical steps were therefore retained. Thus we may study the mechanism of genetic coding in man more readily by studying the nucleic acids of procaryotic or eucaryotic single cells in the test tube or under the electron microscope. We may extract enzymes from all living organisms and find that functionally similar enzymes have regions of identical subunits as well as identical active sites.

The emission of light by inanimate objects or living forms has always held a special significance to man, particu-

larly when this light was not associated with fire or heat. In Exodus, the burning bush that was not consumed and the pillar of fire that guided the children of Israel may have had their origins in electroluminescence, *i.e.* St. Elmo's fire or *ignis lambens* and the brilliant columnar displays of the aurora borealis. The shining face of Moses might have had its origin in the luminescence of sweat or in bioluminescent bacteria. An excellent compendium of these phenomena in history is to be found in Harvey (1957).

Phosphorescence, from *phosphor* or "light bearer" was the name given to the properties of the stone of Bologna, prepared around 1604 by Vincenzo Cascariolo, a cobbler and part-time alchemist who calcined barium sulfate stones containing copper, manganese and silver impurities to the phosphorescent sulfide. Much later Stokes (1852) demonstrated that this property as well as that of fluorescence, coined from the crystal fluorspar, was a self-luminosity produced by the absorption of light. The study of *light emission* has been one of the most prolific and profitable scientific fields of endeavor. The science of spectroscopy, the blackbody theory of radiation, the theories of Bohr, Planck and Einstein evolved from attempts to explain luminescence. In 1895, William Conrad Roentgen, upon making a fluorescent screen of barium platino-cyanide crystals for his cathode ray tube, discovered X-rays. In 1896, Antoine Henri Becquerel, continuing the studies of phosphorescence of uranium salts begun by his distinguished father, Edward Becquerel, discovered the radioactivity of the double sulfate of potassium and uranium.

With the knowledge that radioactivity consisted of the emission of ionizing particles, Sir William Crookes (1903) developed the spintharoscope, capable of detecting single alpha particles by their induced fluorescence (phosphorescence) on a zinc sulfide screen. Rutherford and Geiger (1908) developed the point discharge counter, capable of electrically detecting single alpha particles. Next followed the detection of single beta particles (Geiger, 1913), and finally the Geiger-Muller counter (1928; 1929), capable of detecting not only  $\beta$  and  $\gamma$  rays, but a single photoelectron emitted from the cathode by a UV quantum. It was this type of photoelectric counter that permitted Audubert (1933; 1939) to measure the very low intensity UV chemiluminescence of a large number of chemical oxidation reactions including the oxidation of glucose and the UV luminescence emitted "In certain biological phenomena, also, such as the electrical or mechanical excitation of nerves...". Only recently has it become possible to prepare photocathode materials with the combination of low thermionic emissions and low work functions for the detection of very low-intensities of photons at wavelengths longer than

the UV and violet regions of the spectrum.

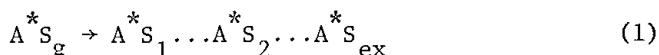
In 1923 Alexander G. Gurwitsch of the Histological Institute of the First Soviet University in Moscow observed a phenomenon that has generated many hundreds of papers and considerable controversy. This observation was that, separate from the extremely efficient functional, visible biological chemiluminescence that we term *bioluminescence*, all oxidizing cells and extracts emit chemiluminescence, although of very low intensity. This fact has been verified by physical detection methods by many independent investigators, beginning with Audubert and is an accepted phenomenon. Gurwitsch discovered this phenomenon many years before it was physically possible to measure low intensity UV radiation. He used a biological detector, the stimulation of mitosis in onion root cells exposed to but physically separated from the growing tip of a second onion root. He reported this action at a distance, characteristic of electromagnetic fields was not inhibited by quartz separating plates but abolished by glass separating plates and induced from this that UV radiation was being emitted by the growing root tip. From the mitotic activity he named this radiation M-rays.

It is this latter hypothesis as to the biological function of M-rays that has remained controversial to the present day. Gurwitsch used onion root tips and later yeast cells and measured an increase in mitotic figures in the former and an increased rate of growth in the latter (Gurwitsch, 1932; Gurvich and Gurvich, 1947; 1948), upon exposure to M-rays whose intensities have been estimated to be between 10-300 photons  $\text{cm}^{-2} \text{ s}^{-1}$  (Glasser and Schott, 1936). In the latest Russian summary of the subject of biological chemiluminescence (Barenboim *et al.*, 1969) it is stated that "there is still no proof that the UV emission appearing in chemiluminescence [of biological systems] contributes to the overall energetics of the cell." Despite the controversial nature of M-rays, no treatment of low intensity biological chemiluminescence would be complete without reference to these original experiments.

## II. PHOTOLUMINESCENT AND CHEMILUMINESCENT REACTIONS

Experimental and theoretical studies of the excited states of molecules have generally proceeded from the electronic description of the ground state to the calculation of the manifold of allowed (stable) energy levels to which the molecule can be raised by the absorption of light of the proper energy. In these photoexcitation descriptions the molecule is already present in its ground state and may be hydrated or complexed with ions in the solution. Since photoexcitation

events and dipole moment changes occur within  $10^{-15}$  s the photoexcited molecule is initially in its ground-state configuration with respect to the solvent or any complexed ions. During the *mean* lifetime  $\langle\tau\rangle$  for spontaneous transition to the ground state, *ca.*  $10^{-9}$  s, solvent relaxation about the new electronic configuration of the photoexcited molecule takes place at rates of  $10^8$ - $10^{12}$   $s^{-1}$  depending on the nature of the solvent. Therefore during the relatively long mean lifetime for fluorescence ( $10^{-9}$ - $10^{-8}$  s) the energy state of the photoexcited molecule-solvent complex *approaches* a new equilibrium through the steps

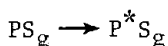


where  $A^*S_g$  represents the initially photoexcited molecule and its solvent interaction in the ground state solvent configuration,  $A^*S_1 \dots A^*S_2 \dots$  represent the photoexcited molecule and its solvent interaction at subsequent times during solvent relaxation and  $A^*S_{ex}$  represents the new equilibrium energy state of the photoexcited molecule and its solvent interaction.

In general it would be expected that each new energy state and configuration  $A^*S_i$  would have a different manifold of transition energies and probabilities. Therefore if  $t_0$  represents the time of photoexcitation, the fluorescence emission spectrum and lifetime  $\tau_i$  of the state  $A^*S_i$  measured at any time  $t < \langle\tau\rangle$ , where  $\langle\tau\rangle$  is the mean lifetime for fluorescence would not necessarily be identical with the fluorescence emission spectrum or the lifetime of  $A^*S_{ex}$ . These phenomena have been studied by the techniques of time-resolved emission spectroscopy (TRES) and have been demonstrated to have utility in physical chemistry and in biological systems in examining the interactions of dye molecules (A) with proteins, nucleic acids and lipid membranes to which binding occurs.

If the molecule A is originally in a weakly-interacting solvent or if the transition from  $A^*S_g$  to  $A^*S_{ex}$  occurs within times  $\ll \langle\tau\rangle$ , the fluorescence emission spectrum measured by TRES will be identical with the statically measured emission spectrum.

In chemiluminescent reactions a chemically different precursor molecule proceeds through a reaction intermediate to a product molecule  $P^*$  in a solvent configuration,  $S_r$ , corresponding to the reaction intermediate. The energy level of the initially formed chemiluminescent molecule,  $P^*S_r$ , is not subject to photoselection rules governing the allowed transitions from



so that in general neither the energy state nor the population density correspond to the photoexcited system:

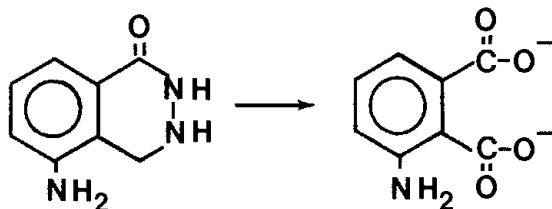
$$P^*S_r \neq P^*S_g \quad (2)$$

It does not follow that the emission spectrum of the chemiluminescence of a substrate molecule M, that produces the sequence of excited state product molecules  $P^*S_r \dots P^*S_{ex}$  will always be identical with the emission spectrum of the photoexcited product  $P^*S_g \dots P^*S_{ex}$ . Therefore the comparison of the photofluorescence emission spectrum of a proposed, synthesized product of a chemiluminescent reaction with the chemiluminescent emission spectrum is an unequivocal method for identifying the product (Seliger and Morton, 1968) only for those cases of weak or extremely rapid solvent interactions with the ground and excited states of the product molecule. Neither does it follow that the fluorescence yield of  $P^*S_r$  will be the same as the photoexcited fluorescence yield of  $P^*S_g$ . Therefore the approximate relationship defining the chemiluminescence quantum yield,  $\phi_{CL}$ , as the product of the chemical yield of product molecule,  $\phi_{chem}$ , and the photoexcited fluorescence yield of the product molecule  $\phi_{f1}$  (Seliger and Morton, 1968; Seliger, 1973)

$$\phi_{CL} = \phi_{chem} \times \phi_{f1} \quad (3)$$

is not valid in all cases.

The chemiluminescence of Luminol (I) can serve as an example of these effects. There is almost quantitative production of the product 3-aminophthalate (II) (White *et al.*, 1964).



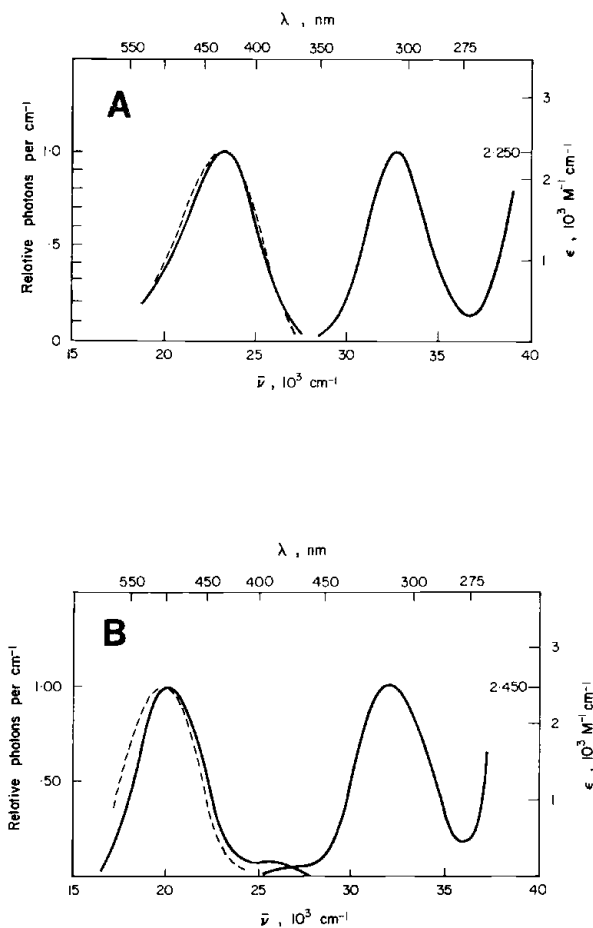


Fig. 1.A. Fluorescence and absorption spectra (full lines) of 3-aminophthalic acid ( $10^{-4} \text{ M}$ ) in aqueous solution (pH 11.6);  $0.1 \text{ M K}_2\text{CO}_3$ ). Chemiluminescent emission spectrum (dashed line) from Luminol ( $10^{-5} \text{ M}$ ) oxidized by  $\text{H}_2\text{O}_2$ /hemin under the same aqueous conditions. B. Fluorescence and absorption spectra (full lines) of 3-aminophthalic acid ( $10^{-4} \text{ M}$ ) in DMSO made basic by the addition of *t*-BuOH (4%) saturated with *t*-BuOK ( $\approx 10^{-2} \text{ M}$ ). Chemiluminescent emission spectrum (dashed line) from Luminol ( $10^{-5} \text{ M}$ ) oxidized by oxygen under the same conditions.

In aqueous solution (pH 11.5, 0.1 M  $K_2CO_3$ ) and in dimethylsulfoxide (4% t-BuOH saturated with t-BuOK) the photoexcited fluorescence yields of II are 0.30 and 0.14 respectively (Lee and Seliger, 1970). However the quantum yields for the chemiluminescence of Luminol in aqueous solution and in dimethylsulfoxide are identical,  $\phi_{CL} = 0.012 \pm 6\%$  (Lee and Seliger, 1972). Therefore it follows that Eq. (3) is incomplete and must be modified to

$$\phi_{CL} = \phi_{chem} \times \phi_{ex} \times \phi_{f1} \times \phi_P \quad (4)$$

where  $\phi_{ex}$  is the fraction of the chemical yield of product molecules that proceed via  $P^*S_r$  rather than through a dark oxidation pathway and  $\phi_P$  is the fraction of the excited state pathway products  $P^*S_r \dots P^*S_g$  that is equivalent to the photoexcited manifold  $P^*S_g \dots P^*S_{ex}$  from which  $\phi_{f1}$  is measured. The identical value of  $\phi_{CL}$  in aqueous and dimethylsulfoxide solutions is striking since the respective chemiluminescence emission spectra are different in these solvents (Fig. 1), implying significant solvent interaction. In addition the chemiluminescence emission spectrum of Luminol in dimethylsulfoxide is not identical with the photoexcited emission spectrum of II in the same solvent, as shown in Fig. 1B (Lee and Seliger, 1970; Roswell and White, 1978).

Despite the questions raised by the apparent identity of  $\phi_{CL}$  in aqueous and dimethylsulfoxide solutions, Luminol remains an excellent material for the standardization of other chemiluminescent reactions and for the calibration of photon detecting equipment (Seliger, 1978). It is stable, readily available and easily purified. The chemiluminescent reaction is simple to carry out and can be repeated with a coefficient of variation of 5%. This will be described in more detail in a later section.

### III. STATISTICS OF LUMINESCENCE

#### *Chemiluminescence*

The probability for the production of any excited product molecule  $P^*S_r$  from any of a large number of substrate molecules,  $M$ , is subject to chance. The assumption is made that the probability  $p$  that a molecule  $M$  shall be transferred within a small interval of time  $\Delta$  is independent of the time that has elapsed since the molecule  $M$  was introduced into the reaction and is the same for all molecules  $M$ . Thus for small values

of  $\Delta$ ,

$$p = k\Delta \quad (5)$$

where  $k$  is a reaction probability characteristic of the constant reaction conditions acting on  $M$ . The probability that any molecule  $M$  will not be transformed in a time period  $t = n\Delta$  will be

$$q = (1 - k\Delta)^n \quad (6)$$

From Eq. (5) and as  $\Delta \rightarrow 0$  we can rewrite Eq. (6) as

$$q = \lim_{\Delta \rightarrow 0} \left[ (1 - k\Delta)^{-\frac{1}{k\Delta}} \right]^{-kt} = e^{-kt} \quad (7)$$

Equation (7) states that, provided the reaction conditions remain constant, *i.e.* concentration of reactants that transform  $M$  to  $P^*S_r$ , the rate of loss of  $M$  will be given by the 1st order rate equation:

$$\frac{dM}{dt} = -kM \quad (8)$$

From Eq. (4), the intensity of chemiluminescence,  $I_{CL}$ , will also exhibit a 1st order decay given by:

$$I_{CL} = -kM\phi_{CL} \quad (9)$$

The conditions for the emission of a chemiluminescence photon during any small time interval  $\Delta$  and the independence of the reaction probability  $k$  of prior events, *i.e.* dependence only on the nature of  $M$  and the constant reaction conditions, are precisely the conditions for a Poisson distribution in time. Therefore, at any time during the course of a chemiluminescent reaction, the probability of finding two or more photons emitted within the resolving time  $\Delta$  of a coincidence circuit will be given by

$$1 - P(0, I_{CL} \Delta) - P(1, I_{CL} \Delta) \quad (10)$$

where  $P(0, I_{CL} \Delta) = e^{-I_{CL} \Delta}$  (probability for zero photons) (11)

and  $P(1, I_{CL} \Delta) = I_{CL} \Delta e^{-I_{CL} \Delta}$  (probability for one photon) (12)

If the efficiencies for photoelectric emission from the photocathodes of the phototubes in the liquid scintillation counter are  $\alpha$  and the geometry is close to 100% efficient, the probability of measuring a coincidence due to the emission of two or more photons during the resolving time  $\Delta$  will be approximately

$$P_c = \left[ 1 - e^{I_{CL} \Delta} (1 + I_{CL} \Delta) \right] \frac{\alpha^2}{2} \quad (13)$$

Nominal values of coincidence resolving times  $\Delta$  and photoelectric efficiencies  $\alpha$  are  $10^{-6}$  s and 0.2 respectively. Thus for chemiluminescence intensities such that  $(I_{CL} \Delta)^3 \ll 1$ , Eq. (13) becomes

$$P_c \approx \frac{(I_{CL} \Delta)^2 \alpha^2}{4} \quad (14)$$

and the fractional coincidence counting rate is:

$$\frac{P_c}{I_{CL} \Delta} \approx \frac{(I_{CL} \Delta) \alpha^2}{4} = 10^{-8} I_{CL} \quad (15)$$

It is evident from Eq. (15) why it is essential for low-intensity chemiluminescent reactions to be counted in the out-of-coincidence mode for measurements made in the liquid scintillation counter. These same statistical arguments apply to fluorescence and phosphorescence measurements in the liquid scintillation counter.

*Luminescence Produced by Short Pulses*

When an ionizing particle such as a  $\beta$  particle traverses the fluor in a liquid scintillation counter all of the excitation is produced within  $\alpha \cdot 10^{-9}$  s, which is also the mean lifetime for fluorescence of the fluor. Since these time intervals are much shorter than the resolving time of the coincidence circuitry of the liquid scintillation counter, each disintegration will produce *all* of the attendant fluorescence emission within the resolving time of the counting system. If  $N$  photons are produced by a  $\beta$  particle, the probability for detection of at least one photoelectron by one of the phototubes is approximately  $1 - (1 - \alpha)^{N/2}$  and the probability for measuring a coincidence is

$$P_c \approx \left[ 1 - (1 - \alpha)^{N/2} \right]^2 \quad (16)$$

This probability is independent of the disintegration rate of the source and of the resolving time of the coincidence circuit, provided the latter is long compared with the fluorescence lifetime of the fluor. Using the same value for  $\alpha$  as the previous section, for  $N = 10$ ,  $P_c \approx 0.45$ , while for  $N = 30$ ,  $P_c \approx 0.93$ , to be compared with Eq. (14) for chemiluminescent reactions.

## IV. MEASUREMENT OF CHEMILUMINESCENT REACTIONS

A. *Signal to Noise Ratio*

The use of the liquid scintillation counter for chemiluminescence in the out-of-coincidence mode, at phototube-amplifier gains such that single photoelectrons are detected also means that the thermionic emission of the phototube photocathode will be counted with equal efficiency. If  $N_1$  and  $N_2$  represent the thermionic electron noise of the phototubes, the signal-to-noise ratio for chemiluminescence measurements will be approximately

$$S/N \approx \frac{I_{CL} \alpha}{N_1 + N_2} \quad (17)$$

It is essential, therefore, that the phototubes be specially selected for low noise at room temperature, since this is the temperature at which most chemiluminescent reactions are

carried out. In the case of detecting ionizing particles in fluors, the signal-to-noise ratio, will be given by

$$S/N = \frac{P_c R}{2\Delta N_1 N_2} \quad (18)$$

where  $P_c$  is given by Eq. (16) and  $R$  is the disintegration rate of the  $\beta$  source in the fluor. To some degree small values of  $\Delta$  can compensate for tube noise.

In all cases in the liquid scintillation counter, pulses from the anode of the phototube are shaped by the electronic circuits so that there is an intrinsic dead-time for pulse counting. There is no reason for the manufacturers of liquid scintillation counters to produce shaped pulses from the phototubes significantly narrower than the coincidence resolving time. Therefore the effective dead-time for counting radioactive sources in the coincidence mode would also apply to the counting rates of chemiluminescent reactions measured in the out-of-coincidence mode. As a rule of thumb any chemiluminescent reaction that is just barely visible to the completely dark adapted eye is too intense to be counted in the liquid scintillation counter without significant dead-time losses and should be measured with a phototube in a D.C. amplifier current measuring circuit. Except for the high efficiency geometry of light collection and the ready availability of liquid scintillation counters in most laboratories there is no *a priori* reason to use pulse counting techniques over D.C. current measurements for the detection of chemiluminescent reactions (Seliger, 1973). There are some practical advantages to the pulse counting technique when for increased light collection it is desired to sit vials and reflectors or color filters directly on the face of the phototube. In these cases the photocathode should be at earth potential to avoid electrostatic field effects on the photocathode material and charging up of the glass envelope. The anode at positive high voltage can be coupled capacitatively to a pulse preamplifier. With the direct anode-coupled, current measuring technique the D.C. amplifier and recording devices would also be at the same high voltage, creating serious isolation problems and shock hazards. For this reason D.C. amplifier current measurements are usually made with the photocathode at negative high voltage, the anode being grounded through the input resistor of the amplifier.

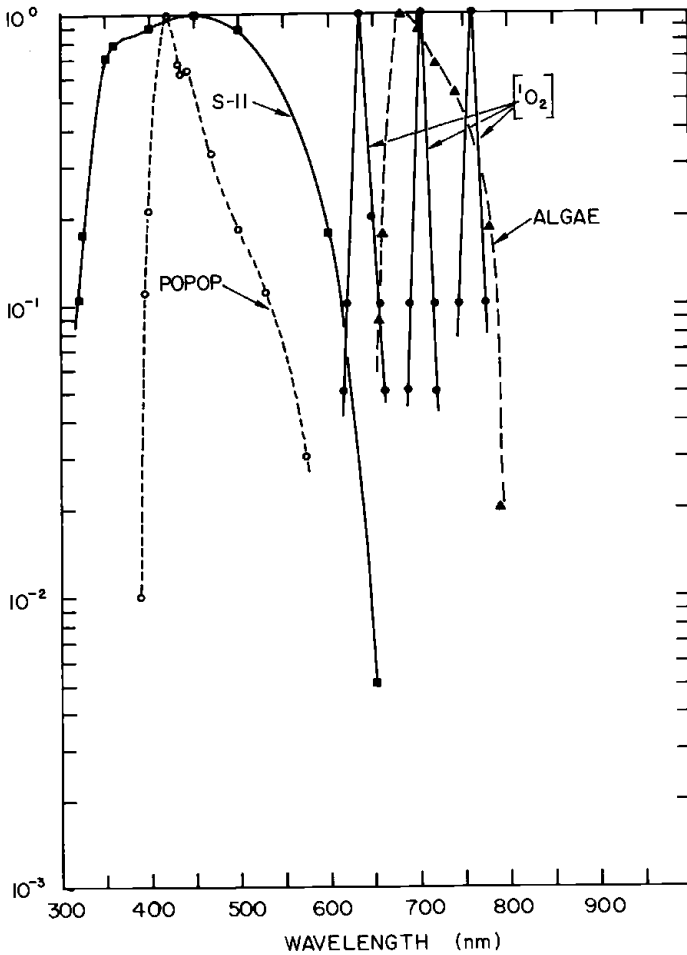


Fig. 2. Relative spectral photoelectric efficiency for an S-11 photocathode and relative spectral emissions of the wavelength shifter POPOP, the major chemiluminescence emission bands of singlet oxygen and the delayed luminescence (light-induced chemiluminescence) of green algae and isolated chloroplasts.

### B. *Photocathode Spectral Sensitivity and Chemiluminescence Emission*

The major function of wavelength shifters or fluors in liquid scintillation counting is literally to shift by sensitized fluorescence, the photon spectral emission of the sample vial to match more closely the spectral sensitivity of the photocathode of the phototubes. This is fixed in the blue region of the spectrum because in this region it is possible to achieve the highest photoelectric efficiency. Therefore phototubes for liquid scintillation counting are made specifically to be blue-sensitive and to have sharply reduced efficiencies at longer wavelengths. This has the effect of reducing thermionic noise by retaining a relatively high work function. While this is optimum for counting of scintillations from fluor solutions, it follows that the phototubes in the liquid scintillation counter may not be very efficient in detecting chemiluminescent reactions whose emissions occur at longer wavelengths. In Fig. 2 are plotted on a logarithmic ordinate versus wavelength, the relative spectral photoelectric efficiency for a typical S-11 photocathode, and the relative spectral emissions of the wavelength shifter POPOP (2,2'-*p*-phenylene-bis-(5-phenyloxazole)) (Ott, 1958), the narrow  $2[{}^1\Delta g]_{(0,0)}$ ,  $2[{}^1\Delta g]_{(0,1)}$  and  ${}^1\Sigma^+g_{(0,0)}$  chemiluminescent emission bands of singlet oxygen (Seilger, 1964; Khan and Kasha, 1970) and the delayed luminescence (chemiluminescence) of green algae and isolated chloroplasts.

### C. *Calibration with Known Sources*

The chemiluminescent reaction of Luminol in dimethylsulfoxide ( $\lambda_{\max} = 480 \text{ nm}$ ) can be used to determine the absolute quantum efficiency of the liquid scintillation counter for the detection of chemiluminescent reactions emitting in this general region of the spectrum. The calibration can be preserved by using a radioactive luminous source consisting of a vial of tritiated water in a scintillation cocktail, very heavily quenched with nitromethane so as to approximate a single photon-per disintegration source. This strongly quenched scintillation solution is a constant (decaying with the 12.3y half life of  ${}^3\text{H}$ ) sealed source of single photons and can be used routinely to check the efficiency of the liquid scintillation counter or any other geometry for the detection of chemiluminescence. In Fig. 3 are plotted on a logarithmic ordinate the relative integral discrimination counting rate curves for phototube noise ( $\blacktriangle$ ), for the chemiluminescence of Luminol (o) and urine ( $\bullet$ ) which are single photon emitters following Poisson statistics and the luminescence of a heavily quenched  ${}^3\text{H}$ -scintillation

cocktail ( $\Delta$ ). The curves are approximately parallel indicating that the  $^3\text{H}$  source emits essentially a single photon per event (disintegration).

A  $^3\text{H}$  scintillation cocktail, quenched with nitromethane from approximately  $6 \times 10^5$  c/M to 300 c/M, as measured in the coincidence counting mode, results in a 30-fold reduction in

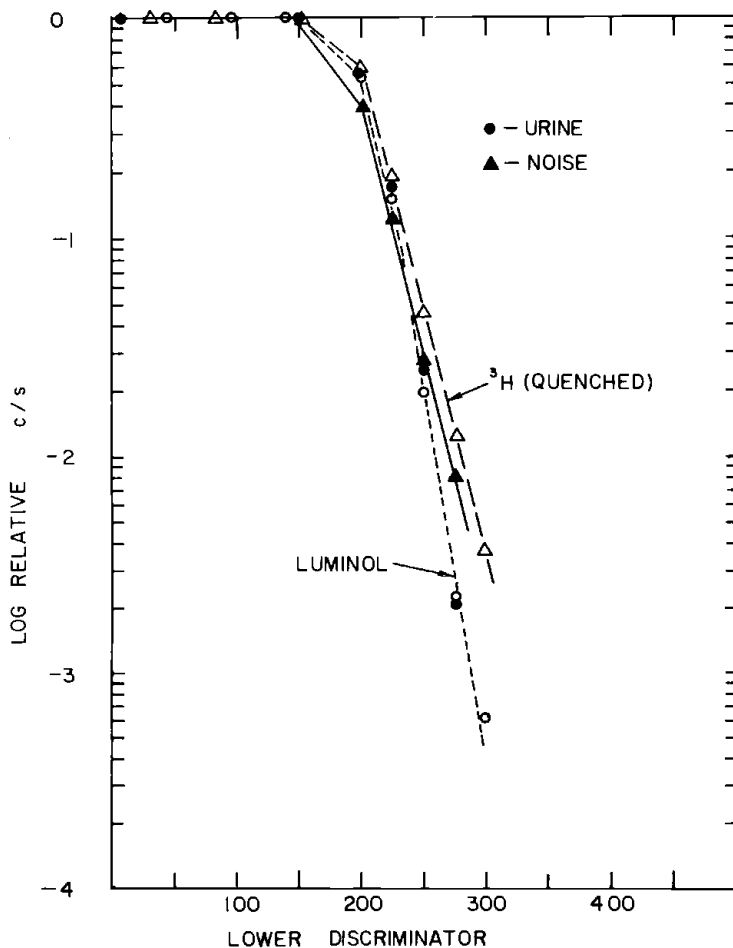


Fig. 3. Relative integral discrimination counting rate curves for phototube noise ( $\blacktriangle$ ), for the chemiluminescence of Luminol ( $\circ$ ) and urine ( $\bullet$ ) and for the luminescence of a heavily quenched  $^3\text{H}$  scintillation cocktail ( $\Delta$ ).

photon emission per disintegration. This is sufficient to insure that most of the  $^3\text{H}$  disintegrations produce 0 or 1 photon, resulting in a constant source of single photons.

The Luminol can be prepared at concentrations of  $10^{-13}$ – $10^{-12}$  M in dimethylsulfoxide by serial dilution from stock solution. The molar extinction of Luminol in dimethylsulfoxide at  $\lambda_{\text{max}} = 359$  nm is  $7760 \pm 190$ . One cubic centimeter of a Luminol solution of OD  $1 \text{ cm}^{-1}$  emits  $9.7 \pm 0.3 \times 10^{14}$  photons upon complete oxidation. All operations should be carried out in dim red light. The reaction is initiated by injecting a small volume of t-butanol containing a large molar excess of potassium t-butoxide relative to Luminol (Lee *et al.*, 1966). The light emission rises to a maximum within a fraction of a second and the subsequent decay is first order. The total light emission can be obtained by counting all photons until the reaction goes essentially to completion, *ca.* 6–10 minutes, or the slope of the 1st order decay curve of  $I_{\text{CL}}$  can be measured. Since

$$\int_0^{\infty} I_0 e^{-kt} dt = \frac{I_0}{k} \quad (19)$$

it is necessary to measure only  $I_0$ , the intensity at  $t = 0$  and the slope of the chemiluminescence decay. If we assume a half-time for decay of approximately 60 seconds,  $k = 0.012$ . We do not want  $I_0\alpha$  to be greater than  $10^5$  c/s. From Eq. (19), the total photon emission should not exceed  $4 \times 10^7$ . If we react 10 ml of the Luminol solution in the liquid scintillation counter, the molarity of the solution should be

$$\frac{4 \times 10^6}{9.7 \times 10} \times 1.3 \times 10^{-4} \text{ M} \approx 5 \times 10^{-13} \text{ M}.$$

If we react this known concentration and volume of Luminol containing M substrate molecules in the liquid scintillation counter the total counts recorded will be

$$\frac{(R_L)_0}{k} = M\phi_{\text{CL}}\Omega_1\alpha_1 \quad (20)$$

where  $(R_L)_0$  is the counting rate at  $t = 0$ ,  $\Omega_1$  is the light collection geometry and  $\alpha_1$  is the mean photocathode photoelectric efficiency for the emission spectrum of Luminol chemiluminescence. If we replace the Luminol reaction with the quenched  $^3\text{H}$  single photon source, the counting rate measured will be

$$R(^3\text{H}) = B(^3\text{H})\Omega_1\alpha_2 \quad (21)$$

where  $B(^3\text{H})$  is the photon emission per second from the  $^3\text{H}$  source,  $\Omega_1$  is the light collection geometry and  $\alpha_2$  is the mean photocathode photoelectric efficiency for the emission spectrum of the POPOP secondary solute in the cocktail. This spectrum does not need to be identical with the chemiluminescence emission spectrum of Luminol. The only requirement is that both emission spectra must have *some* overlap with the photocathode photoelectric efficiency spectrum. From Eq. (21) we can set  $B(^3\text{H})\Omega_1\alpha_2$  equal to  $B(\text{L})\Omega_1\alpha_1$ , where  $B(\text{L})$  is the rate of photon emission *equivalent* to a Luminol chemiluminescent source. Thus

$$B(\text{L}) = \frac{B(^3\text{H})\Omega_1\alpha_2}{\Omega_1\alpha_1} \quad (22)$$

From Eq. (21),

$$B(\text{L}) = \frac{R(^3\text{H})}{\Omega_1\alpha_1} \quad (23)$$

If we now substitute for  $\frac{1}{\Omega_1\alpha_1}$  from Eq. (20)

$$B(\text{L}) = \frac{R(^3\text{H})\text{kM}\phi_{\text{CL}}}{(R_{\text{L}})_0} \quad (24)$$

The right-hand side of Eq. (24) contains only known constants or measured quantities. It is not necessary to know either  $\alpha_1$  or  $\alpha_2$  or the emission spectrum of any of the sources, or the exact light collection efficiency. Since  $R(^3\text{H})$  is constant it follows that once

$$\frac{\text{kM}\phi_{\text{CL}}}{(R_{\text{L}})_0} = K \quad (25)$$

is measured for Luminol in the liquid scintillation counter, any strongly quenched  $^3\text{H}$  scintillation cocktail will produce an *equivalent* Luminol photon emission rate given by

$$B(L) = K \times R(^3\text{H}) \quad (26)$$

A similar derivation for the case of D.C. amplifier current measurements in any arbitrary light collecting geometry is given in Seliger (1978).

If it is desired to use the quenched  $^3\text{H}$  scintillation cocktail as a reference source for the firefly bioluminescent reaction for ATP assays, it is only necessary to react a known number of firefly luciferin molecules with a large excess of luciferase and adenosine triphosphate, to obtain a new constant for Eq. (25)

$$K(\text{firefly}) = \frac{k' M(\text{firefly}) \phi_{\text{CL}}(\text{firefly})}{[R(\text{firefly})]_0} \quad (27)$$

The molar extinction for firefly luciferin at pH 5.0  $\lambda_{\text{max}} = 328 \text{ nm}$  is  $1.82 \times 10^4$  (Morton *et al.*, 1969) and the bioluminescent quantum yield for firefly luciferin is 0.88 (Seliger and McElroy, 1959; 1960). The *equivalent* firefly bioluminescence photon emission rate will be

$$B(\text{firefly}) = K(\text{firefly}) \times R(^3\text{H}) \quad (28)$$

#### D. Emission Spectra of Chemiluminescent Reactions

The extreme sensitivity of photon-detection techniques makes possible the measurement of chemiluminescent reactions whose quantum yields may be as low as  $10^{-16}$ . Therefore it is essential, if these chemiluminescences are to be related to some physiological or biochemical processes, to be able to identify the nature of the reactions and the reactants. In many cases the yields of products are outside of the range of assay by microanalytical chemistry. In these cases, despite the uncertainties described in Section II above, the only remaining analytical technique is the comparison of the precise shapes of the chemiluminescence emission spectra with the photoexcited fluorescence emission spectra of proposed product molecules and the induction from these data of the substrate molecules and the mechanisms or pathways of the oxidation reactions.

In principle the chemiluminescent reaction can be carried out at the entrance slit of a large aperture spectrometer with a phototube detector at the exit slit, in the same manner as fluorescence emission spectra are measured (Lee and Seliger, 1965). If the chemiluminescent reaction emits rapidly decaying flashes a variant of this static measurement, called the Charge Normalization Technique (Seliger, 1960) can be used. In this case a second phototube, viewing the source outside of the entrance slit, is used to normalize the emission flashes in a point-by-point measurement.

At a wavelength the setting,  $\lambda_i$ , the photoelectrons collected at the anode of the spectrometer phototube due to a chemiluminescent flash will be

$$q_1(\lambda_i) = \int_0^t dt I(\lambda_i, t) \Omega_1 T_1(\lambda_i) \alpha_1(\lambda_i) G_1 \quad (29)$$

where  $I(\lambda_i, t)$  is the intensity of photon emission by the chemiluminescent source of photons in a band whose center is at  $\lambda_i$  and whose bandwidth is determined by the slit widths of the spectrometer,  $\Omega_1$  is the light collecting geometry,  $T_1(\lambda_i)$  is the transmission of the spectrometer for the wavelength band denoted by  $\lambda_i$ ,  $\alpha_1(\lambda_i)$  is the photoelectric efficiency of the spectrometer phototube photocathode for photons of wavelength  $\lambda_i$  and  $G_1$  is the multiplication factor of the phototube, determined by the phototube high voltage.

Similarly the photoelectrons collected by the normalization phototube, viewing the complete spectrum, will be

$$q_2 = \int_0^t dt \int_{\lambda_{\min}}^{\lambda_{\max}} d\lambda I(\lambda, t) \Omega_2 \alpha_2 G_2 \quad (30)$$

where  $\Omega_2$  is the light collection geometry and  $G_2$  is the multiplication factor for the normalization phototube. The factor  $\alpha_2$  is the mean photoelectric efficiency of the normalization phototube photocathode for the entire chemiluminescent spectrum.

Since the shape of the emission spectrum is invariant with time

$$\int_0^t I(\lambda_i, t) dt = f(\lambda_i) \int_0^t dt \int_{\lambda_{\min}}^{\lambda_{\max}} d\lambda I(\lambda, t) \quad (31)$$

where  $f(\lambda_i)$  is the fraction of photon emission within the wavelength band denoted by  $\lambda_i$ .

Dividing Eq. (29) by Eq. (30) and solving for  $f(\lambda_i)$  we obtain

$$f(\lambda_i) = \left[ \frac{q_1(\lambda_i)}{q_2} \right] \times \left[ \frac{\Omega_2 \alpha_2 G_2}{\Omega_1 G_1} \right] \times \left[ \frac{1}{\alpha_1(\lambda_i) T(\lambda_i)} \right] \quad (32)$$

The quantities in the first bracket are measured quantities. The quantities in the second bracket are constants of the experiment and need not be measured. The quantity  $\alpha_1(\lambda_i) T(\lambda_i)$  in the third bracket is the efficiency of the spectrometer phototube for producing a photoelectron from a photon of wavelength  $\lambda_i$ . Since only the *relative* value of  $f(\lambda_i)$  is necessary to plot the *shape* of the emission spectrum it can be seen from Eq. (32) that only the ratio of  $q_1(\lambda_i)$  and  $q_2$  need be measured. The relative values of the function  $\alpha_1(\lambda) T(\lambda)$ , characteristic of the spectrometer-phototube, must be determined separately. Eq. (32) is therefore independent of the time course of the chemiluminescent reaction, provided both phototubes measure over the *same* time interval.

Methods and extensive references for measuring absolute and relative spectral sensitivities of spectrometer-phototube detectors are summarized in Seliger (1978). The simplest and most direct method for determining this function is by use of a tungsten-halogen Standard Lamp of Spectral Irradiance and a diffuse reflector. The lamp emission at a fixed power dissipation is specified in units of microwatts per unit wavelength interval per square centimeter at a fixed distance from the lamp filament. This can be converted to photons  $s^{-1} cm^{-2}$  at a fixed distance of wavelengths between  $-\frac{\Delta\lambda}{2} + \lambda_i$  and  $\lambda_i + \frac{\Delta\lambda}{2}$ , where  $\Delta\lambda$  is the wavelength pass band of the spectrometer. A white diffusing plate made of MgO smoke or BaSO<sub>4</sub> (Eastman White Reflecting Paint) is placed near and facing the entrance slit so that the ratio of the distance of the plate from the slit to the projection of the diameter of the plate is less than the numerical aperture of the spectrometer. The diffuser plane is adjusted to be exactly normal to the line joining the lamp to the diffuser. The light source should illuminate the face of the diffuser without the spectrometer housing obscuring any portion of the diffuser. The diffuser face should be close to normal to a line joining the entrance slit to the diffuser. This requirement is not critical since the property of a Lambertian diffuser makes the spectrometer response independent of this angle. The distance of the diffuser plate from the entrance slit is arbitrary so long as the slit aperture is

completely filled. If the distance from the lamp to the diffuser is measured accurately the spectral response function of the spectrometer-phototube can be calibrated absolutely. For most cases, as for Eq. (32), only the relative response function is required.

The current in amperes measured by the spectrometer-phototube combination for any wavelength setting  $\lambda$  and slit width  $\Delta\lambda$

$$I(\lambda) = \frac{B(\lambda)}{D^2} \Delta\lambda \Omega \alpha(\lambda) T(\lambda) G \times \frac{\lambda [\text{nm}]}{198.7} \times 10^{12} \times 1.60 \times 10^{-19} \quad (33)$$

where  $\frac{B(\lambda)}{D^2}$  is the power incident on the diffuser at a distance  $D$  from the lamp, per unit wavelength [ $\mu\text{W cm}^{-2}$ ],  $\Delta\lambda$  is the bandwidth of the spectrometer,  $\Omega$  is the optical geometry for light collection by the spectrometer entrance optics, the product  $\alpha(\lambda)T(\lambda)$  is the photoelectric efficiency of the system,  $G$  is the gain of the phototube,  $\frac{\lambda [\text{nm}]}{198.7} \times 10^{12}$  converts  $\mu\text{W}$  to photons  $\text{s}^{-1}$  and  $1.60 \times 10^{-19}$  converts electrons at the anode to the practical system of coulombs.

The factors  $\Delta\lambda$ ,  $\Omega$ ,  $G$  and  $D$  are constant during the measurement. We can solve Eq. (33) for  $\alpha(\lambda)T(\lambda)$

$$\alpha(\lambda)T(\lambda) = \frac{I(\lambda)}{B(\lambda) \times \lambda} \times K_1 \quad (34)$$

where  $I(\lambda)$  is measured,  $B(\lambda)$  is known from the Standard Lamp specifications and  $\lambda$  is the wavelength setting of the spectrometer. The constants lumped into  $K_1$  need not be measured or known to determine the relative spectral efficiency function in Eq. (34), to be used in Eq. (32).

For exceedingly weak chemiluminescent reactions it is possible to improve the light collection geometry at the expense of spectral resolution and to use a wedge interference filter spectrometer as shown in Fig. 4 (Hamman, Biggley and Seliger, 1979).

In Fig. 4 an opaque plastic sample holder (H) whose 0.5-mm thick base contains a 3 mm wide x 10 mm long slit (S), positions the bottom of a 13 mm I.D. x 45 mm glass vial at 1.5 mm above the top surface of a Verlauf B-60 wedge interference filter (F) (60 mm long x 25 mm wide x 3 mm thick; Schott Optical Glass Co., Inc.). The filter is mounted in a 3-mm thick filter holder which slides horizontally 1 mm above the photocathode of an EMI 9789 QB phototube (effective diameter of photocathode 10 mm). The total distance from the bottom of the solution in the glass vial to the photocathode is 6 mm. A synchronous motor rotates a screw drive which in turn drives the

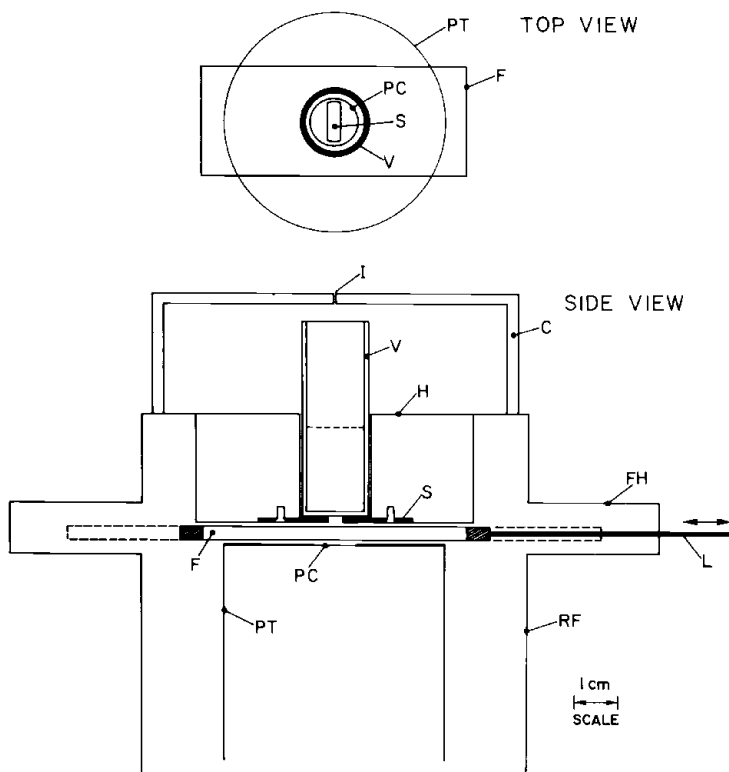


Fig. 4. Schematic drawing of the details of the construction of the wedge interference filter spectrometer. I, injection port; C, light-tight cover; V, sample vial; H, sample vial holder; S, slit; FH, filter housing; F, wedge interference filter; PT, phototube; PC, photocathode; L, linkage to screw drive; RF, RF shielded phototube housing.

interference filter in a horizontal plane at a maximum scanning rate of  $300 \text{ nm min}^{-1}$ . A microswitch closes on each revolution of the screw to give a wavelength position reference signal. The phototube is operated in a single-photon counting mode. Electronic detection is accomplished with an Ortec preamp (Model 9301), amplifier-discriminator (Model 9302) and rate meter (Model 9349). The analog signal from the rate meter and the wavelength markers due to the closing of the microswitch are recorded on a strip chart recorder exhibiting a fast response.

In this case the geometry is quite poor and it is preferable to measure the relative spectral efficiency function by making use of chemiluminescent reactions whose emission spectra are known precisely, *i.e.* the Luminol chemiluminescence and the firefly bioluminescence.

At any wavelength setting of the wedge interference filter spectrometer the current measured due to a known chemiluminescent reaction in exactly the same geometry as any unknown reaction will be

$$I(\lambda) = Cf(\lambda) \times \Omega \times \alpha(\lambda)T(\lambda) \times G \times 1.60 \times 10^{-19} \quad (35)$$

where  $C$  is the total photon intensity of the known chemiluminescent reaction,  $f(\lambda)$  is obtained from the known emission spectrum and the other symbols have the same meaning as before. If  $C$  is kept constant we can solve Eq. (35) for  $\alpha(\lambda)T(\lambda)$  to give the relative spectral efficiency function.

$$\alpha(\lambda)T(\lambda) = \frac{I(\lambda)}{f(\lambda)} \times K_2 \quad (36)$$

It is possible, using a known chemiluminescent reaction such as Luminol to specify the sensitivity of any spectrometer for the measurement of emission spectra in terms of a noise-equivalent signal at a specified wavelength and bandwidth. For example at 490 nm, the approximate peak of emission for Luminol chemiluminescence in dimethylsulfoxide, and at a bandpass of 24 nm, a vial of a Luminol reaction emitting  $2.72 \times 10^7$  photon  $s^{-1}$  isotropically into the light collecting geometry of the wedge filter spectrometer produced  $10^3$  pulses  $s^{-1}$  at 20°C while the phototube dark noise was 20 pulses  $s^{-1}$ . From the known chemiluminescence emission spectrum of Luminol the fraction  $f(490)$  in a 24 nm bandwidth was calculated to be 0.21. The efficiency of the wedge interference filter spectrometer for detecting an isotropic source of 490 nm photons is therefore

$$\frac{10^4}{0.21 \times 2.72 \times 10^7} = 1.8 \times 10^{-3}$$

The dark noise of 20 pulses  $s^{-1}$  is therefore equivalent to an isotropic photon source of  $10^4 s^{-1}$ , which is the noise equivalent signal at 490 nm and a bandwidth of 24 nm:

$$(NES)_{490}^{24} = 10,000 \text{ photons } s^{-1} \quad (37a)$$

This represents an improvement in signal-to-noise by more than a factor of 100 over the most sensitive grating spectrometer that we have used previously (Seliger *et al.*, 1974).

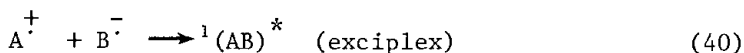
The optimum light collection geometry for chemiluminescent solutions uses an ellipsoidal reflector with the light source at one focus and a cooled phototube photocathode at the other. Individual colored glass filters are placed near the face of the phototube and the emission spectrum is determined by the differences in filter transmissions. With this idealized system the authors report a sensitivity such that their noise equivalent signal at 500 nm and a bandwidth of 30 nm is (Inaba *et al.*, 1979)

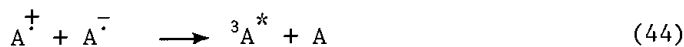
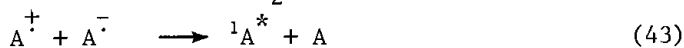
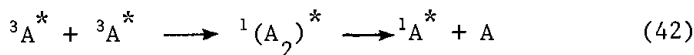
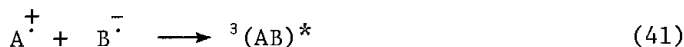
$$(\text{NES})_{500}^{30} = 250 \text{ photons s}^{-1} \quad (37b)$$

## V. CHEMILUMINESCENT REACTIONS IN SOLUTION

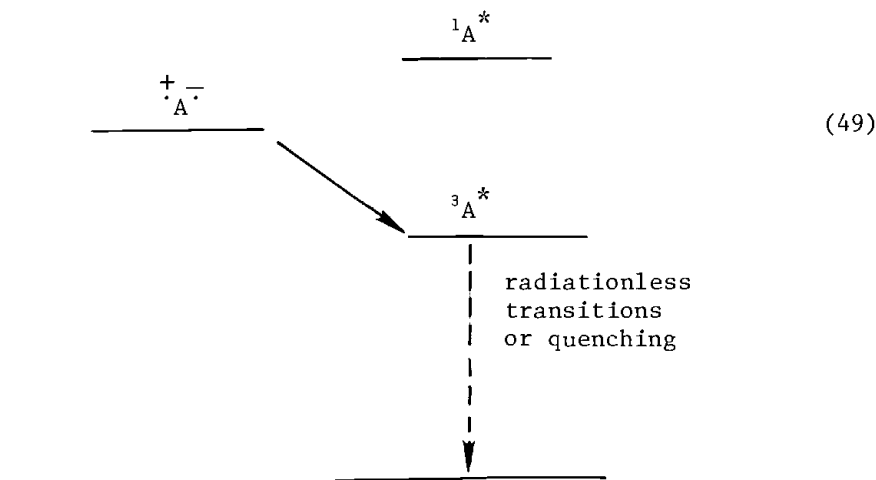
The requirement for luminescence in molecules is an electronic charge displacement corresponding to one of the allowed energy state configurations above the ground state. If these displacements are produced through chemical reactions the population of chemi-excited states is not restricted by photoselection absorption rules and it is in principle possible to observe emissions that are difficult to produce by direct photoexcitation. Some of the weak UV chemiluminescence reported by Audubert in acid-base neutralization reactions and hydration and dehydration reactions may have been emissions from excited hydroxyl ions or radicals or from dissolved  $\text{N}_2$  or  $\text{O}_2$  populated by collisional encounters. All of these have transitions in the UV (Herzberg, 1963 Table 39). Crystalloluminescence of alkali halide solutions in the UV region has been observed.

By far the greatest yield of chemiluminescent reactions in biological systems is produced by oxidation reactions involving the chemical species of oxygen,  $\text{O}_2$ ,  $\text{H}_2\text{O}_2$ ,  $\text{O}_2^{\cdot-}$ ,  $\text{HO}^{\cdot}$  and  $\text{HO}_2^{\cdot}$ . It is possible that all chemiluminescent emissions with the exception of dissociation reactions are the result of precursor radical recombination reactions:

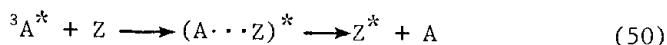




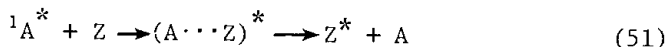
The directions of these reactions will depend on the free energies of the respective states. For example in the simplest case of Eq. (47) and (48)



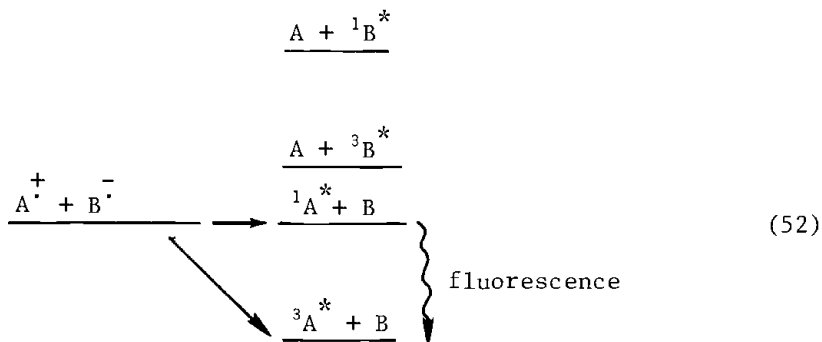
the free energy of the biradical  $\overset{\cdot+}{\cdot-}A$  may not be sufficient to populate the singlet excited state. The oxidation reaction leading to  $\overset{\cdot+}{\cdot-}A$  will therefore not be chemiluminescent. However if a high  $Z$  (efficient intersystem crossing) fluorescent acceptor is present in sufficient concentration it will be possible to observe a *triplet sensitized* chemiluminescence resulting in the fluorescence of  $Z$  which is not a product of the reaction.



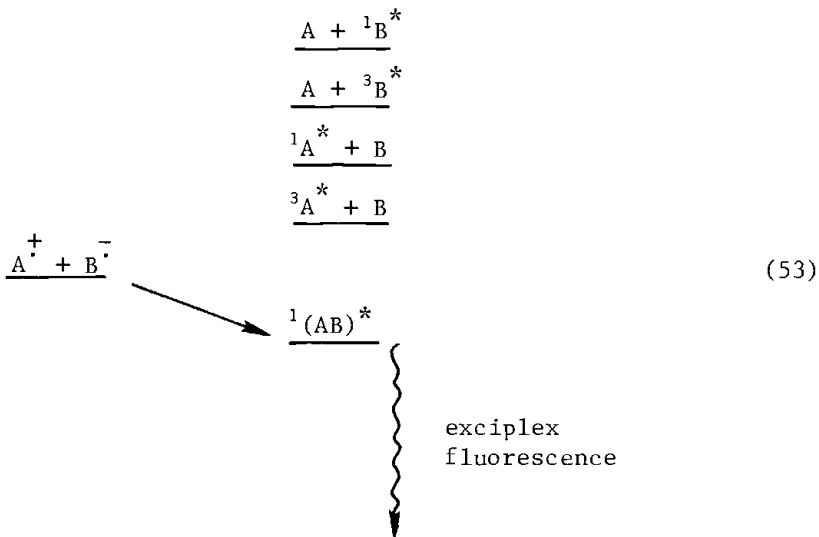
It follows that *singlet sensitized* chemiluminescence is also possible.



In the case of  $A^{\dagger}$  and  $B^{-}$  recombinations it is possible that the free energies are such that the excited state  ${}^1A^*$  is very probable and the excited state  ${}^1B^*$  is not accessible, resulting in an efficient chemiluminescence emission of the product A.

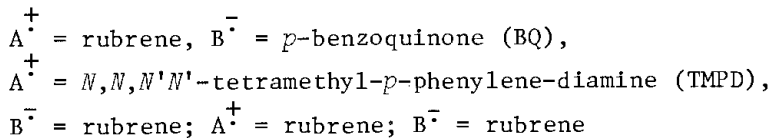


A third case is represented by Eq. (40),



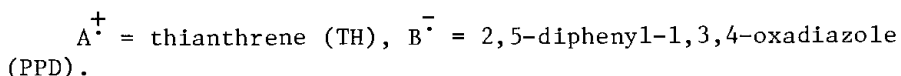
where only the exciplex  ${}^1(AB)^*$  can be populated. The chemiluminescence of Eq. (53) is significantly different from the expected fluorescence of the product A as in Eq. (52).

Faulkner *et al.* (1972) have shown for the cases of



that only singlet rubrene fluorescence (Eq. (43), (53)) is observed.

The 1<sup>st</sup> excited singlet states of both BQ and TMPD are higher in energy than the free energy of the radical pair. A variant of Eq. (52) occurs (Keszthelyi *et al.*, 1972) when



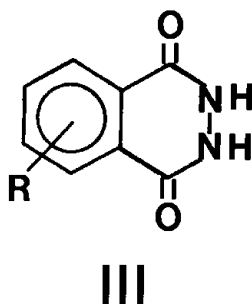
In this case *both* singlet excited states are accessible and radical recombination results in the fluorescences of both TH and PPD.

Radical cations react strongly with nucleophiles. Radical anions can reduce  $O_2$  to  $O_2^{\cdot-}$  or are neutralized rapidly by proton donors *i.e.* protic solutions. It is quite possible that in some cases the very small quantum yields of biological chemiluminescences are the result of quenching of the radical precursors to the excited states. Therefore in Eq. (4) the yield  $\phi_{ex}$  may be the limiting factor in the observation of the products or rates of oxidation reactions by luminescence.

### A. Luminol

The efficient chemiluminescence of Luminol (I) has been used as a tracer for monitoring protein binding reactions. Ligands such as biotin and thyroxin are attached to the free amino residue through an alkyl-bridging group (Schroeder *et al.*, 1978). Under the controlled catalytic decomposition conditions the reaction of Luminol with  $H_2O_2$  is stoichiometric so that the chemiluminescence can be used as an assay for the production of  $H_2O_2$  during the course of enzyme reactions (Seitz and Hercules, 1973; Seitz, 1978).

The chemiluminescence of Luminol proceeds through the decomposition of the 1,4-endoperoxide. The reaction intermediate leading to the excited state may be represented by Eq. (47) and (48). Roswell and White (1978) report that the position of the amino group and the substitution for the amino group of a variety of adducts can significantly affect the value of  $\phi_{CL}$ .



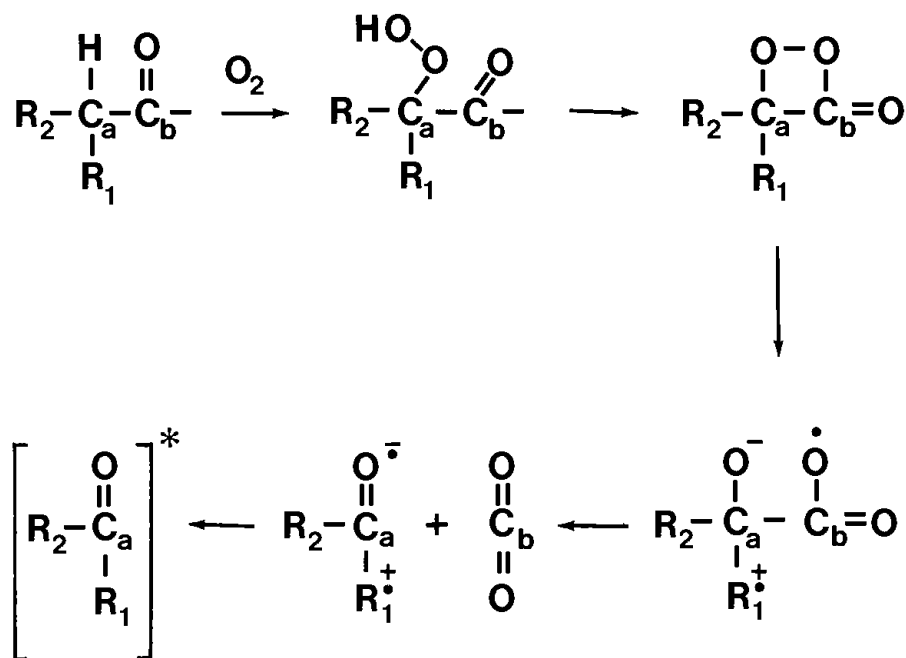
Structure (III), where R = 6-*N*(CH<sub>3</sub>CH<sub>2</sub>)<sub>2</sub> (Gundermann, 1974), R = *f, g, h*-perylene (White and Brundrett, 1973), R = 6-*N*-(3-amino-2-hydroxypropyl)amino and R = *h*-phenyl-3'-{*N*-(4-amino-butyl)-*N*-ethyl}amino (Schroeder *et al.*, 1978) gives higher values of  $\phi_{CL}$  than Luminol (I) (R = 5-amino). Since the fluorescence yield of the aminophthalic acid product (II),  $\phi_{f1} = 0.3$  it is more likely that the R group of (III) affects the efficiency  $\phi_{ex}$  rather than the overall chemical yield  $\phi_{chem}$  of Eq. (4).

### B. Biological Chemiluminescence

Reactions of Eq. (38)–(53) can give rise to luminescence. Dioxetane intermediates responsible for the chemiluminescence of many biological luciferin molecules proceed through Eq. (47) to produce the singlet excited state of the oxidized product molecule (Seliger, 1975; Koo and Schuster, 1978).

H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub> are produced by a large variety of mixed function oxidase reactions. H<sub>2</sub>O<sub>2</sub> is decomposed catalytically by heme, Fe<sup>III</sup>, Co<sup>II</sup> and Cu<sup>II</sup>. H<sub>2</sub>O<sub>2</sub> in the presence of peroxidases and chloride ion exerts strong bactericidal action by lipid oxidation possibly through the production of singlet oxygen or hypochlorite (Krinsky, 1974).

All of these reactions specifically utilize molecular oxygen which reacts with the substrate molecules. The intermediates formed in these reactions are either *a*) hydroperoxides or peroxy adducts, *b*) epoxides or *c*) dioxetanes.



(54)

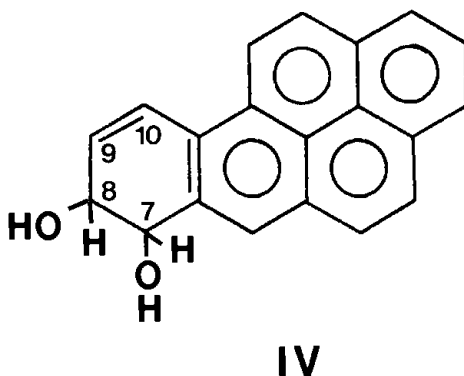
The flavin-peroxy-aldehyde intermediate in bioluminescent bacteria results in the efficient production of the observed chemiluminescence (Balny and Hastings, 1975). Dioxetanes and 1,4-endoperoxides have been demonstrated to be the active intermediates in a wide variety of chemiluminescent reactions.

It is no wonder that chemiluminescence should be observed during biological oxidation reactions! So far as we know the only *function* for chemically produced excited states in biological systems is for bioluminescence, the emission of light for signalling. In these cases there have evolved concurrently, specific enzymes and substrate molecules where  $\phi_{\text{chem}}$ ,  $\phi_{\text{ex}}$ ,  $\phi_{\text{fl}}$  and  $\phi_{\text{p}}$  (Eq. (4)) are close to unity as well as complex regulatory and triggering mechanisms for the controlled emission of this chemiluminescence. Gurwitsch proposed that a low intensity UV chemiluminescence might stimulate cell division. In view of the strong non-specific absorption of this short wavelength UV by all biological material it is unlikely that there is a specific receptor molecule for this radiation. In only one other mechanism in biochemical systems might there be a

function for chemi-excited states, that is in the mechanism of DNA repair by photoreactivating enzymes. Since photoreactivating enzymes similar to those found in yeast cells have been found in mammalian systems where UV light does not normally penetrate, there might be some selective advantage to the dark chemical production of an excited state *equivalent* to the photoexcited state. In this case there would be a selection against high values of  $\phi_{ex}$  or for  $\phi_{fl}$  of the product molecule; the only requirement would be for the  $\phi_{chem}$ , the activation energy for effecting the *equivalent* photochemical reaction. These reactions, if present, would not be expected to be chemiluminescent.

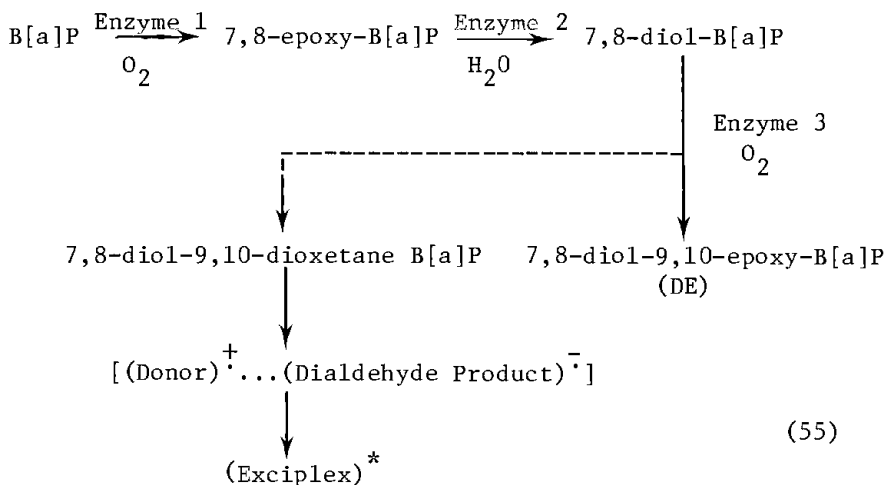
Therefore it can be assumed that biological chemiluminescence, with the exception of bioluminescence, has no function in the energetics of biochemistry or regulation. It is a fortuitous reaction associated with biological oxidations. However this does not preclude its use as an assay technique, *i.e.* for the measurement of rates of certain biological oxidations or for the identification of products that may either be chemiluminescent or reactants in chemiluminescent reactions. Into this category would be placed the delayed luminescence of chloroplasts (Strehler and Arnold, 1951; Arnold and Azzi, 1971), the chemiluminescence of mitochondria (Stauff and Ostrowski, 1967), the chemiluminescence of oxidizing cells and tissues (Barenboim *et al.*, 1969), the chemiluminescence of xanthine oxidase reactions (Totter *et al.*, 1960a; 1960b; Greenlee *et al.*, 1962; Arneson, 1970), the chemiluminescence of liver microsomal extracts (Howes and Steele, 1971; 1972), the chemiluminescence of polymorphonuclear leucocytes (Allen *et al.*, 1972; Allen, 1973; Trush *et al.*, 1978) and other peroxidases (Durán *et al.*, 1977) and the chemiluminescence of carcinogenic polycyclic aromatic hydrocarbons as the result of their metabolism by the aryl hydrocarbon hydroxylase detoxification systems in liver microsomes (Hamman and Seliger, 1976; Seliger and Hamman, 1976; 1977).

1. *Microsomal Chemiluminescence of Carcinogenic Hydrocarbons.* As an example of the utility of spectroscopic identification of chemiluminescent spectra, it has been demonstrated that the chemiluminescence emission of the carcinogen benzo[a]pyrene proceeds through the production of a dioxetane intermediate of the 7,8-diol metabolite of benzo[a]pyrene (IV) that undergoes inter-molecular electron exchange leading to Eq. (53) (Hamman *et al.*, 1979). In Eq. (53)  $B^{\cdot-}$  is the dialdehyde radical anion product of the 7,8-diol-benzo[a]pyrene and  $A^{\cdot+}$  can be any other planar aromatic hydrocarbon, pyrene, perylene, naphthacene or a second molecule of (IV) that has donated an electron to the dioxetane. The chemiluminescence is an exciplex emission. Monomer fluorescence of the dialdehyde product



is not observed. It is conceivable that an electron donor A in Eq. (53) may have an excited singlet state of lower energy than the free energy of  $A^+ + B^-$ , in which case a dual chemiluminescent emission might be observable.

The ultimate chemical carcinogenic metabolite of the parent carcinogen benzo[a]pyrene has been shown to be the 7,8-diol-9,10-epoxide (DE) of benzo[a]pyrene produced from the 7,8-diol precursor. The mechanism study has shown that the observed microsomal chemiluminescence of benzo[a]pyrene arises mainly from the dioxetane of the 7,8-diol. It can be presumed that the enzymatic reactions leading to the carcinogenic DE can be summarized as:

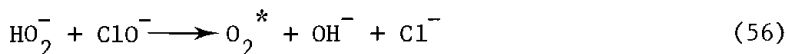


where a fraction of the 7,8-diol proximate carcinogenic metabolites proceeds through a dioxetane intermediate and produces chemiluminescence. If this is the case it can be inferred that the microsomal chemiluminescence of parent polycyclic aromatic hydrocarbons is directly correlated with the production of their carcinogenic diol epoxides and is therefore an assay for these specific enzyme reactions. The converse, that non-carcinogenic polycyclic aromatic hydrocarbons are not transformed into carcinogenic diol epoxides and from Eq. (55) should exhibit much weaker or negligible microsomal chemiluminescence also appears to be valid. We have tested a small number of carcinogenic and non-carcinogenic hydrocarbons for microsomal chemiluminescence with the results shown in Table 1.

TABLE 1

<i>Compound</i>	<i>Carcinogenic</i>	<i>Microsomal Relative Chemiluminescence</i>
<i>Benzo [a]pyrene</i>	+	+
<i>dibenzo [a, h]pyrene</i>	+	+
<i>dibenz [a, i]anthracene</i>	+	+
<i>7, 12-dimethyl benz [a]- anthracene</i>	+	+
<i>3-methyl-cholanthrene</i>	+	+
<i>7, 8-diol-benzo [a]pyrene</i>	+	+
<i>Anthracene</i>	-	-
<i>Pyrene</i>	-	-
<i>Benzo [e]pyrene</i>	-	-
<i>Perylene</i>	-	-
<i>Naphthacene</i>	-	-

2. *Spontaneous Chemiluminescence of Urine.* There are a large number of potentially chemiluminescent metabolic products excreted in urine. Reaction of a 1 ml aliquot of a urine sample in the singlet oxygen generating system



produces chemiluminescent flash intensities greater than  $10^7$  photons  $\text{s}^{-1}$ . The spontaneous chemiluminescence of urine remains constant at a low intensity for many hours and is easily measurable in the liquid scintillation counter. In a sample of 94 non-smokers the urine specific spontaneous chemiluminescence

intensity, corrected for self-absorption, was normally distributed and approximately  $40 \text{ photons s}^{-1} \text{ ml}^{-1}$  with a coefficient of variation of 45%. Since the emission spectrum of this chemiluminescence has not yet been measured the mean of  $40 \text{ photons s}^{-1} \text{ ml}^{-1}$  can be low by as much as a factor of 2. In non-smokers the spontaneous specific chemiluminescence varies only slightly with the time of day of the collection. This variation is within the range of the coefficient of variation.

The urine of cigarette smokers has been shown to contain significantly higher concentrations of mutagens than that of non-smokers (Yamasaki and Ames, 1977) and in general ingested carcinogens result in the excretion of their mutagenic products in the urine (Durstun and Ames, 1974). It is possible that the specific spontaneous chemiluminescence or the induced chemiluminescence of a component of urine may also reflect the increased concentrations of mutagens detected by the Ames bacterial revertant assay and thus provide a convenient method for following the exposures of large populations to environmental carcinogens.

### 3. Spontaneous Chemiluminescence of Cigarette Smoke.

Figure 5 is a contact photograph using Polaroid Type 410 film exposed to the spontaneous chemiluminescence emitted by an extract of the smoke from a single cigarette. The smoke puff by puff was collected on a glass fiber filter and extracted in the dark into 60 ml of *N,N*-dimethylformamide. The extract was initially heated to  $75^\circ\text{C}$  in a glass beaker and the beaker placed on the glass projection slide whose photograph is shown. The glass slide in turn rested on the Polaroid film. Exposure time to the chemiluminescence in the beaker was approximately 10 minutes. The photographed slide shows the spontaneous chemiluminescent emission spectra of *a*) a single puff (35 cc) of cigarette aerosol and *b*) a single puff of cigarette aerosol extracted into *N,N*-dimethylformamide (Seliger *et al.*, 1974). The red-shifted spectrum in Fig. 5a is due to self-absorption of the chemiluminescence by the aerosol particles while the lower curve is the true emission spectrum in solution.

The spontaneous chemiluminescence of cigarette smoke is the result of pyrolysis of the tobacco. It is not present in tobacco extracts. The kinetics of decay of the spontaneous chemiluminescence are higher than 1<sup>st</sup> order. Approximately  $10^{12}$  photons are emitted from the extract of a single puff of cigarette smoke over a period of 16 hours indicating a significant concentration of reactive precursors, produced by burning. The spontaneous chemiluminescence of one puff of cigarette smoke, extracted into *N,N*-dimethylformamide and heated to  $60^\circ\text{C}$ , is visible to the dark-adapted eye. Under constant conditions of mechanical puffing and extraction the observed spontaneous

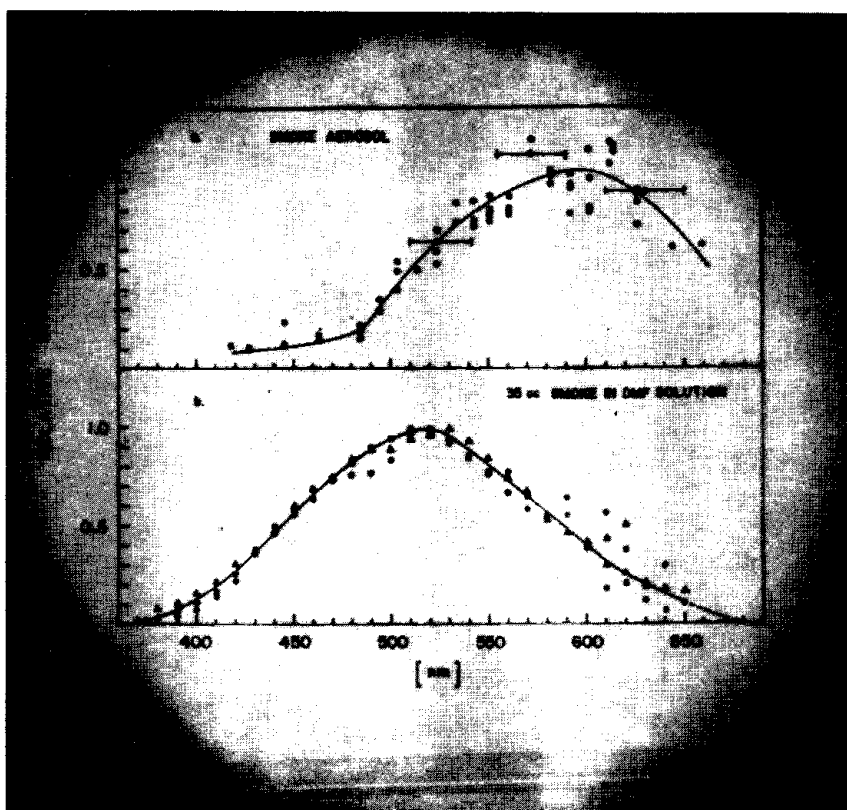
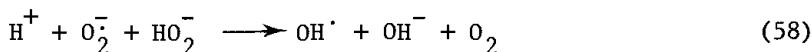
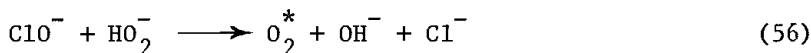
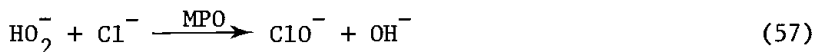


Fig. 5. Contact photograph of a projection slide made on Polaroid Type 410 film by the chemiluminescent emission of the smoke extract of a single cigarette.

chemiluminescence covaried with tar and nicotine content of the cigarette brand. As in the case of urine chemiluminescence the singlet oxygen-induced chemiluminescence intensity of cigarette smoke extracts is orders of magnitude higher than the spontaneous chemiluminescence intensity. As might be expected cigarette smoke aerosol contains significant concentrations of products that can be metabolically activated to produce mutagenesis in the Ames bacterial system (Kier *et al.*, 1974).

4. *Chemiluminescence of Phagocytic Cells.* The bactericidal action of phagocytic cells such as the polymorphonuclear leukocytes (PMN) was shown by Klebanoff (1967; 1968) to result from the reaction of the enzyme myeloperoxidase MPO and  $\text{H}_2\text{O}_2$  in the presence of halide ions. The reactions postulated were



and it was assumed that the chemiluminescence of phagocytizing PMN measured in the liquid scintillation counter was the emission of singlet oxygen. In view of Fig. 2 this is extremely unlikely. It is more probable that the chemiluminescence is a non-specific emission from oxidized membrane lipids and even a sensitized chemiluminescence. The requirement for MPO for both chemiluminescence and bactericidal action is the diagnostic feature of this assay. In genetic myeloperoxidase deficiency phagocytosis occurs but there is negligible destruction of the ingested bacterium and negligible chemiluminescence.

Since the chemiluminescence is an assay for phagocytizing cells and Eq. (56) represents a singlet oxygen generating system it is possible to use the Luminol chemiluminescence reaction to enhance the  $\phi_{\text{CL}}$  (Eq. (4)) and therefore to increase the sensitivity of the assay. Trush *et al.* (1978) report a maximum chemiluminescent intensity of  $3.3 \times 10^{-4}$  count  $\text{s}^{-1}$   $\text{PMN}^{-1}$  from normal PMN measured in the liquid scintillation counter. An increase in specific chemiluminescence by factors of 10-100 is measured when Luminol enhancement of chemiluminescence is used ( $4.5 \times 10^{-8}$  M final concentration assuming a minimum of 10 ml of solution in the scintillation counter vial).

The intensity of Luminol chemiluminescence is maximum above pH 10.4 and decreases markedly at lower pH values (Bostwick and Hercules, 1975). The pH optima of the MPO and the oxidase systems generating  $\text{H}_2\text{O}_2$  are significantly lower. Therefore one must be quite careful to investigate pH dependence in the interpretation of induced Luminol chemiluminescence data. The interpretation is more complex since Luminol chemiluminescence is produced by a variety of peroxidases (Schroeder *et al.*, 1978) in the presence of  $\text{H}_2\text{O}_2$ , although these authors have not apparently investigated the stimulation of the reaction by halogen ions as in the case of MPO.

## VI. CONCLUSIONS

The detection of light quanta emitted by chemical reactions remains the most sensitive analytical technique for measuring reaction rates. The sensitivity can be expressed as a noise-equivalent signal for the phototube detectors. In the liquid scintillation counter light-collecting geometry (close to 100%) the single photoelectron dark-noise can be as low as 120 pulses  $s^{-1}$ . Assuming a photocathode photoelectric efficiency of 0.2 the noise-equivalent-signal for the rate of a chemiluminescent reaction (within the S-11 response curve of Fig. 2) is

$$NES = \frac{10^{-21}}{\phi_{CL}} \text{ mole } s^{-1} \quad (59)$$

For the same chemiluminescent reaction the spectrophotometric determination of the emission spectrum has a noise-equivalent signal at peak emission wavelength,

$$(NES)_{490}^{24} = \frac{2 \times 10^{-20}}{\phi_{CL}} \text{ mole } s^{-1} \quad (60)$$

It should be possible therefore to assay and to identify by chemiluminescence, the rate and nature of almost any strongly oxidizing chemical reaction involving molecular oxygen. One can measure the endogenous low-intensity chemiluminescence or assay for products of the reactions such as  $H_2O_2$  or  $O_2^-$  by the addition of suitable chemiluminescent materials that require these reactants. The extension of single photon chemiluminescence techniques to analytical assays in biochemistry as well as to industrial and environmental processes awaits the ingenuity of the researcher.

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