

LIQUID SCINTILLATION COUNTING
RECENT APPLICATIONS AND DEVELOPMENT
VOLUME II. SAMPLE PREPARATION AND APPLICATIONS

CHARACTERIZATION OF NEUTROPHIL CHEMILUMINESCENCE
USING A LIQUID SCINTILLATION COUNTER¹

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I. INTRODUCTION

Human polymorphonuclear neutrophils (PMN) engaged in oxidative antimicrobial activity chemiluminesce (Allen, 1972). This CL is concomitant with the generation of reactive electron reduction products of molecular oxygen (O_2^- , H_2O_2 , OH^\cdot) and of the singlet excited state 1O_2 (Klebanoff, 1975). The halogenation of microbes by the myeloperoxidase (MPO)-halide- H_2O_2 microbicidal system appears to be one source of light from respiratory active PMN (Rosen, 1976). MPO light generation involves the reaction of hypochlorite with H_2O_2 for the generation of 1O_2 (Rosen, 1977). Spontaneous conversion of 1O_2 to the ground state is accompanied by a pulse of light and this may be the source of PMN CL (Wilson, 1970). Alternatively, 1O_2 may oxygenate areas of high electron density

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resulting in labile polysubstituted dioxetanes (Foote, 1976). As a consequence of the weakness of the peroxidic link dioxetanes spontaneously cleave resulting in carbonyl fragments of high free energy. Light emission is possible from relaxation of excited carbonyls and may be responsible for the CL of PMN (Allen, 1972). However, light generating reactions may be the property of many or all oxidizing transients (Babior, 1978) and an additional complexity is introduced by the interconvertibility of derived species of oxygen (Cundall, 1977). Further insight into the oxidation mechanisms and information regarding the photon emitting species may be gained by considering the spectral properties of the emitted CL (Maugh, 1973). No commercial instruments are available to establish an accurate spectrum because of the low intensity of the emitted light (Stauff, 1964). However, the weak CL emitted by PMN and certain chemical reactions can be routinely detected by the commercial photomultipliers of liquid scintillation counters (Allen, 1977). With suitable manipulation the spectral regions can be analyzed to yield important information on the mechanistic origin and nature of chemiluminescent systems. Utilizing such technology, Cheson (1976) has established the spectral characteristics of activated neutrophils and found transmission by a xanthine oxidase/purine/zymosan system to be similar. Confirmatory evidence was presented by Andersen (1977) using a similar system. In addition, this author reported spectral equivalence between PMN emission and emission from a cell-free MPO-H₂O₂-Cl system. More recently, McPhail (1979) has shown an NADPH-dependent oxidase cell-free system, isolated from human PMN, to produce CL with characteristics like those of intact cells.

It is the purpose of this communication to report on the spectral characteristics of several other chemiluminescent systems which may contribute to a greater understanding of the CL associated with phagocytosis.

II. MATERIALS AND METHODS

A. Reagents

Tetramethyl-1,2-dioxetane, prepared by treating the corresponding bromohydroperoxide with base, was a generous gift from Dr. K.R. Kopecky, University of Alberta, Department of Chemistry, Edmonton, Alberta, Canada.

Potassium peroxychromate (K₃CrO₈) was synthesized according to the method of Risenfeld (1905). Potassium chromate (16.9 g, 9.087 mol) and potassium hydroxide (3.5 g, 0.065 mol) in 300 ml of water was slowly at 5° C to 300 ml of

33% aqueous methanol containing 30 ml of 30% H₂O₂.

L-ascorbic acid (ASC), sodium hypochlorite (NaOCl), hydrogen peroxide (H₂O₂), dimethyl sulfoxide (DMSO), acetone, acetaldehyde, and benzene were obtained from Fisher Scientific Company.

Potassium superoxide (KO₂) was purchased from Alfa Products.

B. Enzymes

Myeloperoxidase was prepared from leukocytes by the weak acid extraction technique of Klebanoff (1965). Approximately 10¹⁰ leukocytes were collected in a volume of 400 ml, during leukapheresis of a patient with chronic granulocytic leukemia and acid extracted. Sephadex G-100 chromatography was used to partially purify the MPO so obtained. MPO containing fractions were eluted from the column after the void volume and concentrated. Peroxidase dependent oxidation of O-dianisidine by H₂O₂, recorded spectrophotometrically as described by Klebanoff (1965), was used to determine peroxidase activity. Xanthine and xanthine oxidase were purchased from Sigma Chemical Company.

C. Preparation of Cells

PMN and mononuclear leukocytes (MNL) were prepared from Ficoll-Hypaque density difference centrifugation as previously described by English (1976). After isolation, the cells were enumerated and diluted to desired concentrations in Hank's balanced salt solution (HBSS), Grand Island Biological Company, Grand Island, New York.

D. Preparation of Opsonized Particles

Zymosan (ICN Pharmaceuticals Incorporated, Life Science Group, Plainview, New York) was opsonized by suspension of 100 mg in 1 ml fresh human serum and 1 ml HBSS for 30 min at 37° C. The opsonized particles were recovered by centrifugation (100 x g, 5 min), subsequently washed twice and resuspended in HBSS at a final concentration of 20 mg/ml.

E. Preparation of Bacteria

Strain 502_A of *Staphylococcus aureus*, and strain K₁₂ of *Escherichia coli*, were prepared from overnight cultures in

Penn-Assay broth. Bacteria were pelleted by centrifugation (1800 x g, 10 min) and opsonized in HBSS containing 10% fresh human serum (0.5 ml/ml pelleted), for 30 min at 37° C. Bacteria were recovered by centrifugation, washed twice with saline and resuspended at a final concentration of 1×10^{12} organisms per ml in HBSS as determined by routine pour plate techniques.

F. Quantitation of Chemiluminescence

To investigate the weak light fluxes of the cells and chemical reaction systems a single-photon method of recording CL was employed. An Isocap 300 liquid scintillation counter was operated at 27° C in the out-of-coincidence mode as previously described (English, 1976). The tritium window was used to record all counts. CL was recorded as counts per minute. The method of Andersen (1977) was used to characterize the CL spectra. This method, schematically represented in Figure 1, is performed by insertion of gelatin spectral absorption filters (Edmund Scientific Company) between the light emitting sources and the photomultiplier tubes. Calculations of the percent transmitted light were made with reference to the light emission rate from an unfiltered vial in accord with Andersen (1977).

RESULTS

Table 1 describes the various oxidative and $^1\text{O}_2$ generating systems analyzed in this study. As reported earlier by English (1976) PMN generated a shorter lived CL during phagocytosis of opsonized bacteria in comparison to zymosan. Similarly, the cell-free MPO-halide- H_2O_2 system generated a greater CL response to zymosan than it did when *S. aureus* was added. CL resulted from the addition of H_2O_2 to suspensions of heat killed micro-organisms, zymosan and enzymically inactivated (boiled) neutrophils. MPO activity in the boiled preparations was not evident using the benzidine dihydrochloride method of Kaplow (1965). During the course of a preliminary experiment we found that the addition of the anti-oxidant ascorbic acid severely impaired the CL of phagocytosing PMN. Ascorbic acid also caused a comparable inhibition of killing of *S. aureus* by PMN (data not shown). Furthermore, ascorbic acid caused a comparable inhibition of CL in the cell-free MPO- H_2O_2 -Cl system. Our findings are in accord with the evidence of Demopolos (1973), Diluzio (1973), and Tappel (1973), that ascorbic acid might function to

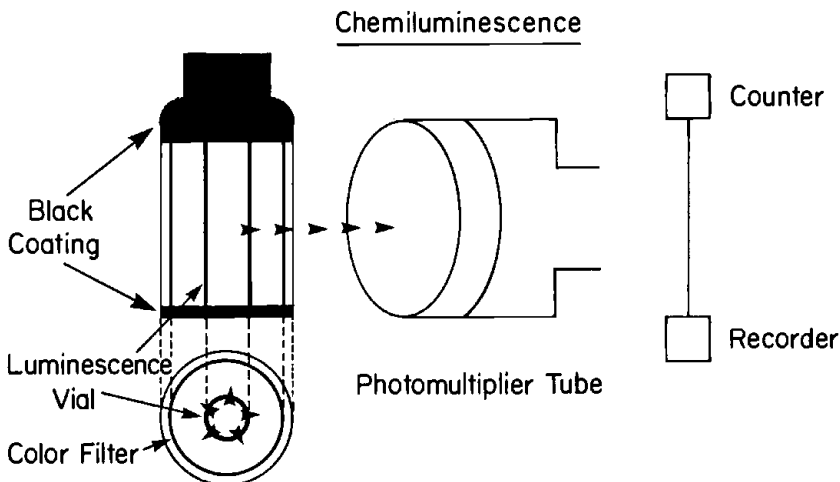


FIGURE 1. Schematic diagram of filter transmission studies. Flexible, gelatin spectral transmission filters lined the inner walls of scintillation vials. Light-emitting reactions took place in smaller vials which were inserted into the filtered vial. A coating of black paint around the top and bottom of the exterior circumference of the filtered vial prevented extraneous non-filtered photons from reaching the phototube. Each sample was measured for 0.1 min and then inserted into the next vial and measured. Compensation for changes in the CL rate was made by alternating sample readings from filtered vials with readings from the same sample in an unfiltered vial. The fraction of incident radiation transmitted through any given filter was evaluated by reference to the light yield, near that point in time, from the unfiltered vial. Only relative, and not absolute, luminescence calculated.

preserve cell integrity by inactivating free radicals and oxidants produced during phagocytosis. Drath (1974) has shown ascorbate and H_2O_2 in the presence of Cupric ions to be markedly bactericidal. This system may be operational in the microbicidal activity of the PMN, Ericsson (1955), Nungester (1948), Olson (1976), and in our experience is chemiluminescent. The addition of bacteria or zymosan to this system greatly increases the photon yield without altering the spectral characteristics.

Preliminary experiments indicate that the addition of either superoxide dismutase or catalase will inhibit light production from the ascorbate system (data not shown). Since

TABLE I. Initial Unfiltered Light Rate of CL Systems at Time of Spectral Comparison

System design	Background (cpm)	Complete system (cpm)
PMN 6 x 10 ⁷ /3 ml (HBSS)	12,000	
Zymosan 1 mg		150,000
Ascorbate pH 7.0 (0.01 M)		32,000
MNL 6 x 10 ⁷ /3 ml (HBSS)	10,000	
Zymosan 1 mg		65,000
PMN 6 x 10 ⁷ /3 ml (HBSS)	12,000	
S. aureus 1 x 10 ⁹		90,000
H ₂ O ₂ 0.7 mM	4,500	
PMN (living) 2 x 10 ⁷		90,000
H ₂ O ₂ 0.7 mM	4,500	
PMN (boiled) 2 x 10 ⁷		234,000
H ₂ O ₂ 0.7 mM	4,500	
S. aureus 1 x 10 ¹¹		65,000
H ₂ O ₂ 0.7 mM	4,500	
E. coli 1 x 10 ¹¹		45,000
Xanthine (9 x 10 ⁻⁴ M) pH 7.8 (PO ₄ ⁼ buffer), 1 x 10 ⁻⁴ EDTA, 0.3 M HCO ₃ ⁻ , Zymosan 5 mg	6,000	
Xanthine oxidase		20,000
Acetaldehyde 10 mM, HCO ₃ ⁻ , 0.3 M, PO ₄ ⁼ , pH 10	9,000	
Xanthine oxidase 4 x 10 ⁻⁷ M		100,000

Table I continued

System design	Background (cpm)	Complete system (cpm)
MPO 1,000 U (HBSS), Zymosan 1 mg	8,000	100,000
H ₂ O ₂ 0.3%		25,000
Ascorbate pH 7.0 (0.01 M)		
Ascorbate pH 7.0 (0.01 M), copper sulfate 1 mM, Zymosan 1 mg	8,000	150,000
H ₂ O ₂ 1 mM		
DMSO 3 ml	7,000	900,000
KO ₂ 0.05 mg		
DMSO 3 ml	7,000	900,000
K ₃ CrO ₈ 0.5 mg		
H ₂ O ₂ (alkaline) 1% 3 ml	7,000	80,000
NaOCl 37% 0.25 ml		
Benzene 3 ml	3,000	
(CH ₃) ₄ -1,2-dioxetane, 1 μ l of a 2 x 10 ⁴ dilution in benzene		900,000
Acetone 3 ml	9,500	900,000
KO ₂ 0.05 mg		
Acetaldehyde 10 mM in H ₂ O	10,000	900,000
KO ₂ 0.05 mg		

removal of either O_2^- or H_2O_2 appears inhibitory to the CL of this system an interaction between the two resulting in a third intermediary with light emitting potential is implicated.

The fraction of light which traversed representative photographic filters during CL reactions is shown in Figure 2 and 3. In this study, ten filters were used, the results of five are illustrated.

Luminescence due to the decomposition of tetramethyl-1,2-dioxetane in benzene yielded a maximum value for wavelength of 430-440 m μ . This is similar to the spectrum of luminescence obtained by Kopecky (1969) for the thermal decomposition of 3,3,4-trimethyl-1,2-dioxetane with a maximum emission of 430-440 m μ . Decomposition of these transient 1,2-dioxetanes form the electronically excited carbonyl compounds acetone and acetone/acetaldehyde, respectively. By excitation of acetone and acetaldehyde either singly, or as a 50% mixture of each with O_2^- generated from KO_2 , we obtained identical photon interference data placing the maximum emission around 435 m μ .

In confirmation of previously reported data (Andersen, 1977) the MPO- H_2O_2 -Cl chemiluminescent system had spectral characteristics nearly identical to that of phagocytosing PMN, placing the maximum emission near 570 m μ , Figure 3. Moreover, the CL which resulted from addition of KO_2 or H_2O_2 to live PMN had nearly identical characteristics. Inactivation of MPO by heating the cells did not change the spectral characteristics nor decrease the intensity of the CL. Indeed, the light yield was increased. The CL that resulted from addition of H_2O_2 to heat killed bacteria (Table 1) had similar transmission characteristics to that produced by the MPO-mediated reactions. Transmission spectrum of phagocytosing MNL's was indistinguishable from PMN's.

Rosen (1977) has presented evidence that formation of hypochlorous acid by the MPO- H_2O_2 -Cl system generates 1O_2 in an identical manner to the reaction of H_2O_2 in sodium hypochlorite. Peters (1972) has shown potassium peroxychromate to liberate 1O_2 upon decomposition while Khan (1970) has shown the evolution of 1O_2 from potassium superoxide in dimethylsulfoxide. Spectral evaluation of these CL sources revealed filter transmission patterns similar to the CL produced by phagocytosing PMN and MNL's and that produced by H_2O_2 oxidation reactions.

This profile was also obtained upon analysis of the ASC + Cu^{++} + H_2O_2 and xanthine/xanthine oxidase/zymosan/bicarbonate CL systems. However, xanthine oxidation of acetaldehyde in the presence of bicarbonate resulted in peak intensity at about 435 m μ ; a value previously reported by Henry (1977).

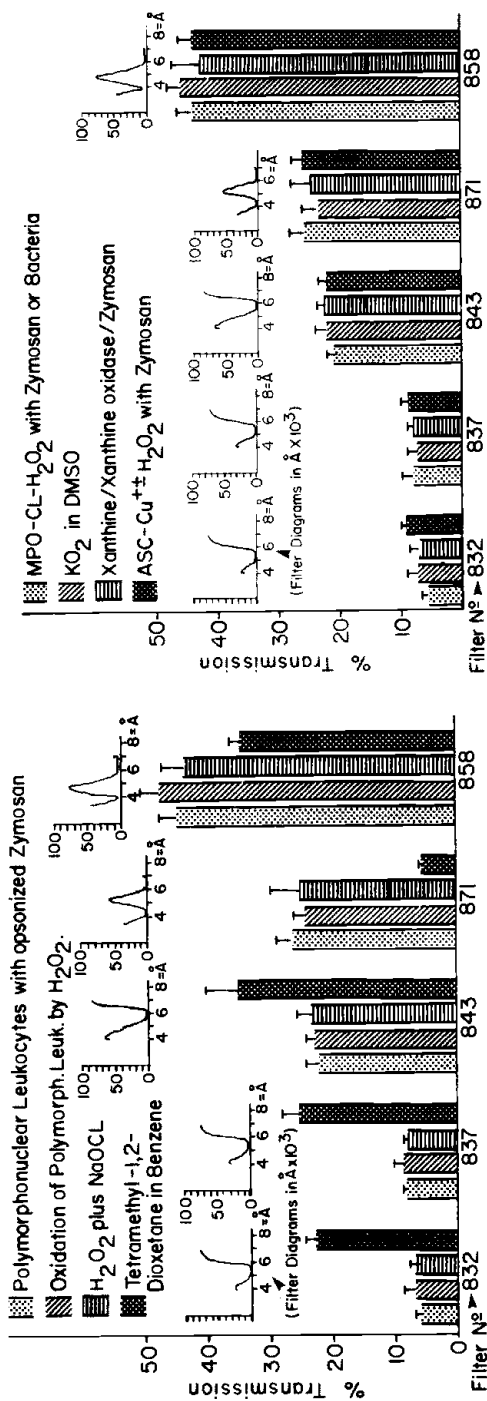


FIGURE 2

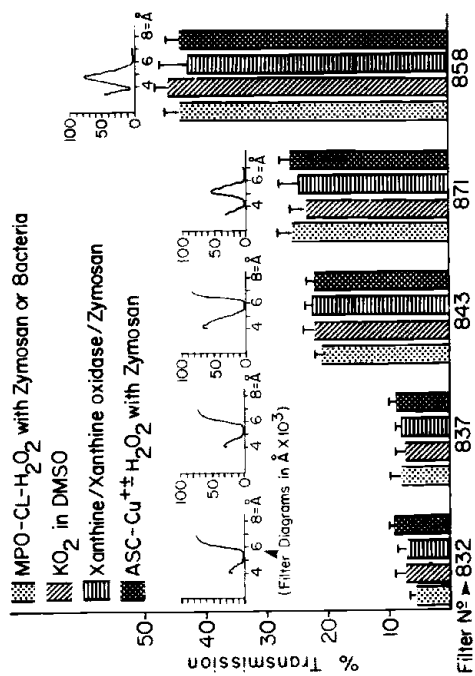


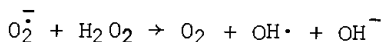
FIGURE 3

FIGURES 2 and 3. Relative luminescence of CL systems as depicted by % of light traversing each filter with reference to unfiltered emission as 100% transmission. The filter number corresponds to the spectral transmission characteristics depicted above each group of reactions. Bar height represents the mean of 5 duplicate experiments and the brackets denote standard deviation.

DISCUSSION

The accumulated data support the conclusion that the CL associated with the generation of oxidizing agents in solution and phagocytosing PMN all share a common photon emitting specie(s). Stauff (1973) has shown that the weak blue-green CL accompanying many redox reactions of H_2O_2 in aqueous solution is due to contaminate CO_2 , carbonate or bicarbonate radical anions. Hodgson (1976) has shown the xanthine oxidase/ acetaldehyde/carbonate formed CL to result entirely from side-reactions involving generation of carbonate radicals. Accordingly, we have observed CL generation during ascorbate oxidation to increase more than one order of magnitude with the addition of bicarbonate to the system without affecting transmission patterns. Furthermore, the addition of bicarbonate anions to the xanthine/xanthine oxidase system enhanced light emission, maintaining the luminescence wavelength maximum at ~ 570 m μ . Since CO_2 is present in many solutions, and many media and cells employ a bicarbonate buffer system, the common emitter may be radical CO_2 carbonate and/or bicarbonate anions.

Spectral characteristics for the xanthine oxidase/ acetaldehyde system in the presence of carbonate anions were shifted from the ~ 570 m μ of xanthine/xanthine oxidase/ bicarbonate to a wavelength maximum of 435 m μ . Comparison of the emission spectrum of the xanthine oxidase/ acetaldehyde/ carbonate system shows similarity to our photon interference data for O_2^- excitation of acetone or acetaldehyde. In addition, spectral emission similar to the above has been obtained for the electronically excited products acetone and acetone/ acetaldehyde from tetramethyl-1,2-dioxetane and 3,3,4-trimethyl-1,2-dioxetane, respectively. The results indicate that carbonate/bicarbonate radicals may react to produce the excited singlet state of acetaldehyde which, in this case, may be the actual photon emitting species. The induction of CL in the ASC + Cu^{++} system by the addition of H_2O_2 may be sensitive to inhibition by either superoxide dismutase or catalase. These findings have been taken to suggest the possibility of another oxidizing intermediate formed in the reaction of O_2^- with H_2O_2 . An explanation may be provided by the work of Taubner (1977) and Weiss (1978) who have implicated $OH\cdot$ production by human neutrophils by the Haber-Weiss reaction:



These authors have shown the release of ethylene from methional in the presence of phagocytizing neutrophils. The oxidizing agent thought responsible for this reaction is $\text{OH}\cdot$ and not O_2 or H_2O_2 . Further support for the production of $\text{OH}\cdot$ in the ASC system may be provided by the fact that ethylene is endogenously produced in plants from methionine by a copper-ascorbate- H_2O_2 non-enzymatic system (Bors, 1974).

CONCLUSIONS

The experimental results described above indicate that formation of excited carbonate radicals or contaminate CO_2 excitation may explain spectral similarity of many CL reactions. Since CO_2 enters into many solutions by exposure to atmosphere this would not be surprising. This hypothesis is particularly pertinent to the CL of neutrophils and other phagocytic blood cells since intracellular pH is maintained by a carbonate buffer system under physiological conditions.

In addition, a significant fraction of PMN CL may be the consequence of autooxidation of cell membrane constituents by leakage of reactive oxidants to the extracellular milieu. This is suggested by the intensity and similarity of CL from peroxidation of enzymically inactivated (heat killed) PMN and bacteria. The results indicate that MPO, while enhancing CL, is not necessary for its production within cells. CL initiated by oxidation of ASC in the presence of Cu^{++} spectrally parallel PMN induced CL. This system, if operational in PMN, may therefore contribute to the intensity of PMN emission.

The decomposition of simple 1,2-dioxetanes yielded wavelengths of a more energetic nature than light emitted by phagocytic cells. However, dioxetanes found in biological systems would be highly conjugated producing excited singlet state carbonyl compounds of much lower energy (Kopecky, 1969). Thus, our results neither lend support, nor detract from the proposal that decomposition of dioxetane compounds is responsible for PMN CL. Finally, the emitting species in the xanthine oxidase/acetaldehyde/carbonate system is indicated to be acetaldehyde.

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