INFECTION AND IMMUNITY, Nov. 1980, p. 385-390
0019-9567/80/11-0385/06$02.00/0

Vol. 30, No. 2

Generation of Chemiluminescence by Human Neutrophils Exposed to Soluble Stimuli of Oxidative Metabolism

MARY ALICE WESTRICK, PAMELA S. SHIRLEY, AND LAWRENCE R. DECHATELET*

Department of Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27103

The detection of chemiluminescence of human polymorphonuclear leukocytes activated by the nonparticulate stimulus phorbol myristate acetate required the presence of suitable substrate, such as protein or luminol, in the reaction medium. This substrate requirement was met by the addition of human serum or various proteins, such as bovine serum albumin, lysozyme, egg albumin, or immunoglobulin, to the reaction vial. Luminol, a chemiluminescent compound, could substitute for protein and markedly enhanced chemiluminescence by phorbol myristate acetate-induced and concanavalin A-induced polymorphonuclear leukocytes. From this work, it appears likely that soluble stimuli activate the polymorphonuclear leukocytes, but this activation, as measured by chemiluminescence, is not detectable in the absence of a secondary interaction with suitable components in the medium.

Phagocytosis is associated with an increase in the oxidative metabolism of polymorphonuclear leukocytes (PMNL). The addition of the nonparticulate stimulus phorbol myristate acetate (PMA) to resting human PMNL results in metabolic alterations similar to those seen during phagocytosis, that is, an increased rate of glucose metabolism via the hexose monophosphate shunt, increased superoxide anion and hydrogen peroxide formation, and iodination of protein material (9). In a study by this laboratory in collaboration with R. B. Johnston, Jr., PMA was also shown to induce chemiluminescence (CL) by resting PMNL (9). The initial slope of the curve and the time course of the PMA-induced CL was similar to that found in the opsonized zymosan-stimulated CL. Later attempts to reproduce the PMA-induced CL were inconsistent, and often no CL could be measured in PMNL stimulated with PMA although other measurements of increased oxidative metabolism done at the same time showed marked stimulation by PMA. Review of the methods revealed that the procedure for the isolation of PMNL in Johnston's laboratory included the addition of 0.2% bovine serum albumin (BSA) (15), suggesting that PMA-induced CL might be dependent upon the presence of protein in the system. Other investigators have noted that the addition of soluble protein or excess nonopsonized zymosan to the reaction increases the amount of CL measured. It has been postulated that these agents produce their effect by providing a source of substrate for secondary light-producing reactions (7, 11). The present studies were undertaken to investigate the protein requirement of the PMA-induced CL.

In addition, we studied the effect of the chemiluminescent compound luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) on the PMA-activated system. Luminol, which reacts with oxidizing agents and subsequently emits light (13, 16), amplifies the detection of CL. Luminol has been especially useful in systems where low levels of CL are produced, such as in macrophage systems, or when the number of PMNL is limited, such as in neutropenic patients (2, 14). We therefore wanted to see whether luminol could enhance CL in the PMA-stimulated system.

These studies demonstrate that PMA will induce CL but that it is detectable only in the presence of protein or an amplification system such as luminol. This may be a general phenomenon for the interaction of soluble stimuli with PMNL.

MATERIALS AND METHODS

Isolation of PMNL. Human PMNL were isolated from venous blood by sedimentation of the erythrocytes with plasma gel (HTI Corp., Buffalo, N.Y.) as previously described (8). Cells were collected by centrifugation and washed, and contaminating erythrocytes were removed by hypotonic lysis for 20 s in cold, deionized water. Total counts were performed in a leukocyte-counting chamber, and the cells were suspended in Dulbecco phosphate-buffered saline (PBS). The concentration of PMNL was usually 5×10^6/ml, although in the experiments using luminol, the concentration was 1×10^5 PMNL/ml. This procedure typically yielded preparations containing greater than 85% PMNL as determined on Wright-stained smears.

Preparation of PMA solutions. PMA (Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 2.0 mg/ml. The stock solution was stored frozen; a portion was diluted to the appropriate concentration with PBS immediately before use. A final concentration in the reaction vial of 1 μg/ml was used in most experiments.

Preparation of luminol. Luminol (Sigma) was dissolved initially in DMSO to a concentration of 10 mg/ml as described by Stevens et al. (14). This solution was stored in a dark bottle at room temperature. On the morning of the experiment, appropriate dilutions of the luminol in PBS were made to achieve the final concentration of 10^{-6} M. The diluted solution was then dark adapted for 5 to 6 h before use.

Materials. Pooled human serum collected from normal donors was stored frozen in equal portions. One portion was thawed and used on the day of the experiment. BSA, lysozyme, egg albumin, immunoglobulin, and concanavalin A (ConA; Sigma) were diluted in PBS to the appropriate concentrations. Defatted BSA was kindly provided by Moseley Waite, Department of Biochemistry, Bowman Gray School of Medicine.

Measurement of CL. CL was measured at ambient temperature in a darkened room with a Beckman LS-100C liquid scintillation spectrophotometer set in the out-of-coincidence mode. The ^{14}C-^{3}H window was used. Glass scintillation vials with a total reaction volume of 6.0 ml were used in experiments without luminol. In experiments with luminol, the system was miniaturized and polyethylene vials with a reaction volume of 2.0 ml were used. Both vials were dark adapted for at least 48 h before use. The PMNL in buffer were brought to room temperature and dark adapted 30 min before the experiment. All other solutions were at room temperature and dark adapted at least 1 h before use. The reaction was started by the addition of PMNL to the reaction vial. The vial was mixed by inversion and placed in the counter for 0.2-min counts. The scintillation counter was set on repeat cycle to achieve repetitive counting. Suspensions of either PMNL in PBS alone or PMNL and protein without PMA were used as controls. With the scintillation counter in the out-of-coincidence mode, these values ranged from 3,000 to 5,000 cpm and did not vary during the course of the experiment. In experiments with luminol, PMNL suspended in luminol and buffer served as the control. The background counts of the cell/luminol system were somewhat higher at 10,000 to 18,000 cpm, but again remained constant in a narrow range throughout a particular run. On multiple occasions, solutions of DMSO (at the concentrations similar to those which would be achieved by the addition of this solvent with either the PMA or luminol) were run. There was no effect by DMSO on the CL at these dilute concentrations (0.002 and 0.05%).

RESULTS

Effect of serum. The effect of increasing amounts of pooled human serum on PMA-induced CL is shown in Fig. 1. When exogenous protein was omitted from the system, addition of 1 μg of PMA per ml to 5×10^6 PMNL resulted in a minimal increase in CL compared with controls. The control counts per minute (5×10^6 PMNL and buffer alone) ranged from 3,000 to 4,000. The PMA-stimulated PMNL exhibited a slow increase in CL measured. The peak activity was measured at 60 to 80 min and was only 4,000 cpm above control. By contrast, the presence of 10% serum in the system resulted in a 10-fold increase in peak counts per minute. The initial slope of the CL curve was steep, with near maximal CL noted at 20 min. Decreasing serum concentrations resulted in proportionally less CL and a slightly decreased initial slope. With the addition of serum to the system, the enhancement was sustained at near peak levels for the duration of the experiment (90 min).

Effect of BSA. When BSA was added to the reaction vial in place of serum, even more striking enhancement of PMA-induced CL was noted. Figure 2 illustrates the marked and rapid increase in CL resulting from the presence of BSA in the medium. At the higher concentrations of BSA (4 and 8 mg/ml), the increase in CL was rapid and peak CL occurred within 10 min. At the highest concentration of BSA (8 mg/ml), the peak response was greater than 90,000 cpm as compared with a base-line value of 3,500 cpm. There was then a rapid decrease in intensity, although at 60 min high levels of CL could still be measured. At lower concentrations of BSA (0.08 to 0.8 mg/ml), the initial slope was less, and peak CL occurred later, at 20 to 30 min. In the absence of BSA, only low levels of CL in PMA-stimulated cells could be detected. A very gradual increase in CL, with peak CL measuring 6,500 cpm above control, occurred at 50 to 60 min. No increase in CL was noted when 8 mg of BSA per ml was added to PMNL in the absence

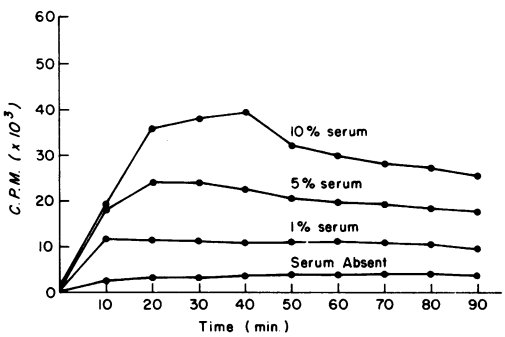

FIG. 1. *Effect of serum concentration on PMA-induced CL in human PMNL. Each reaction vial contained 5×10^6 PMNL and 1 μg of PMA per ml. Control counts per minute (PMNL and buffer alone) were subtracted from the values plotted in the graph.*

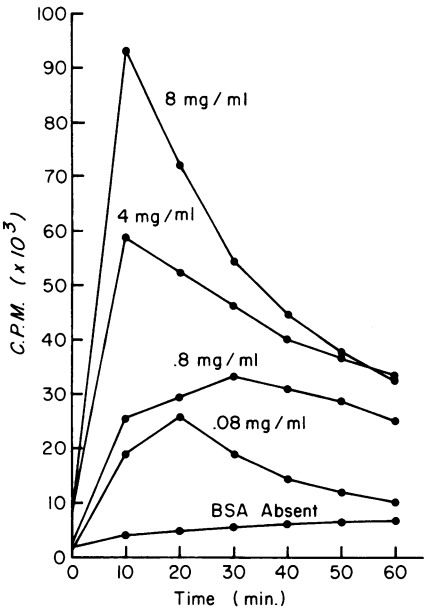

FIG. 2. *Effect of BSA concentration on PMA-induced CL in human PMNL. Each reaction vial contained 5 × 10⁵ PMNL and 1 μg of PMA per ml. Control counts per minute (PMNL and buffer alone) were subtracted from the values plotted in the graph.*

of PMA. Similar results were obtained with commercial albumin and albumin which was especially defatted.

Dose-response curve with PMA. In the presence of 4 mg of BSA per ml, PMA-induced CL was easily detected and noted to be dose dependent (Fig. 3). The increase in CL was rapid and the peak response occurred at 10 min with the higher doses of PMA. At 0.01 μg of PMA per ml, the initial slope was less and the peak CL occurred later, at 30 to 40 min. It is noteworthy that with BSA in the medium, CL was easily measured at 0.01 μg of PMA per ml, whereas without BSA, CL was barely detectable at a 100-fold-higher dose of PMA.

Effect of other proteins. Lysozyme, egg albumin, and immunoglobulin were then evaluated to determine whether these proteins could also augment the CL of the PMA-stimulated system. Equal concentrations (4 mg/ml, final concentration in the reaction vial) of each protein were used, and all three were noted to enhance CL (Fig. 4). BSA and 5% serum were run at the same time for comparison. CL peaked at 10 min with immunoglobulin and at 20 to 25 min with lysozyme and egg albumin. CL fell to near base-line levels by 60 min with these three proteins. There was no enhancement of CL by the various proteins in the absence of PMA.

Luminol-dependent CL. Luminol markedly enhanced CL induced by PMA (Fig. 5). The number of PMNL in each reaction vial was decreased to 10^5, since CL was off-scale (greater than 1,000,000 cpm) with the 5×10^{-6} PMNL originally used (data not shown). Two concentrations of luminol, 10^{-6} and 10^{-7} M, and two

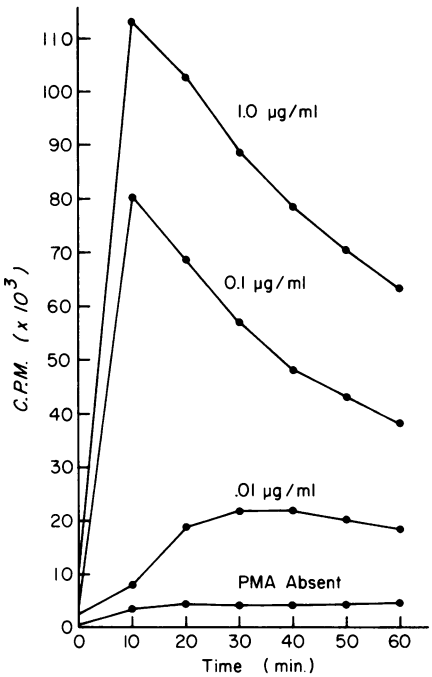

FIG. 3. *Dose response of PMA-induced CL. Each reaction vial contained 5 × 10⁵ PMNL and 4 mg of BSA per ml. Control counts per minute (PMNL and buffer alone) were subtracted from values plotted in the graph.*

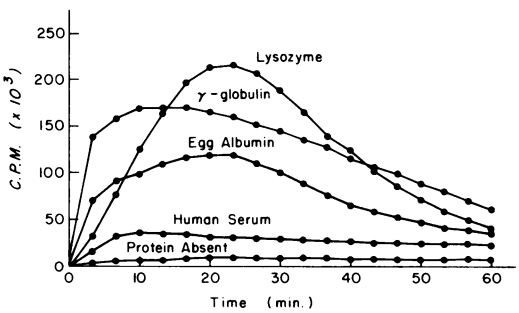

FIG. 4. *Effect of various proteins on PMA-induced CL in PMNL. A 4-mg/ml concentration of lysozyme, egg albumin, or immunoglobulin or 5% human serum was present in the reaction vial. Each reaction vial contained 5 × 10⁵ PMNL and 1 μg of PMA per ml. Control counts per minute (PMNL and buffer alone) were subtracted from the values plotted in the graph.*

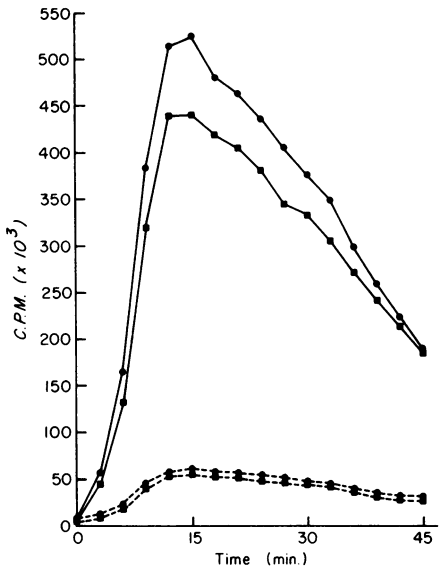

FIG. 5. *Dose response of luminol and PMA on CL of PMNL induced by PMA. Two doses (10^{-6} and 10^{-7} M) of luminol and two doses (0.1 and 1.0 μg/ml) of PMA were compared. PMNL (10^5) were added to each reaction vial. Symbols: Solid lines, 10^{-6} M luminol; dashed lines, 10^{-7} M luminol; circles, 1.0 μg of PMA per ml; squares, 0.1 μg of PMA per ml.*

doses of PMA, 1.0 and 0.1 μg/ml, were compared. The concentration of luminol was the more important variable, since the intensity of CL measured was quite similar with either 0.1 or 1.0 μg of PMA per ml. Decreasing the luminol concentration from 10^{-6} to 10^{-7} M resulted in a marked decrease in CL. At both concentrations, however, peak CL occurred within 10 to 15 min. At 10^{-6} M luminol, CL intensity declined rapidly from the peak response, although CL was still considerable at 45 min. PMNL suspended in luminol and buffer served as the control. PMNL in 0.002% DMSO or PMA and luminol alone showed no enhancement.

A different soluble stimulus, ConA, was also tested in the luminol system (10^{-6} M luminol). Measurement of CL induced by ConA was enhanced by luminol, and the intensity of CL was dependent upon the dose of ConA used to stimulate the PMNL (Fig. 6). At the highest dose of ConA (150 μg/ml), CL increased rapidly and peaked within 15 to 20 min. At the lower doses (25 and 50 μg/ml), the initial slope was less and the peak CL occurred later, at 20 to 25 min. In the absence of luminol, addition of 150 μg of ConA per ml resulted in a minimal increase in CL compared with controls.

DISCUSSION

This study demonstrates that the measurement of CL induced by the nonparticulate stimulus PMA is dependent upon the presence of suitable substances, such as protein or luminol, in the reaction medium to permit detection of the light emission. CL by PMA-stimulated neutrophils is an event which parallels increased cellular oxidative metabolism, although the nature of the light-emitting species is not certain (9, 12, 15). Reactive species, such as O_2^-, H_2O_2, $^1O_2$, and hydroxyl radicals may be generated by metabolically active PMNL (5). Relaxation of $^1O_2$ to the ground state was originally suggested by Allen et al. (3) as the mechanism of light production. On the other hand, microbicidal oxidation of an ingested organism with production of excited carbonyl groups which subsequently relax to the ground state could account for the light production (4).

Several investigators have noted that modification of the reaction medium altered the measurement of CL. Addition of excess nonopsonized zymosan increased CL in both cellular and noncellular CL systems (7, 11). The addition of soluble protein directly to the medium increased CL (11). Furthermore, the slope of the CL curve was proportional to the amount of serum present in the medium (1, 11). In view of these observations, it appears likely that the phenomenon of CL is dependent upon both (i) stimulation of the PMNL by phagocytosis or a soluble stimulus with resulting production of reactive species and (ii) suitable substrate for secondary light-producing reactions.

When a particle such as a bacterium or opsonized zymosan is used as the stimulus, the par-

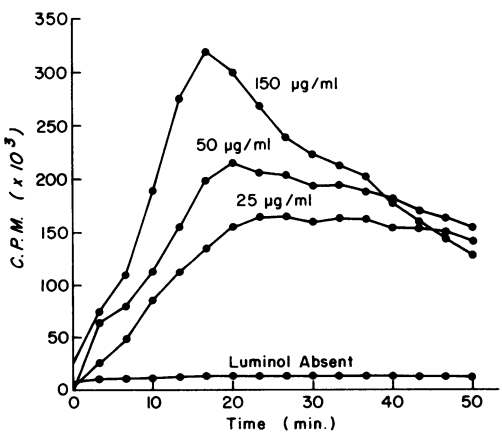

FIG. 6. *Dose response of ConA-induced CL in human PMNL in a luminol-containing medium. In experiments with luminol, each reaction vial contained 10^5 PMNL and 10^{-6} M luminol. Countrol counts per minute (PMNL in luminol and buffer) were subtracted from values plotted in the graph. In the experiment without luminol, the reaction vial contained 10^5 PMNL and 150 μg of ConA per ml suspended in buffer.*

ticle itself may serve as the substrate for secondary light-producing reactions (2). However, the nonparticulate stimulus PMA is evidently unable to serve as substrate for such secondary light reactions. The results of this work point out the key role of the particle in the phenomenon of CL. Minimal CL was detected in PMA-stimulated PMNL when the reaction was performed in PBS alone. However, the addition of serum or several different proteins all resulted in detection of CL. Enhancement of the CL by various proteins in this study suggests a nonspecific reaction with protein. The nature of this interaction is not clear; all proteins tested augmented CL induced by soluble stimuli; in addition, the amino acids tyrosine and tryptophan showed some enhancement of activity (data not shown), but this was not nearly as great in magnitude as that seen with albumin. These results also suggest that in measuring CL induced by PMA, one must consider carefully the amount of protein in the system.

Luminol greatly enhanced CL of PMA-activated PMNL such that far fewer PMNL were necessary for the reaction. Luminol is a chemiluminescent compound which is oxidized by reactive species such as those generated by the respiratory burst of the PMNL. The oxidation of luminol results in the production of an excited aminophthalate anion which relaxes to the ground state with the production of light (2, 6). When luminol was present in the medium, PMA-induced CL was easily measured and protein was not required. When another soluble stimulus of PMNL oxidative metabolism, ConA, was studied, similar results were obtained. In the absence of luminol, ConA-induced CL was barely measurable. Luminol markedly enhanced the measurement of CL produced by ConA-stimulated PMNL.

The results of this study provide further evidence that the relaxation of $^1O_2$ to the ground state is not the major factor in the production of light by activated neutrophils. The lack of measurable CL in the PMA-stimulated system in the absence of protein or luminol argues strongly for the theory that the medium components play a major role in the detection of CL. This is further substantiated by the observation that another soluble stimulus, ConA, likewise requires an amplification mechanism (e.g., luminol) in order to detect CL. It appears likely that soluble stimuli in general activate the cell, but this activation (as CL) is not detectable unless there is some type of secondary interaction. Such a phenomenon has previously been suggested by Cheson et al. (7).

The nature of the oxygen species involved is not known. It is possible that one species, e.g., O_2^-, interacts with protein whereas a different species, e.g., H_2O_2, interacts with luminol, but it seems more plausible that a single, as yet undefined species is involved in both reactions. Previous experiments using inhibitors have suggested the involvement of both superoxide anion and hydrogen peroxide but have been unclear regarding the possible involvement of singlet oxygen or hydroxyl radical (10). Resolution of this question will require more definitive ways of detecting these highly reactive species.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-10732 from the National Institute of Allergy and Infectious Diseases.

The excellent editorial assistance of Gwen Charles is gratefully acknowledged.

LITERATURE CITED

1. **Allen, R. C.** 1977. Evaluation of serum opsonic capacity by quantitating the initial chemiluminescent response from phagocytizing polymorphonuclear leukocytes. Infect. Immun. **15**:828–833.
2. **Allen, R. C., and L. D. Loose.** 1976. Phagocytic activation of a luminol-dependent chemiluminescence in rabbit alveolar and peritoneal macrophages. Biochem. Biophys. Res. Commun. **69**:245–252.
3. **Allen, R. C., R. L. Stjernholm, and R. H. Steele.** 1972. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. **47**:679–684.
4. **Allen, R. C., S. J. Yevich, R. W. Orth, and R. H. Steele.** 1974. The superoxide anion and singlet molecular oxygen: their role in the microbicidal activity of the polymorphonuclear leukocyte. Biochem. Biophys. Res. Commun. **60**:909–917.
5. **Babior, B. M.** 1978. Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med. **298**:659–668 and 721–725.
6. **Campbell, A. K., and J. S. A. Simpson.** 1979. Chemi- and bio-luminescence as an analytical tool in biology, p. 1–7. *In* H. L. Kornberg, J. C. Metcalfe, D. H. Northcote, C. I. Pogson, and K. F. Tipton (ed.), Techniques in the life sciences B213. Elsevier North-Holland Inc., New York.
7. **Cheson, B. D., R. L. Christensen, R. Sperling, B. E. Kohler, and B. M. Babior.** 1976. The origin of the chemiluminescence of phagocytosing granulocytes. J. Clin. Invest. **58**:789–796.
8. **DeChatelet, L. R., and M. R. Cooper.** 1970. A modified procedure for the determination of leukocyte alkaline phosphatase. Biochem. Med. **4**:61–68.
9. **DeChatelet, L. R., P. Shirley, and R. B. Johnston, Jr.** 1976. Effect of phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leukocytes. Blood **47**:545–554.
10. **McPhail, L. C., L. R. DeChatelet, and R. B. Johnston, Jr.** 1979. Generation of chemiluminescence by a particulate fraction isolated from human neutrophils: analysis of molecular events. J. Clin. Invest. **63**:648–655.
11. **Nelson, R. D., M. J. Herron, J. R. Schmidtke, and R. L. Simmons.** 1977. Chemiluminescence response of human leukocytes: influence of medium components on light production. Infect. Immun. **17**:513–520.
12. **Sagone, A. L., Jr., D. S. Mendelson, and E. N. Metz.** 1977. The effect of sodium azide on the chemiluminescence of granulocytes—evidence for the generation of

multiple oxygen radicals. J. Lab. Clin. Med. **89**:1333–1340.

13. **Seliger, H. H.** 1961. Some aspects of the luminol light reaction, p. 200–205. *In* W. D. McElroy and B. Glass (ed.), Light and life. The Johns Hopkins Press, Baltimore, Md.
14. **Stevens, P., D. J. Winston, and K. Van Dyke.** 1978. In vitro evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: utility in patients with severe neutropenia and cellular deficiency states. Infect. Immun. **22**:41–51.
15. **Webb, L. S., B. B. Keele, Jr., and R. B. Johnston, Jr.** 1974. Inhibition of phagocytosis-associated chemiluminescence by superoxide dismutase. Infect. Immun. **9**: 1051–1056.
16. **White, E. H.** 1961. The chemiluminescence of luminol, p. 183–199. *In* W. D. McElroy and B. Glass (ed.), Light and life. The Johns Hopkins Press, Baltimore, Md.