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Chemiluminescent Detection of Bacteria: Experimental and Theoretical Limits

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The limit of sensitivity of the chemiluminescent assay for detection of bacteria by hemeprotein catalysis of luminol oxidation was determined, both experimentally and theoretically, to be no lower than 10^5 to 10^6 viable bacteria per ml.

The heme-containing proteins of bacteria have been shown (1, 5) to catalyze the light-emitting oxidation of luminol, and this chemiluminescent response has been reported to be proportional to the number of bacteria present in water (5) and urine (1). These reports have indicated this assay to be extremely sensitive and capable of detecting as few as 10^3 to 10^4 bacteria per ml. However, experimental analyses in our laboratory, combined with calculations of the theoretical sensitivity limit of the chemiluminescent assay, suggest that the bacterial detection limit of this assay may more accurately exist at 10^5 to 10^6 viable bacteria per ml.

The experimental procedures for the heme-catalyzed chemiluminescent assay reported herein were similar to those previously published (2) in that a stock solution of luminol was prepared by dissolving 0.2 g of luminol (K and K Laboratories) and 3.2 g of anhydrous glucose in 20 ml of 0.2 M NaOH and diluting to 1 liter with charcoal-adsorbed, deionized, doubly distilled water; the luminol stock was refrigerated for at least 2 days prior to use. The oxidant stock, 0.05 M $NaBO_3$, was prepared fresh prior to use, whereas the 0.2 M NaOH stock was used for several days. All stock reagents were 20 mM in ethylenediaminetetraacetic acid. The control hemeproteins, hemin and human hemoglobin (Nutritional Biochemicals), cytochrome *c* (Miles Labs, Inc.), and peroxidase and catalase (Worthington Biochemicals) were prepared as sterile stocks of 1.0 mM concentration. The final concentration of each assay reagent was: luminol, 2.0×10^{-6} to 2.0×10^{-7} M; NaOH, 7.0×10^{-2} M; $NaBO_3$, 7.0×10^{-3} M. Assay reactions were carried out in disposable glass tubes (6 by 50 mm; Kimble 73500) by mixing 20 μl of heme-containing liquid with 20 μl of 0.2 M NaOH for 3 to 5 min; 25 μl of stock luminol and 30 μl of 0.2 M NaOH were then added, and after 1 min, 20 μl of $NaBO_3$ was injected. The light response was monitored using a 760 luminescence biometer (Dupont Instruments) with sensitivity settings at 820. A light response 10 times the mean background (>3 standard deviation) was considered significant. Assay reactions (10-fold in volume) were also carried out in disposable plastic tubes (12 by 75 mm) and monitored with a photodiode light detector composed of a light-excluded cell containing a UDT-500 photodiode (United Detector Technology) with built-in op-amp; this instrument was connected to a recording voltmeter.

Bacteria (*Escherichia coli* [CDC 0111a: K58(B4) and CDC 06:K2:H1], *Staphylococcus aureus* ATCC 12600, and *Proteus vulgaris* ATCC 6380) were cultured in brain heart infusion broth (Difco) or in urine (previously filter sterilized) to mid-log phase. Cell enumeration on brain heart infusion agar was conducted using broth and water dilutions prepared with calibrated pipettes. Surface inoculation was completed using a calibrated paltinum loop. The chemiluminescent response from bacteria, cultured in brain heart infusion broth or urine, was monitored directly in the culture fluid or after sedimentation and resuspension of the cells in water.

Results (Table 1) of investigations carried out to determine the sensitivity of hemeprotein detection by the chemiluminescent assay demonstrate that the procedures employed in this laboratory yield detection limits, for the hemeproteins studied, very similar to those reported previously (2). Furthermore, comparable results were obtained with two different light-detection instruments. Because of these findings, and because theoretical considerations regarding the bacterial hemeprotein detection capacity of the chemiluminescent assay are inconsistent with prior reports (1, 5) of detection limits, studies were carried out to experimentally determine the lower limit for bacterial detection of the chemiluminescent assay system.

Employing the procedures of Ewetz and Strangert (1) and Ewetz and Thore (2), which use sodium hydroxide pretreatment and perbor-

ate preincubation to eliminate chelated transition metal catalysis of luminol oxidation, we were unable to reduce the dampening influences (high background) of the culture medium upon the response to bacterial hemeproteins in a model system where bacteria were cultured in bacteriological broth. Only when the bacterial cells were freed of the culture medium by sedimentation and reconstitution or wash in water (Fig. 1) was this background chemiluminescent

TABLE 1. *Comparison of chemiluminescent detection limits for representative hemeproteins using two different light detectors*

Hemeprotein assayed	Sensitivity limit (M) ± standard deviation	
	Photodiode with op-amp (n)	760 Luminescence detector (n)
Hemin	$0.5 \pm 0.2 \times 10^{-9}$ (20)	$1.0 \pm 0.5 \times 10^{-10}$ (6)
Peroxidase	$1.0 \pm 0.2 \times 10^{-9}$ (12)	$1.0 \pm 0.6 \times 10^{-10}$ (5)
Catalase	$1.0 \pm 0.6 \times 10^{-11}$ (12)	$0.5 \pm 0.4 \times 10^{-12}$ (8)
Hemoglobin (human)	$1.0 \pm 0.4 \times 10^{-10}$ (12)	$1.0 \pm 0.5 \times 10^{-12}$ (8)
Cytochrome *c*	$0.3 \pm 0.2 \times 10^{-8}$ (12)	$1.0 \pm 0.3 \times 10^{-8}$ (4)

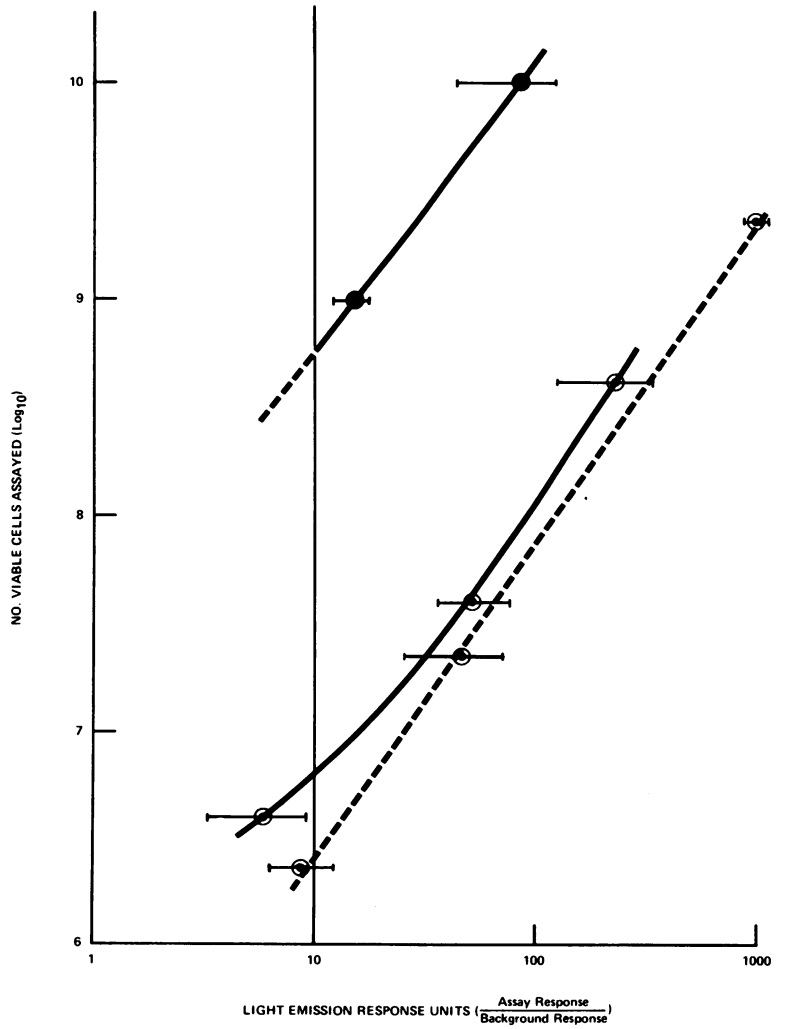

FIG. 1. *Linear relationship of light emission response catalyzed by hemeproteins of E. coli 06.* (●——●) *assay of bacteria in culture fluid diluted with water;* (⊙- - -⊙) *assay of bacteria diluted in water after sedimentation from culture broth;* (⊙——⊙) *assay of bacteria diluted in water after sedimentation, and washed with water. Lateral bars represent ± standard deviation of the mean.*

response eliminated. As noted by Ewetz and Strangert (1), such background responses probably arise as a result of catalysis by heme moieties present in culture fluids. In their study, these moieties were suggested to be present as the result of contamination of the culture fluid, urine, with hemoglobin. In this study, the bacteriological culture medium prepared from animal organ infusions can be expected to contain heme moieties from similar degraded hemeproteins. Therefore, when experimental determinations of detection limits for bacteria are undertaken, consideration must be given to the presence of a response to contaminating heme moieties in a culture fluid. We have shown that this response cannot be eliminated by simple dilution with water.

Having determined that bacterial cells must be separated from their culture fluid prior to conducting experiments to define the sensitivity limit of the chemiluminescent assay for bacterial detection, we employed this technique throughout the remaining investigations. However, as shown in Table 2, the results of our investigations do not compare with the sensitivity limits previously established (1, 5) for bacterial detection by this assay system; the lower limit for bacterial detection was found to be between 10^5 and 10^6 cells per ml rather than 10^3 to 10^4 cells per ml. These detection limits apply for bacteria originally cultured in both bacteriological broth and urine.

Furthermore, as shown in this same set of data, our results indicate that a somewhat more sensitive detection limit is achieved if viable counts are determined from dilutions of bacterial suspensions in water as opposed to bacteriological culture medium. We have concluded that this discrepancy arises as the result of lysis of or hypertonic shock to the cells in the hypertonic diluent, since we observed a range between 0.5 to 1 log discrepancy in viable counts for bacterial suspensions diluted, in parallel, in water and in bacteriological culture fluid. Water-diluted specimens yielded the lower number of viable counts.

This effect was additionally investigated and substantiated by subjecting water- and broth-diluted specimens to Coulter-type electronic analysis (3) to determine total cell number and cell volume. As shown in Fig. 2, bacterial cultures diluted in water yielded an initially lower total cell count than those diluted in broth. Further, as illustrated by the representative plots in this figure, we consistently observed a 30 to 40% decrease in total cell count within 5 min and a 40 to 50% decrease in cell number within 10 min after initial dilution of the cell culture into water. The effect was observed for all bacterial species all bacterial species employed in this investigation. The decrease in cell

TABLE 2. *Chemiluminescent assay detection limit for bacteria cultured in broth or urine and diluted in broth or water*

Microorganism	Viable count (cells per ml) ± standard deviation	
	Broth dilution (*n*)	Water dilution (*n*)
Broth culture		
S. aureus	$7.0 \pm 3.5 \times 10^6$ (9)	$0.5 \pm 0.2 \times 10^6$ (4)
E. coli 06	$3.6 \pm 1.2 \times 10^6$ (8)	$5.9 \pm 2.8 \times 10^6$ (7)
E. coli 0111	$2.0 \pm 0.3 \times 10^7$ (6)	$1.3 \pm 0.4 \times 10^6$ (6)
P. vulgaris	$5.8 \pm 0.9 \times 10^8$ (4)	$8.8 \pm 5.7 \times 10^5$ (5)
Urine culture		
E. coli 0111	$1.4 \pm 0.7 \times 10^6$ (6)	$3.5 \pm 2.3 \times 10^5$ (6)
P. vulgaris	$1.0 \pm 0.8 \times 10^8$ (6)	$3.0 \pm 2.0 \times 10^7$ (8)

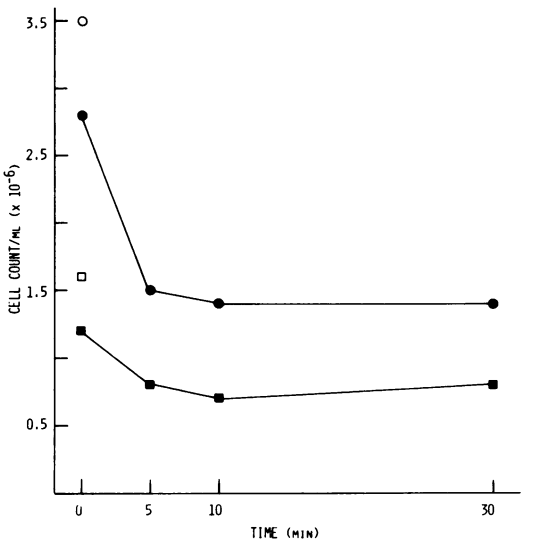

FIG. 2. *Electronic analysis of total cell count of bacteria diluted in brain heart infusion broth or distilled water.* (○) *S. aureus diluted in broth;* (●) *S. aureus diluted in distilled water;* (□) *E. coli diluted in broth;* (■) *E. coli diluted in distilled water. Min designations indicate length of time cells remained in diluent prior to electronic counting; plots represent the mean of quadruplicate determinations with range in precision of 0.4 to 3.7%.*

count stabilized by 10 min; no further decrease in total cell count was observed for cells held for 30 min in the diluent. Decrease of total cell count was not attributed to clumping of cells placed in the hypertonic medium.

This statement is based upon the observation that although the cell volume of the total cell count increased during the initial 5-min holding period, presumably as the result of water intake, cell volume of the total cell count decreased thereafter. Cell volume of bacteria diluted in water and held for 10 to 30 min was identical to the volume of cells diluted and held in broth for the same time periods. We conclude, therefore,

that the proportionately lower sensitivity limit seen for bacteria diluted in water is achieved as the result of an additional response to hemeproteins originating from nonviable or lysed bacteria. In investigations to determine the detection limit of the chemiluminescent assay for viable bacteria, it is therefore advisable to employ diluents of constant osmolarity to establish viable bacterial counts. Alternatively, account must be made for the response to hemeproteins arising from nonviable cells.

The above-noted, experimentally determined detection limit of the chemiluminescent assay for bacteria is more consistent with the following theoretical calculations of this limit. These calculations are based upon certain generally accepted approximations of bacterial composition, including nonstructural protein (6), molecular weight constants, and chemiluminescent assay detection limits set for specific hemeproteins, e.g., catalase (detection limit, 4.0×10^{-16} mol; 2.5×10^5 molecular weight) and cytochrome *c* (detection limit, 1.9×10^{-12} mol; 1.3×10^4 molecular weight). These hemeproteins were selected for this discussion since they are known to exist in bacteria. For the following calculations, we have considered that all 10% (10^{-14} g) of the nonstructural protein content of a bacterial cell is composed of a heme-containing substance. Although this is, in all likelihood, an overestimation of the hemeprotein content of bacteria, use of this figure serves to demonstrate the minimum theoretical limit that can be calculated for the detection of bacteria by the chemiluminescent assay.

Using the above constants, we calculate that 4.0×10^{-20} mol of catalase or 7.7×10^{-19} mol of cytochrome *c* may be present in one bacterial cell. Consequently, to achieve the detection capacity reported by Neufeld et al. (4) for either of these two hemeproteins, one may calculate that 1.0×10^4 cells containing 4.0×10^{-16} mol of catalase or 1.0×10^7 cells containing 1.0×10^{-12} mol of cytochrome *c* are required for a minimum detectable chemiluminescent response. However, the hemeprotein content of a bacterium is probably 10 to 100 times less than that (10^{-14} g) employed for our theoretical calculations. Hence, we conclude, based upon these calculations and the experimental data reported herein, that the detection limit can exist no lower than between 10^5 and 10^6 viable bacterial cells per ml.

Finally, we postulate that a lower detection limit could be achieved only if the chemiluminescent response monitored arises from an additional reponse to hemeproteins originating from nonviable bacteria. Such an additional response may have affected an earlier report (5) of the chemiluminescent assay's sensitivity for detection of bacteria, since viable count dilutions were conducted in a hypertonic medium in that study. Dilution of bacterial suspensions in a hypertonic medium, as noted herein, will affect experimental determinations of the detection limit of the chemiluminescent assay for viable bacteria by yielding an apparent increase in sensitivity.

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