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Chemiluminescence by *Listeria monocytogenes*

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Listeria monocytogenes cells suspended in brain heart infusion broth or in carbonated saline solution emitted light (chemiluminescence) that could be detected by a liquid scintillation spectrometer. This chemiluminescence was inhibited by superoxide dismutase and catalase but not by the hydroxyl radical scavengers mannitol and benzoate; it was also dependent upon and proportional to the carbonate ion concentration in the medium. Organisms suspended in carbonated saline solution which had ceased to chemiluminesce immediately began to chemiluminesce again when acetaldehyde was added but not when glucose, sucrose, or xanthine was added. Acetaldehyde-induced chemiluminescence was inhibited by superoxide dismutase and catalase but not by allopurinol. Our data indicate that the superoxide anion, hydrogen peroxide, and the carbonate ion are involved in chemiluminescence by *L. monocytogenes*. Chemiluminescence is apparently initiated by the extracellular generation of superoxide anion by this organism. The mechanism for the production of the superoxide anion is not known, but xanthine oxidase does not appear to be involved.

Light emission by living cells is not an uncommon phenomenon. For example, there are many species of luminous bacteria in the marine environment; all of these are gram-negative motile rods, which emit substantial amounts of visible (λ_{max}, ~490 nm) light as a result of an enzyme (bacterial luciferase)-catalyzed reaction (10, 14). However, not all light emission by living cells is due to luciferase systems; neutrophilic leukocytes of blood emit light which is generated during the burst of oxidative metabolism that occurs during phagocytosis. This light (λ_{max}, ~570 nm) is of very low intensity and is thought to be due to the generation of highly reactive oxygen moieties, with superoxide anion (O_2^-) playing a central role (1, 3). Biochemical reactions which produce O_2^-, such as the action of xanthine oxidase on its substrate, are known to produce chemiluminescence under the proper conditions (7). The extracellular production of superoxide radicals by certain strains of aerobic bacteria has been reported recently (15); however, spontaneous chemiluminescence by these organisms was not evaluated. In this paper we report our observations on chemiluminescence by *Listeria monocytogenes*.

MATERIALS AND METHODS

Growth and preparation of organisms. Laboratory isolates of *L. monocytogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Salmonella minnesota* were grown in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, Mich.) at 37°C. For the determination of chemiluminescence during growth, 5.0 × 10^7 cells from an overnight culture were added to scintillation vials containing 10.0 ml of BHI diluted 1:2 with 0.15 M phosphate-buffered saline solution (pH 7.2) (PBS). The effects of various compounds on spontaneous chemiluminescence by *L. monocytogenes* were studied by adding each compound and 1.0 ml of a log-phase culture of *L. monocytogenes* to a scintillation vial containing 9.0 ml of BHI diluted 1:2 with PBS. A standardized suspension of washed organisms was prepared by centrifuging a log-phase culture of the organisms at 1,500 × *g* for 20 min, washing the pelleted bacteria once in PBS by repeating the centrifugation, and then suspending the bacteria in PBS to a concentration such that a 1:10 dilution in PBS yielded an optical density of 0.2 at 600 nm with a 1.0-cm light path. Chemiluminescence by washed bacteria in PBS containing different quantities of various compounds was studied by adding each compound and 1.0 ml of a standardized suspension of cells to 9.0 ml of PBS in a scintillation vial. Heat killing was accomplished by heating the standardized cell suspensions to 60°C for 30 min. Killing was confirmed by failure of growth after the inoculation of a blood agar plate.

Reagents. Catalase (Sigma Chemical Co., St. Louis, Mo.) was repeatedly washed on an XM 100A Diaflo ultrafiltration membrane (Amicon Corp., Lexington, Mass.) to remove any potential contamination by superoxide dismutase (11). The remaining catalase activity was assayed at 25°C by the method of Beers and Sizer (6). Superoxide dismutase (Cu^{2+}- and Zn^{2+}-containing enzyme; bovine blood origin; 3,000 U/mg; Sigma Chemical Co.) was dissolved in PBS to a concentration of 1.0 mg/ml. A portion of this stock solution of superoxide dismutase was inactivated by autoclaving for 15 min at 15 lb/in^2. A 1.0 M stock solution of acetaldehyde (Mallinckrodt, Inc., Paris, Ky.) in PBS was prepared fresh daily. Allopurinol (Sigma Chemical Co.) was dissolved in PBS to a concentration of 10^{-3}

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M for use as a stock solution. Solutions containing added carbonate ions were prepared by adding the appropriate amount of sodium bicarbonate and adjusting the pH to 7.2.

Determination of chemiluminescence. Chemiluminescence was measured at ambient temperature (≈24°C) with a Beckman model DPM 100 liquid scintillation spectrometer which had one photomultiplier tube switched off. In all cases, the caps were left loose on the scintillation vials, and the number of counts per minute from a control vial containing the appropriate medium was subtracted from the number of counts per minute from the reaction vials.

Determination of chemiluminescence during growth. The growth of the organisms was monitored by removing samples from the scintillation vials and determining the number of colony-forming units by standard plate count techniques.

RESULTS

Chemiluminescence by *L. monocytogenes* during growth. *L. monocytogenes* displayed increasing chemiluminescence during growth in BHI until it reached a maximum of 130,000 cpm at approximately 6 h after inoculation (Fig. 1). After this, there was a precipitous drop in chemiluminescence despite the continued growth of the organism. When allowed to grow under the same conditions, *K. pneumoniae*, *E. coli*, and *S. minnesota* displayed no detectable chemiluminescence.

Effect of superoxide dismutase. Superoxide dismutase inhibited the chemiluminescence produced by *L. monocytogenes* (Fig. 1 and 2) in a concentration-dependent manner. A concentration of 0.1 μg of superoxide dismutase per ml decreased the chemiluminescence by approximately 50%; 10 μg of superoxide dismutase per ml essentially eliminated the chemiluminescence. Inactivated superoxide dismutase at a concentration of 10 μg/ml had no significant effect on chemiluminescence. Neither active nor inactivated superoxide dismutase had any significant effect on the growth of *L. monocytogenes*.

Effect of catalase. Catalase at a concentration of 1,000 U/ml inhibited the chemiluminescence produced by *L. monocytogenes* in BHI by about 50% (Fig. 2). Increasing the catalase concentration to 6,000 U/ml did not increase the inhibition of chemiluminescence.

Effect of mannitol and benzoate. The addition of the hydroxyl radical scavengers mannitol (10^{-2} or 10^{-3} M) and benzoate (10^{-3} or 10^{-4} M) did not inhibit chemiluminescence by actively growing *L. monocytogenes* cultures in BHI (Table 1).

Effect of carbonate concentration. Chemiluminescence was enhanced by the addition of carbonate to actively growing *L. monocytogenes* cultures in BHI (Table 2). Actively growing *L. monocytogenes* cultures which were washed and placed into PBS containing 0 to 0.4 M carbonate ions displayed chemiluminescence which was dependent upon and proportional to the carbonate concentration (Fig. 3). When the saline solution contained no added carbonate, chemiluminescence was negligible. Maximal chemiluminescence was obtained when the highest concentration of carbonate was present (0.4 M).

Effect of adding acetaldehyde, glucose, sucrose, or xanthine to washed *L. monocytogenes* cells. When an overnight culture of *L. monocytogenes* was washed and allowed to equilibrate for 1 h in PBS containing 0.4 M carbonate, chemiluminescence dropped to a very low level. When acetaldehyde was added at a concentration of 0.1 M, a rapid and dramatic increase in chemiluminescence occurred (Table 3 and Fig. 4). When the *L. monocytogenes* culture was first heated to 60°C for 30 min, acetaldehyde did not induce chemiluminescence (Table 3). The addition of glucose, sucrose, or xanthine did not stimulate chemiluminescence by washed *L. monocytogenes* cells (Table 3).

Effect of superoxide dismutase and catalase on acetaldehyde-induced chemiluminescence by *L. monocytogenes*. Superoxide dismutase at a concentration of 10.0 μg/ml markedly inhibited acetaldehyde-induced chemiluminescence of *L. monocytogenes*. Catalase (6,000 U/ml) also inhibited chemiluminescence, but not as efficiently as superoxide dismutase (Fig. 4).

Effect of allopurinol. Allopurinol at a concentration of 10^{-4} M did not inhibit the chemiluminescence produced by *L. monocytogenes* in BHI (Fig. 2) or acetaldehyde-induced chemiluminescence by *L. monocytogenes* (Fig. 4).

Ability of spent medium to support chemiluminescence. Spent medium (BHI in which *L. monocytogenes* had been grown overnight at 37°C and then removed by filtration through a 0.22-μm filter) was able to support chemiluminescence nearly as well as fresh BHI when actively growing *L. monocytogenes* cells were added (Table 4).

DISCUSSION

L. monocytogenes produced light during growth in BHI. This light production was not directly dependent upon components in the BHI (except perhaps carbonate ions) because chemiluminescence continued for a short time after the cells were washed and placed into a carbonated saline solution. After the washed cells had

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ceased to chemiluminescence, they could be induced to chemiluminescence once again by adding acetaldehyde to the carbonated saline solution (Table 3 and Fig. 4).

The light produced by *L. monocytogenes* was not perceptible by sight and was only detectable with sensitive equipment. This fact distinguishes chemiluminescence by *L. monocytogenes* from previously reported spontaneous light emission by bacteria, which involved visible light apparently produced via the action of bacterial luciferase (10, 14).

The observed inhibition of chemiluminescence by superoxide dismutase and catalase and

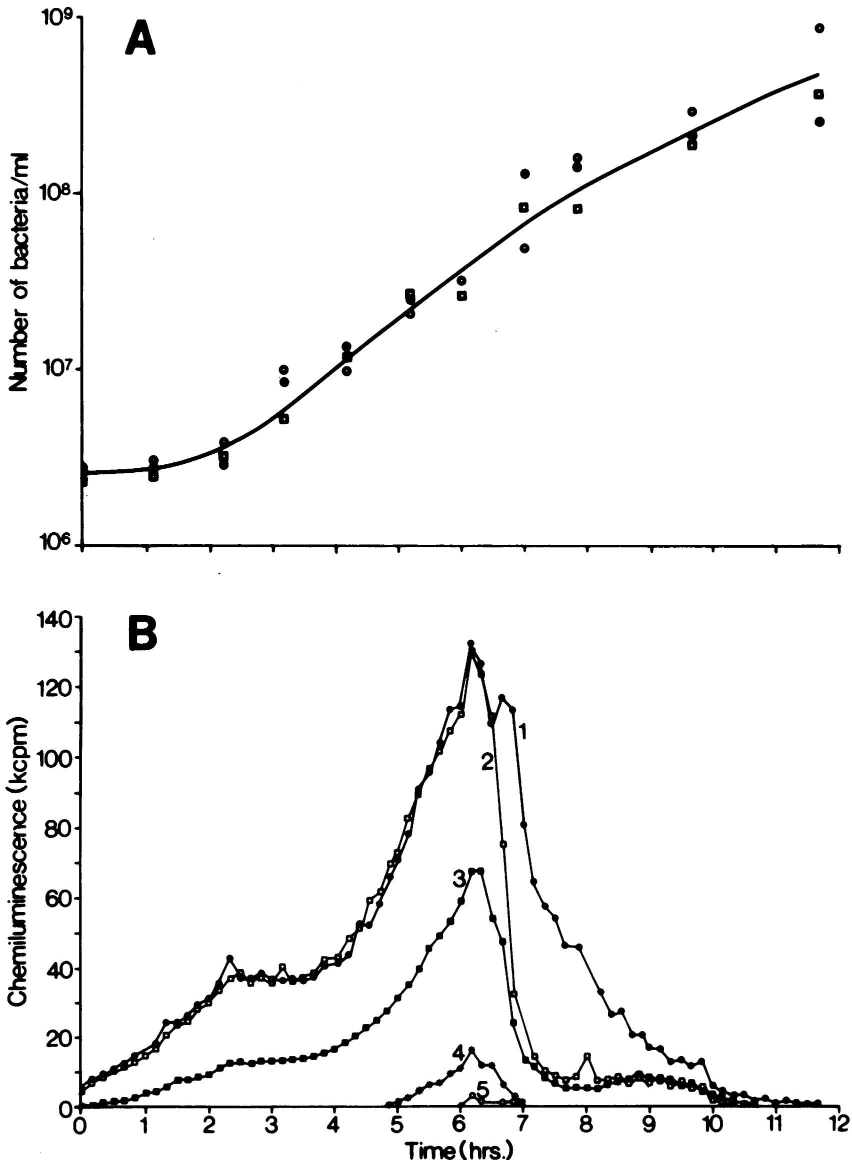

FIG. 1. *Chemiluminescence by L. monocytogenes during growth and the effect of superoxide dismutase. (A) Growth of L. monocytogenes during evaluation of chemiluminescence. The graph illustrates the number of bacteria per milliliter of medium when no superoxide dismutase (●), 10.0 μg of superoxide dismutase per ml (○), or 10.0 μg of heat-inactivated superoxide dismutase per ml (□) was present during growth at ambient temperature. (B) Chemiluminescence by L. monocytogenes during growth when no superoxide dismutase (line 1), 10.0 μg of heat-inactivated superoxide dismutase per ml (line 2), 0.1 μg of superoxide dismutase per ml (line 3), 1.0 μg of superoxide dismutase per ml (line 4), or 10 μg of superoxide dismutase per ml (line 5) was present.*

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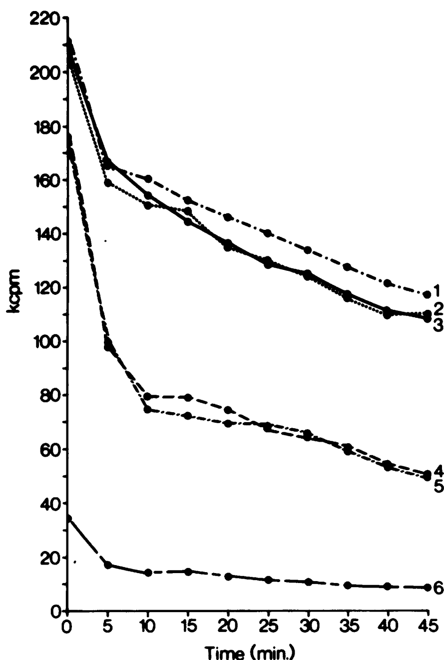


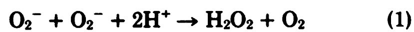
FIG. 2. *Effects of superoxide dismutase, catalase, and allopurinol on light production by L. monocytogenes. A 1.0-ml amount of a log-phase culture of L. monocytogenes was added to 9.0 ml of diluted BHI containing 10^{-4} M allopurinol (line 1), no additional compounds (line 2), 10.0 μg of heat-inactivated superoxide dismutase per ml (line 3), 1,000 U of catalase per ml (line 4), 6,000 U of catalase per ml (line 5), or 10.0 μg of superoxide dismutase per ml (line 6).*

TABLE 1. *Effect of inhibitors of the hydroxyl radical on chemiluminescence by L. monocytogenes in BHI*

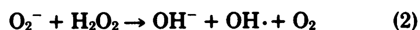
Compound added	Concn (M)	Chemiluminescence (cpm) 10 min after the reactants were combined[a]
None		69,000
Mannitol	10^{-2}	70,000
Mannitol	10^{-3}	68,000
Benzoate	10^{-3}	68,000
Benzoate	10^{-4}	66,000

[a] Background counts per minute have been subtracted.

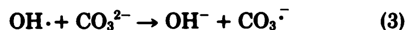
the dependence of chemiluminescence on the carbonate ion concentration suggest the O_2^- H_2O_2, and the carbonate ion are all involved in light production by *L. monocytogenes*. This is somewhat similar to the observed properties of chemiluminescence in the xanthine oxidase system (11). The following reaction scheme has been proposed to explain the chemiluminescence of the xanthine oxidase system (11):



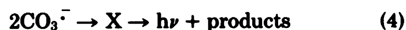
$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$



$$O_2^- + H_2O_2 \rightarrow OH^- + OH\cdot + O_2 \quad (2)$$



$$OH\cdot + CO_3^{2-} \rightarrow OH^- + CO_3^{\cdot -} \quad (3)$$



$$2CO_3^{\cdot -} \rightarrow X \rightarrow h\nu + \text{products} \quad (4)$$

Two O_2^- molecules may react to form H_2O_2 (7). This H_2O_2 may react with an additional O_2^- molecule to yield the hydroxyl radical ($OH^{\cdot -}$), and then the $OH^{\cdot -}$ reacts with carbonate ion (CO_3^{2-}) to yield carbonate radicals ($CO^{\cdot -}$). It has been proposed that two carbonate radicals react to produce the light-emitting species (X). It has also been proposed that the reactions shown above are spontaneous after the production of O_2^-. Superoxide dismutase is an enzyme that catalyzes the dismutation of O_2^- to H_2O_2, as in reaction 1. Enzymatically catalyzed dismutation of O_2^- occurs 10,000 times faster than spontaneous dismutation (7). Therefore, in the presence of adequate amounts of superoxide dismutase, O_2^- is rapidly converted to H_2O_2, resulting in insufficient amounts of O_2^- for reaction 2 to occur.

One apparent difference between the mechanism for chemiluminescence by the xanthine oxidase system and the mechanism for chemiluminescence by *L. monocytogenes* is that hy-

TABLE 2. *Effect of added carbonate ion on chemiluminescence by L. monocytogenes in diluted BHI*

Concn of carbonate added (M)	Chemiluminescence (cpm) 10 min after the reactants were combined[a]
0.0	120,000
0.05	158,000
0.1	177,000
0.2	208,000

[a] Background counts per minute have been subtracted.

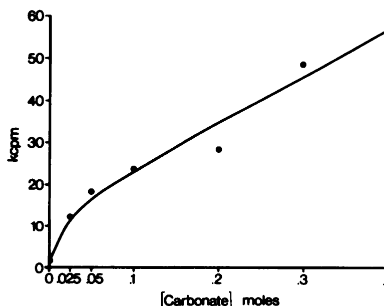


FIG. 3. *Effect of carbonate ion (CO_3^{2-}) concentration on chemiluminescence by L. monocytogenes in PBS. The graph shows chemiluminescence 5.0 min after standard suspensions of washed cells were added to vials containing different concentrations of CO_3^{2-}.*

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droxyl radical scavengers inhibit chemiluminescence in the xanthine oxidase system (11). The failure of the hydroxyl radical scavengers mannitol and benzoate to inhibit chemiluminescence by *L. monocytogenes* in BHI seems to indicate that the hydroxyl radical is not involved. However, these data must be interpreted with caution (8). The relative rates of reaction of the hydroxyl radical with the carbonate ion, with the other components in the BHI, and with the inhibitors mannitol and benzoate under the conditions of this experiment are not known. The hydroxyl radical is capable of reacting with a wide variety of organic compounds (1). Undefined organic compounds in BHI may interfere with the hydroxyl radical-scavenging properties of mannitol and benzoate, or they may react with the hydroxyl radical and result in the generation of secondary radicals (8), which may substitute for the hydroxyl radical in chemiluminescence.

The superoxide anion is a common intermediate of oxygen reduction, and it is probable that all respiring cells produce significant amounts of O_2^- in their cytoplasm. The superoxide anion is toxic to cells; therefore, it is essential that all respiring cells contain superoxide dismutase in their cytoplasm to protect themselves from the toxic effects of O_2^- (7). All aerobic species of microorganisms tested have been found to contain intracellular superoxide dismutase. Those

TABLE 3. *Effects of acetaldehyde, glucose, sucrose, and xanthine on chemiluminescence by selected bacteria*

Reactant(s) added to 10 ml of saline containing 0.4 M CO_3^{-2} and 0.15 M phosphate (pH 7.2)	Chemiluminescence (cpm) 10 min after the reactants were combined[a]
Acetaldehyde (0.1 M)	600
L. monocytogenes	3,300
L. monocytogenes + 0.1 M acetaldehyde	101,400
L. monocytogenes + 0.01 M acetaldehyde	67,800
L. monocytogenes + 0.1 M glucose	3,300
L. monocytogenes + 0.01 M glucose	3,900
L. monocytogenes + 0.1 M sucrose	−4,400
L. monocytogenes + 0.01 M sucrose	1,300
L. monocytogenes + 10^{-4} M xanthine	3,800
L. monocytogenes + 10^{-5} M xanthine	4,700
L. monocytogenes + 10^{-6} M xanthine	3,600
L. monocytogenes + 10^{-7} M xanthine	3,800
Heat-killed *L. monocytogenes*	3,900
Heat-killed (60°C, 30 min) *L. monocytogenes* + 0.1 M acetaldehyde	4,400
E. coli	2,400
E. coli + 0.1 M acetaldehyde	8,200
E. coli + 0.01 M acetaldehyde	3,100
S. minnesota	1,800
S. minnesota + 0.1 M acetaldehyde	7,400
S. minnesota + 0.01 M acetaldehyde	4,100

[a] Background counts per minute have been subtracted.

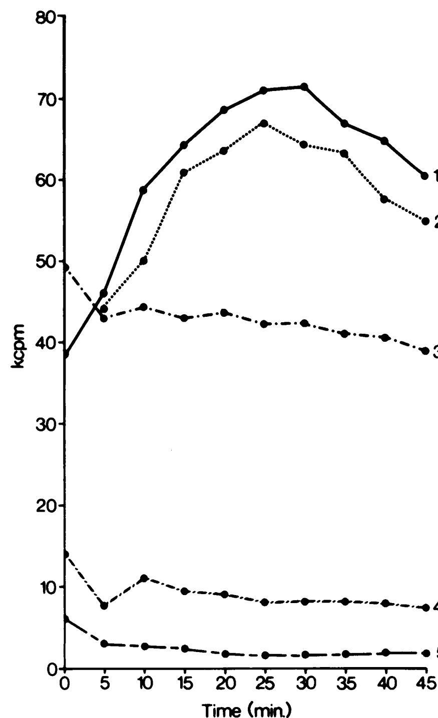

FIG. 4. *Effect of superoxide dismutase, catalase, and allopurinol on acetaldehyde-induced chemiluminescence by L. monocytogenes. Portions (1.0-ml) of a washed, standardized suspension of L. monocytogenes cells were added to scintillation vials containing 9.0 ml of PBS and 0.4 M CO_3^{2-}. The vials were allowed to stand in the scintillation counter until the chemiluminescence subsided to low levels. The following compounds were then added to different vials: line 1, 0.1 M acetaldehyde plus 10^{-4} M allopurinol; line 2, 0.1 M acetaldehyde; line 3, 0.1 M acetaldehyde plus 6,000 U of catalase per ml; line 4, 0.1 M acetaldehyde plus 10.0 μg of superoxide dismutase per ml; line 5, no compounds added.*

bacteria that have been found to lack superoxide dismutase are obligate anaerobes (13). *L. monocytogenes*, as an aerobic organism, was assumed to contain superoxide dismutase in its cytoplasm to protect itself from endogenous O_2^- production. The presence of superoxide dismutase in *L. monocytogenes* was confirmed by using the activity-staining procedure described by Beauchamp and Fridovich (5), as applied to polyacrylamide gel electrophoresis of a lysed suspension of *L. monocytogenes*. Any intracellular O_2^- should be quenched efficiently by this superoxide dismutase and should not contribute to chemiluminescence. In addition, it has been reported that O_2^- is not able to cross bacterial cell envelopes (9). Therefore, *L. monocytogenes* must have a mechanism for producing O_2^- extracellularly. Extracellular production of O_2^- by a number of aerobic organisms after exposure to

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TABLE 4. *Ability of BHI in which L. monocytogenes cells had been grown overnight and then removed by filtration to support chemiluminescence by log-phase L. monocytogenes cultures*

Reactants	Chemiluminescence (cpm) 10 min after the reactants were combined[a]
BHI + *L. monocytogenes*	239,000
Spent BHI + *L. monocytogenes*	182,500

[a] Background counts per minute have been subtracted.

sucrose or acetate has been demonstrated (15). However, this differs from the production of O_2^- by *L. monocytogenes* because sucrose failed to stimulate chemiluminescence by washed *L. monocytogenes* cells (Table 3).

The mechanism of production of extracellular O_2^- is not known. The facts that the properties of the chemiluminescence of *L. monocytogenes* are somewhat similar to those of the xanthine oxidase system and that the addition of acetaldehyde (which is known to produce O_2^- when acted on by xanthine oxidase) induces chemiluminescence suggested the involvement of xanthine oxidase. However, the addition of xanthine to resting *L. monocytogenes* cultures failed to induce chemiluminescence, and allopurinol (a specific inhibitor of xanthine oxidase [4]) failed to inhibit spontaneous or acetaldehyde-induced chemiluminescence by *L. monocytogenes*. Therefore, xanthine oxidase apparently is not involved in the production of extracellular O_2^-. It is not known whether the induction of chemiluminescence by acetaldehyde is due to the direct interaction of a bacterial enzyme with acetaldehyde or whether it is due to a general stimulation of bacterial metabolism by acetaldehyde acting as a carbon source. However, glucose and sucrose failed to induce chemiluminescence by washed *L. monocytogenes* cells. The significance of the low level of chemiluminescence which occurred when acetaldehyde was added to washed *E. coli* and *S. minnesota* cells is not clear; however, this level was much lower than that observed when acetaldehyde was added to washed *L. monocytogenes* cells (Table 3).

Figure 1 shows that detectable chemiluminescence by growing *L. monocytogenes* cultures stops despite the continued multiplication of this organism. This was not due to the build-up of an inhibitor of chemiluminescence or to the depletion of an essential component for chemiluminescence in the growth medium because when a preparation of multiplying organisms was added back to the filtered, spent medium, the level of chemiluminescence was only slightly lower (Table 2). The reason for the cessation of detectable chemiluminescence despite continued growth is not known. It may be due to a decrease in the oxygen tension in the media or to a change in the metabolism of the bacterial cells. The decreased chemiluminescence may also be partially due to the increased turbidity of the growth media.

Superoxide anion and its derivatives are believed to be responsible for oxygen toxicity in living systems. They are capable of damaging erythrocyte membranes and bacterial cells (7, 9, 12, 15). The extracellular production of O_2^- by *L. monocytogenes* may be important as a virulence factor, which results in tissue damage or inhibition of competing bacteria.

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