

A RADIOMIMETIC EFFECT OF HYDROGEN PEROXIDE ON BACTERIAL LUMINESCENCE

A. P. JACOBSON and K. A. McDERMOTT

*Department of Environmental and Industrial Health, The University of Michigan,
Ann Arbor, Michigan 48104, U.S.A.*

Received 17 November 1970; revised manuscript received 24 January 1971

Hydrogen peroxide (H_2O_2) in appropriate doses was found to mimic the reversible X-ray suppression of bacterial luminescence. Catalase protected against this suppression both by X-rays and H_2O_2 , but albumin did not. Luminescence did not recover until H_2O_2 was destroyed. These results support our hypothesis that reduced intermediates of the luminescent pathway are reversibly oxidized by both X-rays and H_2O_2 . Cyanide enhanced the effects of both X-rays and H_2O_2 and this result suggests that endogenous catalase is important to the survival of cells exposed to ionizing radiation.

1. Introduction

Radiation immediately suppresses bacterial luminescence to a lowered steady state. After irradiation, luminescence recovers within minutes to a new steady state^{1,2}). See fig. 1. At least a part of this effect could be due to

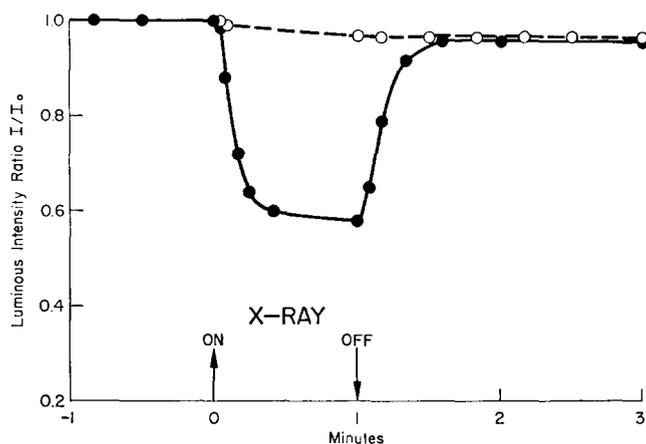


Fig. 1. The reversible suppression of bacterial luminescence by X-rays^{1,2}). Solid curve: no catalase. Dashed curve: 100 Sigma Units of catalase added before irradiation. For these experiments, bacterial cells were suspended in buffered saline at $23.0 \pm 0.3^\circ C$. The X-ray dose rate was 6500 rad/min.

hydrogen peroxide (H_2O_2) formed as a product of water radiolysis³⁻⁷). The dashed curve of fig. 1 shows the protective effect of catalase added to bacterial samples prior to X irradiation. Since most cells contain catalase⁸), it is not surprising that most attempts to imitate radiation injury by the addition of H_2O_2 have not been successful⁹). However, some studies have been successful (for examples, refs. 10 and 11).

This study describes how H_2O_2 mimics the immediate, reversible, X-ray suppression of bacterial luminescence in doses analogous to X-ray doses from 2×10^3 to 2×10^5 rad¹²).

2. Methods and materials

Photobacterium fischeri were washed free of nutrient material, centrifuged, re-suspended in 3 ml of non-nutrient, buffered saline (10^{-1} M, pH 7.2) at 2×10^9 cells per milliliter, and stored at 4°C until used. Details of sample preparation have been reported¹³).

The photometer for measuring luminescence has been described¹³). The waterbath at 12°C surrounded the photometer housing and ensured adequate temperature control of samples during, as well as before, experiments.

To begin an experiment, a luminous sample was placed in the photometer and allowed to reach 12°C . After initial measurements of luminous intensity and stability, $10 \mu\text{l}$ of H_2O_2 at various concentrations were added to the sample. To ensure rapid mixing of H_2O_2 with cells, the following procedure was devised: First, a 15 cm, 20 gauge needle was inserted through a hole in the photometer until it rested on the bottom of the test tube in front of the photocathode. Ten μl of H_2O_2 were placed in the needle's breech. Next, a 1 ml syringe was fitted to the breech and some sample medium was drawn quickly into the syringe. The mixture was moved rapidly into and out of the syringe three times to ensure prompt and thorough mixing. Suppression of luminescence began immediately.

Since it was desirable to know whether luminescence recovered in the presence of H_2O_2 by some process of adaptation or only after H_2O_2 was destroyed by hydrolysis, experiments were designed to measure residual H_2O_2 at various times after addition. These values were then compared to the time-course of luminescence after H_2O_2 addition. A modification of the method of Savage¹⁴) was used for measurements of H_2O_2 : a 6 ml sample of luminous bacteria was treated with $10 \mu\text{l}$ of 8.8×10^{-2} M H_2O_2 (final concentration 1.5×10^{-4} M) and a timer started. At various intervals after addition, this mixture was pulled into a syringe, then the syringe was fitted with a filter of $0.65 \mu\text{m}$ pore size, and 4 ml of sample were filtered into a test tube. The timer was stopped here to measure contact time between H_2O_2

and cells. To this filtrate, 0.4 ml of 0.001 M ammonium molybdate in 1 N H₂SO₄, 0.4 ml of a 5% starch solution, and 0.4 ml of 1 M potassium iodide were added. Color was allowed to develop for 10 min and the sample was read at 600 nm in a Beckman DU spectrophotometer.

Catalase activity was assayed by the Sigma method and is reported in Sigma units (SU)¹⁵. Purity of reagent H₂O₂ was assayed with potassium permanganate¹⁶.

3. Results

Various measurements from the luminescence curves useful for analysis are: *initial luminescence*, the luminous intensity immediately prior to treatment; *maximum suppression*, the lowest point reached by luminescence after

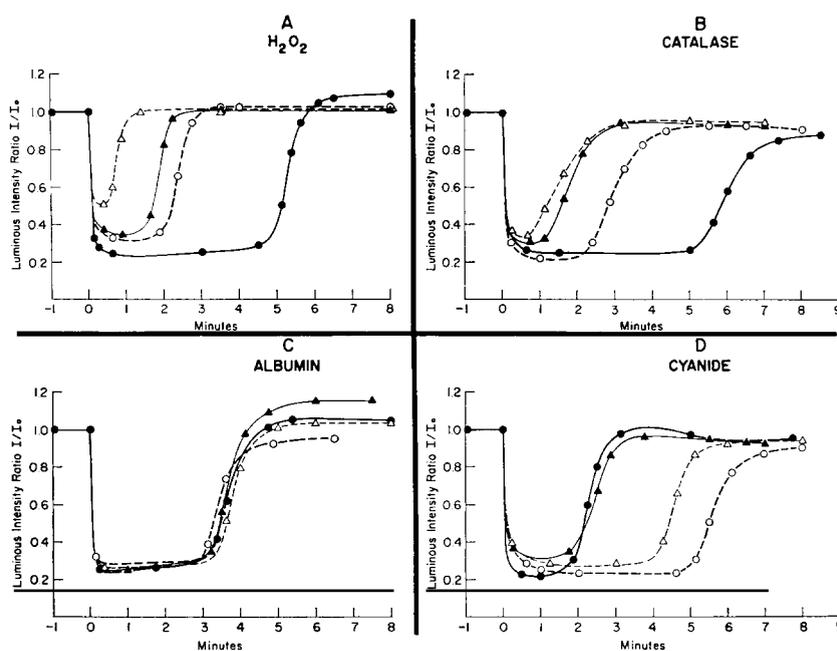


Fig. 2. The reversible suppression of bacterial luminescence by H₂O₂. H₂O₂ was added at 0 min. All other reagents were added previous to the addition of peroxide:

- (A) H₂O₂ alone, ● 2.9×10^{-4} M H₂O₂, ○ 2.9×10^{-5} M H₂O₂, ▲ 1.5×10^{-5} M H₂O₂, △ 2.9×10^{-6} M H₂O₂;
 (B) 2.9×10^{-4} M H₂O₂ plus catalase, ● no catalase, ○ 9.3 S.U. catalase, ▲ 56 S.U. catalase, △ 112 SU catalase;
 (C) 2.9×10^{-4} M H₂O₂ plus albumin, ● no albumin, ○ 0.1 mg/ml albumin, ▲ 0.2 mg/ml albumin, △ 0.4 mg/ml albumin;
 (D) 2.9×10^{-5} M H₂O₂ plus KCN, ● no cyanide, ▲ 3.33×10^{-5} M KCN, △ 1.67×10^{-4} M KCN, ○ 3.33×10^{-4} M KCN.

treatment; *maximum recovery*, the maximum luminous intensity achieved after treatment; and *recovery-half-time* ($RT_{\frac{1}{2}}$), the time after treatment where luminescence regains $\frac{1}{2}$ the value between maximum suppression and maximum recovery.

Fig. 2A shows the effects of adding H_2O_2 to luminous cell samples in the range from 2.9×10^{-4} M to 2.9×10^{-6} M final concentration. The effect produced by H_2O_2 was strikingly similar to that produced by X rays (refs. 1, 2 and fig. 1). With X rays the level of suppression depends upon the dose rate while the duration of suppression depends on total dose¹). Changes of H_2O_2 concentration produced marked changes on the level of suppression and on the recovery half time. Neither the initial rate of suppression nor the rate of recovery were significantly changed by altering the H_2O_2 concentration. At 2.9×10^{-4} M H_2O_2 , luminescence after recovery exceeded the initial value. Lesser concentrations than this produce little or no such "stimulation" and, in fact, sometimes inhibit full recovery (fig. 2A). No effects were observed after 10 μ l additions of buffered saline or distilled water.

It was postulated that these effects from X rays and H_2O_2 were produced by chemical oxidations and that catalase added to cell samples before treatment should diminish or eliminate such effects. Fig. 2B illustrates the changes in the basic response of luminescence to 2.9×10^{-4} M H_2O_2 by the addition of catalase. Concentrations of catalase greater than 168 SU were not studied since they produce turbidity and dim luminescence. In contrast to increased amounts of H_2O_2 , the addition of catalase markedly shortened $RT_{\frac{1}{2}}$. Neither the rate of suppression nor recovery were changed significantly.

There is some variability in the amount of response to H_2O_2 between different batches of bacteria, but comparisons between batches are valid since the response to H_2O_2 is measured for each batch before other reagents are studied. The effects of these reagents are then compared to the basic response produced by H_2O_2 alone.

If the attenuated effects of H_2O_2 produced by catalase (fig. 2B) were produced by specific catalytic lysis of H_2O_2 and not simply by reaction with catalase protein, albumin in weights equivalent to the weight of catalase added should not change the basic response. No significant difference was found between samples treated only with 2.9×10^{-4} or with albumin plus this amount of peroxide (fig. 2C).

Catalase activity is poisoned by cyanide which inhibits reduction of the ferric ions by H_2O_2 ¹⁷). To study whether the action of H_2O_2 on luminescence could be modified by endogenous catalase, KCN was added to luminous samples in the range from 3.33×10^{-4} M to 3.33×10^{-5} M final concentration. Again, $RT_{\frac{1}{2}}$ was altered more than other luminescent characteristics (fig. 2D). The rate of recovery was slightly reduced in the cyanide-treated

samples and the time required for luminescence to reach the lowest point was lengthened. The data are summarized in table 1.

The experiments summarized by fig. 3 show that luminescence recovered only after H₂O₂ concentration diminished considerably. Some slight re-

TABLE 1

A summary of the effect of various treatments on bacterial luminescence with standard errors

Treatment	Concentration	Percent suppression	$RT_{\frac{1}{2}}$ (min)
H ₂ O ₂	2.9×10^{-6} M	46.0 ± 5.0	0.73 ± 0.19
	1.5×10^{-5} M	66.4 ± 1.3	1.98 ± 0.24
	2.9×10^{-5} M	69.7 ± 3.6	2.66 ± 0.39
	2.9×10^{-4} M	76.3 ± 5.9	5.06 ± 1.09
Catalase + 2.9×10^{-4} M H ₂ O ₂	19 units/ml	68.5 ± 4.9	1.92 ± 0.27
	56 units/ml	68.0 ± 4.7	1.47 ± 0.35
	112 units/ml	63.5 ± 6.4	1.02 ± 0.43
	168 units/ml	59.8 ± 7.2	0.75 ± 0.19
Albumin + 2.9×10^{-4} M H ₂ O ₂	0.1 mg/ml	72.2 ± 0.8	3.66 ± 0.26
	0.2 mg/ml	74.7 ± 0.6	3.94 ± 0.18
	0.3 mg/ml	73.5 ± 1.5	3.98 ± 0.50
	0.4 mg/ml	74.8 ± 0.7	4.00 ± 0.60
KCN + 2.9×10^{-5} M H ₂ O ₂	1.67×10^{-5} M	66.5 ± 4.8	3.08 ± 0.78
	3.33×10^{-5} M	67.8 ± 1.8	3.61 ± 1.11
	1.67×10^{-4} M	73.9 ± 3.2	5.51 ± 1.27
	3.33×10^{-4} M	75.2 ± 1.9	6.01 ± 1.64
X ray	(6500 rad/min)	44.0 ± 6.1	1.15 ± 0.20
X ray + catalase	(100 units/ml)	6.1 ± 0.1	∞

covery of luminescence began early after H₂O₂ treatment, but vigorous recovery did not begin until H₂O₂ concentrations fell to the micromolar level. The technique used may not stop all reactions with H₂O₂ after filtration and before iodide was added. This error would displace the H₂O₂ curve to the right of its position in fig. 3.

4. Discussion

In former studies²), an X-ray dose of 6500 rad produced between 40 and 80% suppression of luminescence. Recovery began within seconds after exposure. The present study has shown that appropriate amounts of H₂O₂

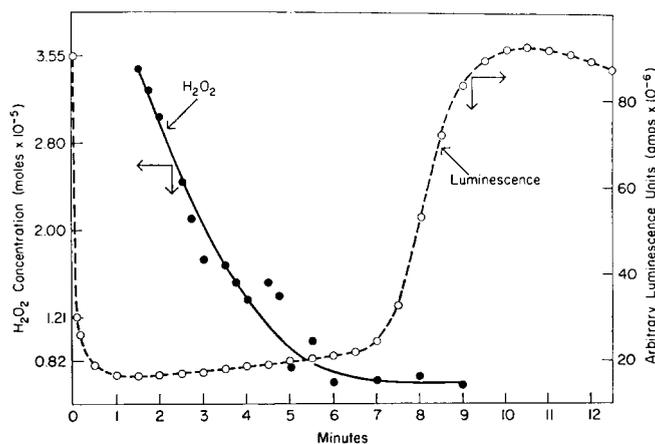


Fig. 3. The time-course of luminescence after H_2O_2 addition to luminous cell samples compared with the rate of destruction of H_2O_2 in similar samples.

mimic these radiation effects. For example, 1.5×10^{-5} M H_2O_2 very closely matched the X-ray effect and was roughly analogous to 8500 rad (fig. 2A)¹².

The difference between the X-ray dose of 6500 rad and the H_2O_2 dose analogous to 8500 rad needed for 75% suppression of luminescence is not significant because X rays produce H_2O_2 randomly throughout the irradiated volume at a finite rate while H_2O_2 is added to samples all at once and at least initially is all outside the cell membrane. Compare the initial rates of suppression in figs. 1 and 2. Also, X rays undoubtedly produce a wider array of effects than does H_2O_2 .

As expected, increased amounts of catalase produced the same effect as reduced amounts of H_2O_2 (figs. 2A and 2B). Catalase added to cell suspensions prior to X irradiation completely obliterated the reversible suppression of luminescence (see fig. 1 and table 1). Further, since added catalase attenuated the effects of H_2O_2 , the amounts of H_2O_2 added here overwhelmed endogenous catalase.

The hypotheses ¹) that radiation effects on luminescence are mediated by H_2O_2 and 2) that endogenous catalase activity protects this system to some extent, were tested by treating the cell samples with cyanide prior to the addition of H_2O_2 . Fig. 2D shows that the results agree with the hypotheses since nearly micromolar concentrations of cyanide were measurably effective in enhancing the effects of H_2O_2 . It can be inferred from these data that H_2O_2 actually produces its effects inside the cell. Cyanide added to cell suspensions prior to X irradiation produces effects similar to those described here¹⁸). Since cyanide inhibits cytochrome as well as catalase

activity, the mechanism by which cyanide sensitizes luminescence to H₂O₂ may involve more than just the inactivation of catalase.

The mechanism for the reversible suppression of luminescence by X rays or H₂O₂ is not known. However, it seems clear from fig. 3 that luminescence does not recover until the H₂O₂ concentration is significantly reduced. Since luminescence does recover, the biochemical target of peroxide oxidation must be under continuous production in some cellular pathway. We suggest three intermediates as likely peroxide targets: reduced nicotine adenine dinucleotide (NADH), reduced flavin mononucleotide FMNH₂, and reduced luciferase. All these compounds are essential for bacterial luminescence and must be chemically reduced in sequence for steady-state luminescence¹⁹). According to our hypothesis, H₂O₂ added or produced by X rays reversibly oxidizes one or possibly all of these intermediates and suppresses luminescence.

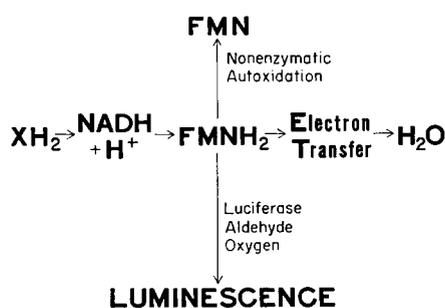


Fig. 4. The relation of various biochemical intermediates to luminescence and electron transport.

After H₂O₂ is hydrolyzed, luminescence recovers in response to the return to normal of substrate concentrations. The relation of these compounds to luminescence and electron transport is presented in fig. 4 which shows the central importance of reduced flavin mononucleotide. NAD reduction precedes while luciferase reduction follows the reduction of FMN.

Cyanide inhibits cytochrome oxidase and probably reduces competition for NADH and FMNH₂. This might explain why the time required for maximum suppression by H₂O₂ is increased by cyanide (fig. 2D). Also, cells suspended in growth medium instead of non-nutrient saline or cells in buffered saline *with micromolar glucose* do not exhibit reversible suppression of luminescence by X rays¹⁸). When luminescence of a sample is low, recovery is neither as rapid nor as complete as in samples with bright luminescence. For example, the recovery rate for the bright samples (fig. 2A) is about 3.5 times faster than for dim samples (fig. 2B).

Failure of luminescence to return to the pre-treatment level may be due to cell death or enzyme denaturation¹¹) caused by momentarily high concentrations of H₂O₂. Experiments are being designed to add H₂O₂ at various rates to match irradiation experiments.

These results show that H₂O₂ does mimic radiation effects. Further, this radiomimetic effect is immediate and reversible. If measurements are not made continuously throughout the treatment, reversible events may not be seen. Perhaps some attempts to show radiomimetic effects have failed because the measurements were not made until these effects had disappeared.

Acknowledgements

The critical review of Drs. G. Hoyt Whipple and Isadore A. Bernstein is acknowledged and appreciated.

References

- 1) C. H. Burns, A. P. Jacobson and G. H. Whipple, *Radiation Res.* **29** (1965) 494.
- 2) A. P. Jacobson, *J. Luminescence* **3** (1970-1971) 203.
- 3) H. Fricke, *J. Chem. Phys.* **2** (1934) 556.
- 4) P. Bonet-Maury and M. Lefort, *Nature* **162** (1948) 381.
- 5) P. Bonet-Maury and M. Lefort, *J. Chim. Phys.* **47** (1950) 179.
- 6) H. Fricke, *J. Chem. Phys.* **3** (1935) 364.
- 7) E. S. G. Barron, L. Seki and P. Johnson, *Arch. Biochem. Biophys.* **41** (1952) 188.
- 8) H. I. Adler, *Radiation Res. Suppl.* **3** (1963) 110.
- 9) A. M. Kuzin, in: *Radiation Biochemistry* (Israel Program for Scientific Translations Ltd., Jerusalem, 1964) p. 24.
- 10) Z. M. Bacq and J. Moutschen, *Compt. Rend. Soc. Biol. Belg.* **150** (1956) 2262.
- 11) E. D. Willis, *Biochem. Pharmacol.* **2** (1959) 276.
- 12) M. Frilley, *Brit. J. Radiol. Suppl.* **1** (1947) 50.
- 13) C. H. Burns, A. D. Nevill and G. H. Whipple, *Anal. Biochem.* **5** (1963) 393.
- 14) D. J. Savage, *Analyst* **76** (1951) 224.
- 15) *Sigma Chemical Co. catalog 1969*, p. 70. One Sigma unit (SU) will decompose 1 μ M H₂O₂ per minute at pH 7.0 and 25°C, while the H₂O₂ concentration falls from 10.3 to 9.2 μ M per milliliter of reaction mixture. The rate of disappearance of H₂O₂ is followed by observing the rate of decrease in optical density at 240 nm.
- 16) H₂O₂ is diluted to 100 ml with distilled water in a volumetric flask. Twenty ml of this solution is treated with 50 ml of dilute H₂SO₄ (S.G. 1.110-1.114) and diluted further with 50 ml distilled water. This solution is titrated with 0.1 N KMnO₄ to stable red color. Each milliliter of KMnO₄ equals 1.7008 mg H₂O₂.
- 17) D. Keilin and E. F. Hartree, *Proc. Roy. Soc. (London) B* **121** (1936) 173.
- 18) A. P. Jacobson, unpublished data.
- 19) J. W. Hastings, W. H. Riley, and J. Massa, *J. Biol. Chem.* **240** (1965) 1473.