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Oxidative Stress Detection with *Escherichia coli* Harboring a *katG'*::*lux* Fusion

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A plasmid containing a transcriptional fusion of the *Escherichia coli katG* promoter to a truncated *Vibrio fischeri lux* operon (*luxCDABE*) was constructed. An *E. coli* strain bearing this plasmid (strain DPD2511) exhibited low basal levels of luminescence, which increased up to 1,000-fold in the presence of hydrogen peroxide, organic peroxides, redox-cycling agents (methyl viologen and menadione), a hydrogen peroxide-producing enzyme system (xanthine and xanthine oxidase), and cigarette smoke. An *oxyR* deletion abolished hydrogen peroxide-dependent induction, confirming that *oxyR* controlled *katG'*::*lux* luminescence. Light emission was also induced by ethanol by an unexplained mechanism. A marked synergistic response was observed when cells were exposed to both ethanol and hydrogen peroxide; the level of luminescence measured in the presence of both inducers was much higher than the sum of the level of luminescence observed with ethanol and the level of luminescence observed with hydrogen peroxide. It is suggested that this construction or similar constructions may be used as a tool for assaying oxidant and antioxidant properties of chemicals, as a biosensor for environmental monitoring, and as a tool for studying cellular responses to oxidative hazards.

Active oxygen species are highly damaging to all living organisms, and aerobically grown cells cannot survive without adequate protection against the toxic effects of these molecules (9). The importance of oxidative stress and damage in biology, agriculture, and medicine is gaining more recognition, as is the significance of antioxidant activity for cellular well-being (5, 7, 9).

Of the various active oxygen species, the free radicals are usually considered the most hazardous. Hydrogen peroxide, although chemically less reactive, is nevertheless a threat to the structure and function of proteins, nucleic acids, lipids, and membranes, whether it is added externally or produced intracellularly (9). The damage inflicted by hydrogen peroxide, as well as many organic peroxides, is due either to intrinsic oxidative activity or to production of hydroxyl radicals.

Several enzymatic systems have evolved to eliminate peroxides and combat their deleterious effects. In both *Escherichia coli* and *Salmonella typhimurium*, the *oxyR* regulon is the best-studied of these defense circuits. Exposure to H_2O_2 leads to induction of at least 30 proteins, 9 of which are under direct positive control of the OxyR protein (7, 17, 18). These include a catalase (HPI) that is encoded by the *katG* gene.

In the framework of a project aimed at the generation of a panel of bacterial biosensors for environmental stress, several *E. coli* strains which respond to oxidative stress by the production of light were designed and constructed (2, 3, 20). One group of these strains, which are responsive to the presence of superoxide radicals, will be described elsewhere (3a). In this paper we describe the construction and characterization of a peroxide-responsive biosensor. To construct this biosensor, the *oxyR*-activated promoter of the *katG* (catalase) gene, under positive control of *oxyR*, was fused to *Vibrio fischeri luxCDABE* genes so that *katG* induction led to light emission.

MATERIALS AND METHODS

Plasmids and *E. coli* strains. Plasmid pKatGLux2 containing a transcriptional fusion of the *katG* promoter region to the *luxCDABE* genes was constructed by a method similar to the method described previously (21). The *katG* promoter was obtained by PCR amplification (GeneAmp PCR reagent kit; Perkin-Elmer Cetus, Norwalk, Conn.) of a 654-bp segment, using lambda phage 538 of the Kohara collection (4, 10) as the template. The primers used were 5′-ACTTA AGGATCCCGAAATGAGGGCGGGAAA-3′ and 5′-AGCAGCGAATTC GAACGTTGCTGACCACGA-3′; these primers included nucleotides −235 to −218 and +406 to +390 relative to the start of *katG* transcription (19), as well as additional nucleotides containing *Bam*HI and *Eco*RI restriction sites, respectively. Following digestion with *Bam*HI and *Eco*RI, the PCR products were directionally ligated to *Bam*HI- and *Eco*RI-digested pUCD615, a plasmid containing the promoterless *luxCDABE* operon of *V. fischeri* (14), which created the desired operon fusion. Plasmid pKatGLux2 was moved into *E. coli* RFM443 (12) by CaCl₂-mediated transformation (15) to yield strain DPD2511. For further studies plasmid pKatGLux2 was also placed in a pair of isogenic hosts, one of which was an *oxyR* mutant (1), by CaCl₂ transformation (15). The host strains and the resulting transformants are listed in Table 1. Table 1 also lists strains containing other *lux* fusion plasmids, detailed descriptions of which have been or will be published elsewhere.

Insertion verification. The sequence, size, and orientation of the *katG* promoter in pKatGLux2 were verified by using the two approaches described below.

The size and orientation of the inserted DNA were verified by PCR amplification, using the isolated plasmid DNA as the template and a pair of primers; one of the primers was from the *luxI* region of pUCD615 (23), and the other was the 5′ primer used for amplification of the original promoter fragment from *E. coli* DNA. The size of the amplified fragment was very similar to the calculated size (1,454 bp).

The sequence of 480 bp in the *katG* promoter region in plasmid DNA isolated from DPD2511 was determined in one direction by using the Promega Silver Sequence kit. The following modifications were made to the procedure outlined in the manufacturer's November 1993 manual: the long glass plate was treated each time with Sigmacote; the 6% Long Ranger gels contained 1.2× TBE and were electrophoresed with 0.6× TBE electrophoresis buffer; amplification profile 1 was used; DNA samples were heated to 75°C for 5 to 8 min just prior to loading; and the developing solution was maintained at 4 to 6°C until just prior to use, split into 1-liter aliquots, and used in succession. The illumination times required for transfer of the silver-stained banding image from the air-dried gel to EDF film were 5 to 20 s. Development of the EDF film with an automated film processor was made possible by attaching a 2- to 3-in. (ca. 5.08- to 7.62-cm) strip

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TABLE 1. *E. coli* plasmids and strains used in this study

Plasmid or strain	Description	Reference
Plasmids		
pUCD615	Amp^r Kan^r, multiple cloning site upstream of *luxCDABE*	14
pKatGLux2	Same as pUCD615, but *katG'::luxCDABE*	This study
pMicFLux1	Same as pUCD615, but *micF'::luxCDABE*	Unpublished data
pGrpELux5	Same as pUCD615, but *grpE'::luxCDABE*	21
pRecALux3	Same as pUCD615, but *recA'::luxCDABE*	Unpublished data
Parental strains		
RFM443	*galK2 lac74 rpsL200*	12
K-12		1
GS08	Same as K-12, but *oxyR::Kan*	1
Strains used in this study		
DPD2511	pKatGLux2/RFM443	This study
TV1061	pGrpELux5/RFM443	21
DPD2794	pRecALux3/RFM443	2, 3
DPD 1554	pKatGLux2/GS08	This study
DPD1560	pKatGLux2/K12	This study
DPD1553	pMicFLux1/GS08	This study

of previously exposed and processed type XAR film to the EDF film with labeling tape.

One mismatch with the previously published *katG* (19) sequence, which was not near or in any known control region, was found.

Cell culture and experimental procedure. All *E. coli* strains were grown in Luria-Bertani (LB) medium (13) at 26°C. To assay for luminescence, cells grown overnight in the presence of kanamycin sulfate (25 mg/liter) were diluted to a concentration of approximately 10^7 cells per ml in fresh LB medium without kanamycin and were allowed to grow at 26°C for 2 to 3 h. A 50-μl portion of the culture was added to each well of an opaque white microtiter plate (Dynatech) that contained a series of twofold dilutions of the compounds to be tested in 50 μl of LB medium. Luminescence was monitored with a temperature-controlled (26°C) microtiter plate luminometer (model ML3000; Dynatech) as described previously (21).

Luminescence values were expressed in the instrument's arbitrary relative light units (RLU). The maximal response ratios were the highest ratios of luminescence in the sample-containing wells to luminescence in wells containing untreated cells determined during a specified period.

Cigarette smoke effect. To study the effect of cigarette smoke on *katG* expression, exponentially growing cells in LB medium at 26°C were split into two 10-ml samples, each in a screw-cap 50-ml Erlenmeyer flask. Into one of the flasks a single puff of smoke from a commercially available cigarette was exhaled, and both flasks were then incubated at 26°C with shaking for an additional 10 min. Samples (50 μl) were then added to the wells of a microtiter plate that contained 50 μl of fresh LB medium, and the plate was immediately placed in the luminometer.

Enzymes and chemicals. All of the chemicals used were analytical grade. Hydrogen peroxide and isopropanol were obtained from J. T. Baker Chemical Co., ethanol was obtained from Quantum Chemical Corp., organic peroxides were obtained from Aldrich, and the remaining compounds (menadione, methyl viologen, and xanthine) and enzymes (catalase, superoxide dismutase, and xanthine oxidase) were obtained from Sigma Chemical Co.
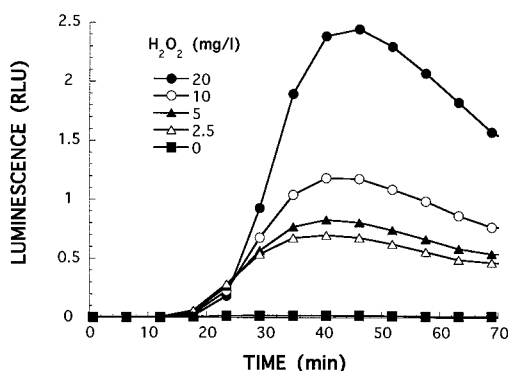
RESULTS

Responses to peroxides. The response of strain DPD2511, the *katG'::lux*-containing construction, to different hydrogen peroxide concentrations is shown in Fig. 1. A lag phase approximately 20 min long was followed by an increase in luminescence, which peaked after approximately 45 min and then declined. The response was dose dependent, with concentrations of more than 50 mg/liter resulting in inhibition of luminescence (data not shown). Other experiments (data not shown) revealed that significant induction of the *katG* fusion can be observed at hydrogen peroxide concentrations as low as 0.1 mg/liter.

Strain DPD2511 also exhibited a clear response to organic peroxides (Fig. 2), with luminescence kinetics very similar to those observed with hydrogen peroxide. Four different organic peroxides were tested at a broad range of concentrations. Two of these compounds, cumene hydroperoxide and *tert*-butyl hydroperoxide, elicited responses similar in magnitude to the response induced by H_2O_2. *tert*-Butyl perbenzoate elicited a clear but small response at the higher end of the concentration range tested (1 g/liter), and DL-tertbutylperoxide resulted in no induction at any of the concentrations tested (data not shown).
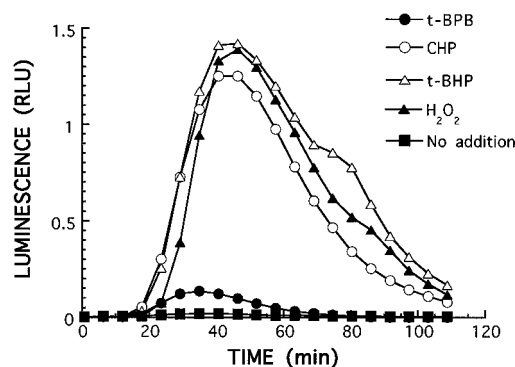

FIG. 1. Effects of H_2O_2 on bioluminescence of *E. coli* DPD2511.

FIG. 2. Response of strain DPD2511 to organic peroxides. The concentrations used are the concentrations which elicited the maximal response for each compound, as follows: *tert*-butyl perbenzoate (t-BPB), 1 g/liter; cumene hydroperoxide (CHP), 125 mg/liter; *tert*-butyl hydroperoxide (t-BHP), 22 mg/liter. The H_2O_2 concentration used was 38 mg/liter.
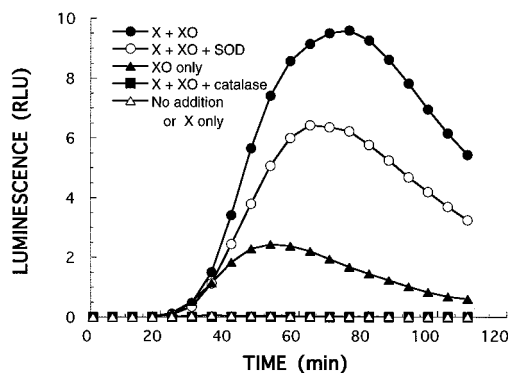
FIG. 3. Xanthine and xanthine oxidase effects on DPD2511 luminescence. The following concentrations were used: xanthine (X), 1 mM; xanthine oxidase (XO), 0.125 U/ml; catalase, 640 U/ml; and superoxide dismutase (SOD), 138 U/ml.

Figure 3 shows the response of strain DPD2511 to peroxides generated enzymatically by the action of xanthine oxidase on xanthine. Very strong induction in the presence of both enzyme and substrate was observed, while xanthine alone elicited no response. Interestingly, induction of luminescence occurred repeatedly in response to xanthine oxidase when there was no externally introduced substrate. Luminescence in response to the presence of both xanthine and xanthine oxidase was partially sensitive to the addition of superoxide dismutase and was completely abolished by catalase (Fig. 3). Of the two active oxygen species potentially generated by xanthine oxidase activity, superoxide and H_2O_2 (6), the latter appeared to be the ultimate inducer of the *katG* promoter. While the actual peroxide concentrations to which the cells were exposed were not calculated, it is possible that the relatively gradual generation of the oxidants allowed induction stronger than the induction observed with H_2O_2 or organic peroxide treatments (Fig. 1 and 2).

Responses to other treatments. While peroxides elicited the strongest response from DPD2511, other compounds also induced luminescence in this strain. These compounds included redox cycling agents, such as methyl viologen or menadione, which produce superoxide radicals. The response to these compounds was similar in magnitude to the response elicited by H_2O_2, a phenomenon which may be explained by the intracellular conversion of superoxide radicals to hydrogen peroxide by superoxide dismutase. Indeed, menadione-induced lumines-
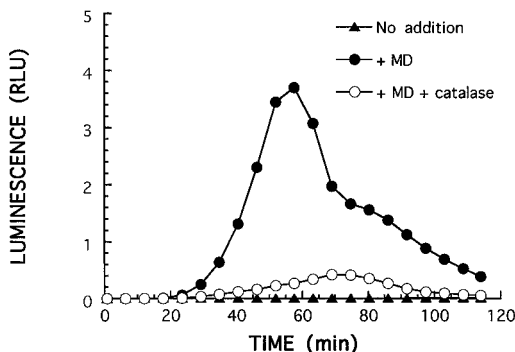
cence was dramatically diminished in the presence of catalase (Fig. 4).

Alcohols, such as isopropanol and ethanol, were another class of compounds which induced significant luminescence in strain DPD2511. Furthermore, the presence of both ethanol and H_2O_2 appeared to have a marked synergistic effect on this induction (Fig. 5); the luminescence observed in the presence of both inducers was far greater than the luminescence expected from the sum of the responses to the individual compounds. It is yet to be determined if the synergy occurred at the level of gene activation or whether the effect was on luciferase activity.

Specificity of the peroxide response. Strain DPD2511 is one of a series of *E. coli* constructions (2, 3, 20–23) containing plasmids on which *lux* genes are fused to various stress promoters. Several of these constructions also responded to oxidative stress exerted by various agents, including peroxides. In order to highlight the enhanced specificity of the *katG'::lux* fusion for H_2O_2, its reactivity and the reactivities of two other strains which exhibited significant responses were compared (Fig. 6). Strain TV1061 carries a plasmid with a *grpE'::lux* fusion and has been shown to be a sensitive reporter of heat shock induction (21). DPD2794 contains a *recA'::lux* fusion and as such is a potential biosensor for SOS-inducing agents (2, 3, 24). Figure 6 clearly shows that DPD2511 was indeed induced to much higher levels than the other strains; at 50 mg/liter, a concentration which led to maximal induction in all three strains, the luminescence of DPD2511 increased 800-fold, compared with 70-fold for TV1061 and approximately 20-fold for DPD2794. Figure 6 also shows that the *katG'::lux* fusion was much more sensitive than the other fusions; at 1.6 mg/liter, DPD2511 exhibited 20-fold induction, compared with the 2.5-fold induction exhibited by DPD2794 and the lack of induction exhibited by TV1061.

To demonstrate that the induced luminescence observed in strain DPD2511 was indeed under the control of the *oxyR* regulatory circuit, the plasmid bearing the *katG'::lux* fusion was inserted into an isogenic pair of *E. coli* hosts, one of which was defective in the *oxyR* circuit (Table 1). Figure 7A shows that the *oxyR* mutation eliminated peroxide induction. To show that this effect was specific to the *katG'::lux*-bearing plasmid, induction of luminescence by an *oxyR*-independent reporter was examined in the same *oxyR* host. As shown in Fig. 7B, induction of the *micF'::lux* fusion, which was responsive to oxygen radicals (under *soxRS* control [7, 8]), by the redox-cycling agent methyl viologen was not affected by the *oxyR* null mutation.

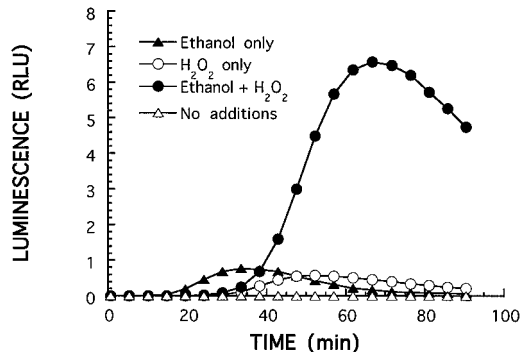**Response to cigarette smoke.** Several potential uses can be

FIG. 4. Effect of catalase (640 U/ml) on DPD2511 luminescence induced by menadione (MD) (4 mg/ml).

FIG. 5. Synergistic effect of H_2O_2 (6 mg/liter) and ethanol (2%) on DPD2511 luminescence.
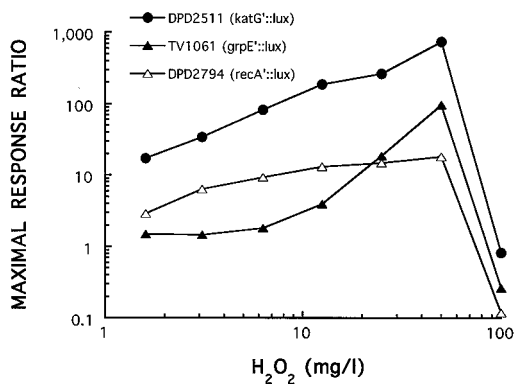
FIG. 6. Responses to H_2O_2 of three *E. coli* constructions, each carrying a different promoter::*lux* fusion. Each point represents the maximal response ratio observed during 120 min of exposure of the cells to the given peroxide concentration. The maxima were reached after 40 to 60 min in strains DPD2511 and TV1061 and after 100 to 120 min in strain DPD2794.

envisioned for bacterial strains bearing *katG'::lux* and similar fusions; these organisms could be used as reporters of intracellular oxidative stress, as a convenient assay system for antioxidant activities, or as biosensors for potential environmental oxidative hazards. Strain DPD2511 has been shown to be responsive to different types of environmentally significant pollutants and chemical industrial wastewater (2, 3, 3a). A somewhat different use is shown in Fig. 8, which shows the induction of luminescence by exposure to a puff of cigarette smoke for 10 min. While this experiment could not provide quantitative data concerning the oxidative effects of cigarette smoke or any of its numerous constituents, it very clearly demonstrated that oxidative hazards were present and suggested a rapid way for detecting them.

DISCUSSION

In this paper we describe an *E. coli* strain that carries a plasmid in which the *V. fischeri lux* structural genes are fused to the promoter for a catalase-specifying gene (*katG*). Luminescence was induced in this strain by the presence of H_2O_2, which was added externally (Fig. 1, 2, 5, and 6), produced extracellularly by xanthine oxidase (Fig. 3), or perhaps generated internally by superoxide dismutase (Fig. 4). The strain which we used was also highly responsive to organic peroxides

(Fig. 2), to redox-cycling agents (Fig. 4), and to cigarette smoke (Fig. 8). The response of the strain was shown to be under the control of the *oxyR* regulon (3) (Fig. 7), the main *E. coli* circuit for defense against peroxide stress. Luminescence in strain DPD2511 was also induced by short-chain alcohols, such as ethanol (Fig. 5). The nature of this effect is being investigated; preliminary results indicate that the effect of ethanol, a known heat shock inducer, may also be at least partially oxidative and dependent on *oxyR* function (3a).

In most previously described studies of the use of bacterial luminescence as a genetic reporter, the workers used only two genes, *luxA* and *luxB*, which encode the two subunits of luciferase (11, 16). In these studies, addition of a long-chain aldehyde as a substrate for the luciferase was necessary. The plasmid used in our study contains a single transcriptional unit which also includes *luxCDE*, which simplifies the experimental procedure by eliminating the need for external aldehyde addition. A danger inherent in such a construction is an increase in the probability that light emission will be affected by parameters that are not related to the promoter-driven expression of *lux*. Such parameters, however, tend to inhibit luminescence rather than enhance it. The fact that the responses of strain DPD2511 are consistently dose dependent suggests that if such inhibitory effects are present, they are relatively insignificant.

To satisfy future experimental and diagnostic needs, increased sensitivity to oxidative agents may be required. One possible approach may be to modify or replace the host bacterium (21), which would allow increased permeability or intracellular accumulation. Another possible avenue is suggested by the synergistic effect demonstrated in Fig. 5. The presence of a small amount of an "enhancer" compound (in this case ethanol) may dramatically increase the inductive effects of compounds whose influence is otherwise hardly noticeable. The nature of the synergy in this case is not clear. There are indications that ethanol-dependent synergy of heat shock induction may occur on a transcriptional level (22), but it remains to be determined whether this is true for *oxyR*-dependent induction.

In summary, strain DPD2511 appears to be a sensitive tool for detecting stress exerted by different types of oxidative agents, as well as for quantifying the effects of the agents. The following three major uses can be envisioned for this construction or similar constructions. (i) They may be used to study the regulation of cellular responses to oxidative hazards; in contrast to most other genetic reporter systems, *lux* expression can be monitored in vivo, which allows real-time monitoring of the
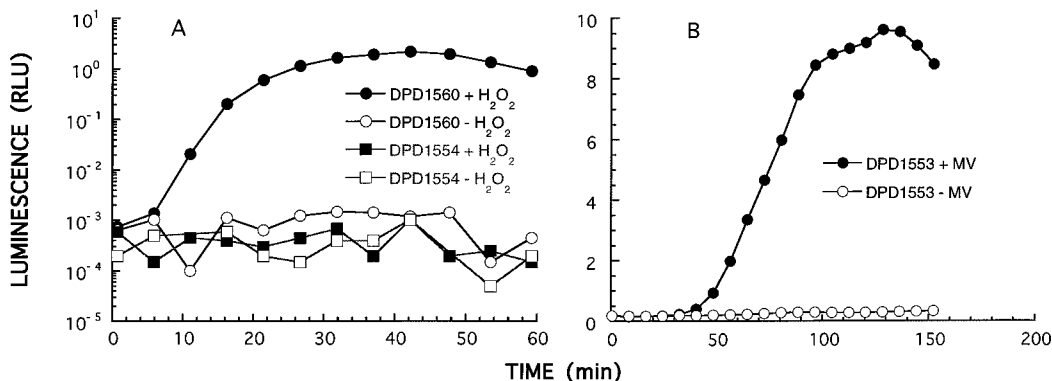

FIG. 7. H_2O_2-induced luminescence in the *katG'::lux* fusion is controlled by *oxyR*. (A) Strains DPD1554 (*oxyR::kan*) and DPD1560 (*oxyR+*), both containing pKatGLux2, challenged with H_2O_2 (3 mg/liter). (B) Strain DPD1553, containing pMicFLux1 in the *oxyR* mutant, challenged with methyl viologen (MV) (1 mg/ml). See Table 1 for strain descriptions.
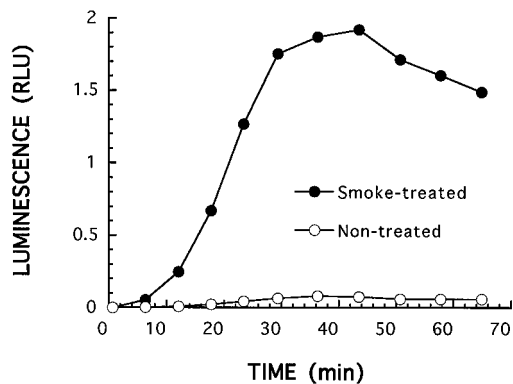
FIG. 8. Cigarette smoke induces DPD2511 luminescence. The experimental conditions used are described in Materials and Methods. Time zero was the end of direct exposure to smoke and the beginning of luminescence monitoring.

cellular events involved. (ii) They may be used as very convenient tools for assaying the oxidant and antioxidant properties of chemicals, food additives, etc. (iii) They may be used as sensitive tools for monitoring and assessing the hazards in environmental samples in different media, including drinking water, wastewater, soil, and air; this can be accomplished by using isolated batch samples or may be part of an on-line early warning system.

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