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Toxicity and Mutagenicity of Plumbagin and the Induction of a Possible New DNA Repair Pathway in *Escherichia coli*

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Actively growing *Escherichia coli* cells exposed to plumbagin, a redox cycling quinone that increases the flux of O_2^- radicals in the cell, were mutagenized or killed by this treatment. The toxicity of plumbagin was not found to be mediated by membrane damage. Cells pretreated with plumbagin could partially reactivate lambda phage damaged by exposure to riboflavin plus light, a treatment that produces active oxygen species. The result suggested the induction of a DNA repair response. Lambda phage damaged by H_2O_2 treatment were not reactivated in plumbagin-pretreated cells, nor did H_2O_2-pretreated cells reactivate lambda damaged by treatment with riboflavin plus light. Plumbagin treatment did not induce lambda phage in a lysogen, nor did it cause an increase in β-galactosidase production in a *dinD*::Mu d(*lac* Ap) promoter fusion strain. Cells pretreated with nonlethal doses of plumbagin showed enhanced survival upon exposure to high concentrations of plumbagin, but were unchanged in their susceptibility to far-UV irradiation. *polA* and *recA* mutants were not significantly more sensitive than wild type to killing by plumbagin. However, *xth-1* mutants were partially resistant to plumbagin toxicity. It is proposed that *E. coli* has an inducible DNA repair response specific for the type of oxidative damage generated during incubation with plumbagin. Furthermore, this response appears to be qualitatively distinct from the SOS response and the repair response induced by H_2O_2.

Most, if not all, living organisms require defensive systems to protect against active oxygen species. Substantial attention has been paid to the relative roles of superoxide (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^·$), and singlet oxygen in mediating, directly or indirectly, damage to cellular components (4, 6, 12, 23, 25, 32, 37). Parallel attention has been paid to the roles of enzymes that may function as protective scavengers of active oxygen species. These enzymes include superoxide dismutases (SODs), catalases, peroxidases, and others (1, 7, 12, 15, 23, 36). Recent in vitro studies have shown that O_2^-, when accompanied by H_2O_2, can cause DNA strand scission (4). In vivo studies (32) point to the mutagenicity of paraquat, believed to increase the intracellular flux of O_2^-.

Given the apparent ubiquity of O_2^- and H_2O_2 in organisms growing aerobically, along with evidence that both O_2^- and H_2O_2 can damage DNA, it appears that two general types of defensive systems must exist: predamage protective mechanisms, most notably SOD and catalase, and postdamage repair mechanisms. Evidence for one type of postdamage repair has been reported by Demple and Halbrook, who demonstrated an inducible DNA repair response to H_2O_2 that is distinct from either the SOS or the adaptive response (10).

Plumbagin is a quinone that is capable of abstracting electrons from electron transfer components and thereby diverting electron flow to molecular dioxygen to form superoxide radicals (8, 16). There are several compounds that have the ability to "redox-cycle" electrons intracellularly to form O_2^- radicals. Plumbagin was of particular interest to us because it is a potent inducer of manganese SOD (MnSOD) in *Escherichia coli* and because it has been shown to mediate O_2^- production in vitro in cell-free systems (16).

Although substantial evidence points to the toxicity of superoxide, questions have been raised concerning the direct reactivity of O_2^- radicals with biomolecules in vivo (12, 34). It has been suggested that O_2^- gives rise to $OH^·$ radicals and H_2O_2 by enzymatic and nonenzymatic reactions and that the latter two are responsible for observed damage to macromolecules, including DNA damage (4, 12, 32).

Certain DNA repair-deficient strains, including *polA*, *recA*, and *xthA* mutants, are quite sensitive to killing by hydrogen peroxide and other agents that generate forms of active oxygen (1, 7, 11, 38). In this report, we describe the effects of subjecting these repair-deficient mutants to the O_2^- radical generator plumbagin. We also describe the toxic and mutagenic consequences of exposing *E. coli* cells to different levels of plumbagin, as well as the inducible responses to such exposure. Finally, we present evidence that the damage and the repair response induced in cells exposed to plumbagin are qualitatively distinct from the damage and repair brought about by exposure to H_2O_2 or to agents that induce the SOS response.

MATERIALS AND METHODS

Chemicals. Mitomycin C, riboflavin, *o*-nitrophenyl-β,D-galactopyranoside, plumbagin (5-hydroxy-2-methyl-1, 4-napthoquinone), ampicillin, and X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside) were from Sigma Chemical Co., St. Louis, Mo. H_2O_2 (30%) was from Mallinckrodt, Inc., St. Louis, Mo.

Bacterial strains and growth conditions. Bacterial strains are listed in Table 1. Unless stated otherwise, cells were grown overnight in minimal medium with glucose (M9+G), supplemented with essential amino acids and vitamins (M9+Gs, our designation), as described by Miller (31). Cells were diluted approximately 100-fold and allowed to grow to the specified density. Luria broth (LB) and λ broth were as described elsewhere (31).

Measurement of plumbagin-induced toxicity. Cells were grown overnight at 37°C in M9+Gs. Overnight cultures were diluted with fresh medium and allowed to grow to approxi-

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TABLE 1. *E. coli* strains

Strain designation	Genotype	Source or reference
AB1157	*argE3 his-4 leu-6 proA2 thr-1 thi-1 ara-14 galK2 lacY1 mtl-1 xyl-5 tsx-33 sup-37 str-31*	(17)
AB1157 *recA*	As above but *recA56*	P1 JC10240 (9) × AB1157, selected for UV sensitivity
AQ550	F⁻ *thyA709 his metD trpA lacY* (λ *c*I857 *S7*)	λ *c*I857 *S7* (33) lysogen of CSH26 (31)
AQ624	F⁻ *thyA argH ilv met thi-1 his-29 trpA9605 deo-29 rpoB340 proB*	(21)
AQ634	As for AQ624 but *arg*⁺ *rpoB*	(21)
AQ685	*proA3 metD88 lac-3 metB1 thyA his-29 trpA9605 deo-29 rpoB340*	(21)
BW9091	*xth-1 thr leuB6 his-4 proA2 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 str-31 tsx-33 supE-37*	Weiss (30)
GW1040	*sfiA11 tif-1 dinD1*::Mu d(*lac* Ap) *thr-1 leu-6 his-4 lacΔ*(*U169*)	Walker (20)
JW164	F⁻ *thyA1 lacY rha malB str-31 polA1*	Wechsler (40)

mately 10^8 cells per ml. A small sample was removed, diluted 10^{-4} in M9+G, and allowed to stand for 40 min at room temperature. Plumbagin or M9+G was then added to 0.5 ml of cells to the final concentrations indicated, and the cells were shaken at 37°C for 1 h. Small samples were removed and plated on either M9+Gs or L plates. Plates were counted after 24 or 48 h of incubation at 37°C.

Assay for membrane damage. *E. coli* AB1157 cells were treated as described above except that small samples of plumbagin-treated cells were plated on M9+Gs and M9+Gs with 200 mM NaCl. Plates were counted after 48 h at 37°C. The positive control for membrane damage was near-UV light as described elsewhere (18).

Plumbagin-induced mutagenesis. Plumbagin-induced mutagenesis was assayed with *E. coli* AQ634 cells by the method described by Witkin and Kogoma (43).

Plumbagin pretreatment and cell survival in subsequent far-UV light and high concentrations of plumbagin. Strain AB1157 cells were grown overnight at 37°C in LB. Cells were diluted into fresh LB and allowed to grow to approximately 10^8/ml. The culture was then divided, and one sample was brought to a final concentration of 0.075 mM plumbagin. Cells were shaken at 37°C for 1 h. Samples were diluted and plated on L plates for viability (0.075 mM plumbagin treatment had no lethal effect upon cells). To test for induced UV resistance, 1 ml was removed from each culture and centrifuged, and the cells were suspended in M9+G. Cells were then diluted 10^{-4} in M9+G. Samples (5 ml) of cells were placed in sterile plastic petri dishes. Control samples (100 μl) were removed, diluted, and plated before irradiation. Cells were irradiated with a germicidal lamp at a fluence of 54 J/m^2, and 100-μl samples were diluted and plated. Colonies were counted after 24 h. To examine for induced resistance to plumbagin, 5-ml samples from control and 0.075 mM-plumbagin cultures were centrifuged and suspended in 1 ml of fresh LB. Control samples (50 μl) were diluted and plated. One-half of the remaining samples were brought to 2 mM plumbagin and incubated for 2 h at 37°C with shaking. At the end of 2 h, samples from treated and control cultures were diluted and plated. Colonies were counted after 24 h.

Plumbagin exposure and induction of λ prophage in AQ550. Strain AQ550, a lysogen of λ *c*I857 *S7*, was grown at 30°C in M9+Gs plus 10 mM $MgSO_4$ and 0.1% maltose. When cells reached early exponential phase, they were treated with 0.25 and 0.5 mM plumbagin for 1 h at 30°C. Cells were then centrifuged, suspended in fresh M9+Gs containing 10 mM $MgSO_4$ and 0.1% maltose, and allowed to grow for an additional 2 h at 30°C, after which time they were collected in a microcentrifuge (Eppendorf) for 3 min. Samples (0.1 ml) of the supernatant were mixed with 0.2 ml of strain AB1157 indicator cells and plated as described elsewhere (31). Incubation with mitomycin C at 1 μg/ml was the positive control.

Induction of β-galactosidase in *dinD*::Mu d(*lac* Ap) promoter fusion strain. Cells were grown in M9+Gs at 30°C. At 10^8 cells per ml, plumbagin was added to final concentrations of 0.0, 0.05, 0.075, and 0.1 mM. The positive control was 1 μg of mitomycin C per ml. At time zero and each hour thereafter for 4 h, 0.75-ml samples were removed and quickly frozen. Samples were thawed, 1 ml of Z buffer (31), 100 μl of 1% sodium dodecyl sulfate, and 150 μl of chloroform were added, and the cells were shaken at 37°C for 30 min. Samples of 50 μl were used to assay for protein by the method of Lowry et al. (27). To the remaining solution, 0.75 ml of additional Z buffer and 0.4 ml of 4-mg/ml *o*-nitrophenyl-β,D-galactopyranoside were added. This solution was incubated at 30°C for 1 h, and absorbance was measured at 550 and 420 nm. Enzyme activity per microgram of protein was calculated as described elsewhere (20, 30).

Riboflavin/light treatment of λ phage. A 0.1-ml volume of λ *vir* (31) stock in SM buffer (28) was added to 1.9 ml of the same buffer with and without 0.3 mM riboflavin. The control was kept in the dark. Treated samples were placed 2 cm from a white, fluorescent light source (GE 15W Cool White) for the time of exposure indicated in each experiment. Oxygenation was accomplished by bubbling air through the solution continuously during illumination.

Protection experiments with catalase, SOD, and anoxia were performed by diluting λ *vir* phage into SM buffer containing SOD (120 U/2-ml reaction volume), catalase (1,600 U/2 ml), or SOD plus catalase (120 and 1,600 U, respectively, per 2-ml reaction volume). Experiments in which oxygen was kept to a minimum were conducted by bubbling N_2 gas through all solutions for 10 min before illumination.

Assay for riboflavin/light-induced single-strand nicks in supercoiled plasmid DNA. pBR325 plasmid DNA in DNA buffer (10 mM Tris hydrochloride–10 mM NaCl, pH 8.2) or water was brought to a final concentration of 0.3 mM riboflavin and then placed 2 cm from the same light source used to inactivate λ phage. Samples were removed at intervals, mixed with tracking dye, and then kept on ice in the dark until all samples had been collected. DNA samples were electrophoresed in 1% agarose and photographed as described elsewhere (28).

Infection of plumbagin- and H_2O_2-pretreated cells by λ phage inactivated by treatment with riboflavin/light or H_2O_2. Strain AB1157 cells were grown overnight in λ broth or M9+Gs (including maltose and $MgSO_4$). Cells were diluted

into fresh medium and grown to approximately 5×10^8 cells per ml. Either plumbagin or H_2O_2 was then added, and the cells were incubated with shaking at 37°C for 1 h with plumbagin or for 40 min with H_2O_2. After treatment with H_2O_2 as described elsewhere (10) or with riboflavin/light as described above, phage (0.1 ml) was added to cells (0.1 ml) and allowed to adhere for 25 min at 37°C. Lambda soft agar (3.0 ml; 0.5% agar) was added immediately before plating on λ plates. Plaques were counted after 8 h of incubation at 37°C.

Assay for single-strand breaks in cellular DNA by exposure to H_2O_2 and plumbagin. The assay for single-strand breaks was that described by Anathaswamy and Eisenstark (1).

RESULTS

Sensitivity of DNA repair mutants to killing by plumbagin. Wild-type *E. coli* is very sensitive to killing by plumbagin. In our experiments, strain AB1157 cells were 99.9% killed by exposure to 1.0 mM plumbagin for 1 h at 37°C (Fig. 1). Previous studies have shown that *polA*, *recA*, and *xthA* mutants are hypersensitive to killing by H_2O_2 (1, 7, 11). Because H_2O_2 is a possible by-product of plumbagin exposure, we were interested in determining whether these mutants were also hypersensitive to plumbagin. *polA* and *recA* strains were only slightly more sensitive to killing by plumbagin than was the isogenic wild type. Surprisingly, the *xth-1* mutant was quite refractive to the lethal effects of plumbagin as compared with the wild type (Fig. 1). We examined *polA*, *recA*, and *xth* mutants for the ability to induce MnSOD in the presence of plumbagin, using a gel-staining assay (2, 16) and scanning densitometry. In all cases, plumbagin-treated cells exhibited at least a fourfold increase in MnSOD activity over uninduced cells (data not shown).

Plumbagin is slightly mutagenic. To determine the mutagenicity of plumbagin, we measured the $Trp^- \rightarrow Trp^+$ reversion frequency in actively growing AQ634 cells and in stationary-phase cells. The results show that plumbagin was not mutagenic in stationary-phase cells but was moderately mutagenic in exponential-phase cells (Table 2).

Plumbagin toxicity is not mediated by cell membrane damage. Certain species of active oxygen are thought to cause membrane damage through lipid peroxidation (12, 14). To determine whether exposure to plumbagin affected membrane integrity, we exposed cells to plumbagin and then plated them onto normal M9+Gs and onto high-salt M9+Gs (200 mM NaCl). Figure 2 shows that even at concentrations of plumbagin that induce 95% killing there was no difference in the number of colonies formed on normal and hypertonic media, suggesting that plumbagin toxicity was not a function of membrane damage. As a positive control comparison (18), the Fig. 2 inset shows the effects of near-UV upon plating efficiency on normal and high-salt media.

Preexposure to plumbagin increases resistance to subsequent plumbagin treatment, but not to far-UV irradiation. *E. coli* cells pretreated with far-UV irradiation at low fluences or with low concentrations of known SOS inducers withstand subsequent challenge doses of far UV better than cells that have not been treated (26, 39). To determine whether pretreatment with low concentrations of plumbagin enhanced survival upon exposure to subsequent challenge doses of plumbagin or far UV, cells were first incubated with plumbagin at a nonlethal concentration (0.075 mM) and then exposed to lethal doses of plumbagin or far UV (Table 3). The results indicate that plumbagin pretreatment enhanced survival against high concentrations of plumbagin at least threefold. The same pretreatment had no effect upon survival of cells subjected to subsequent far-UV irradiation, however. This suggested that the resistance to plumbagin acquired during pretreatment was not due to expression of the SOS response.

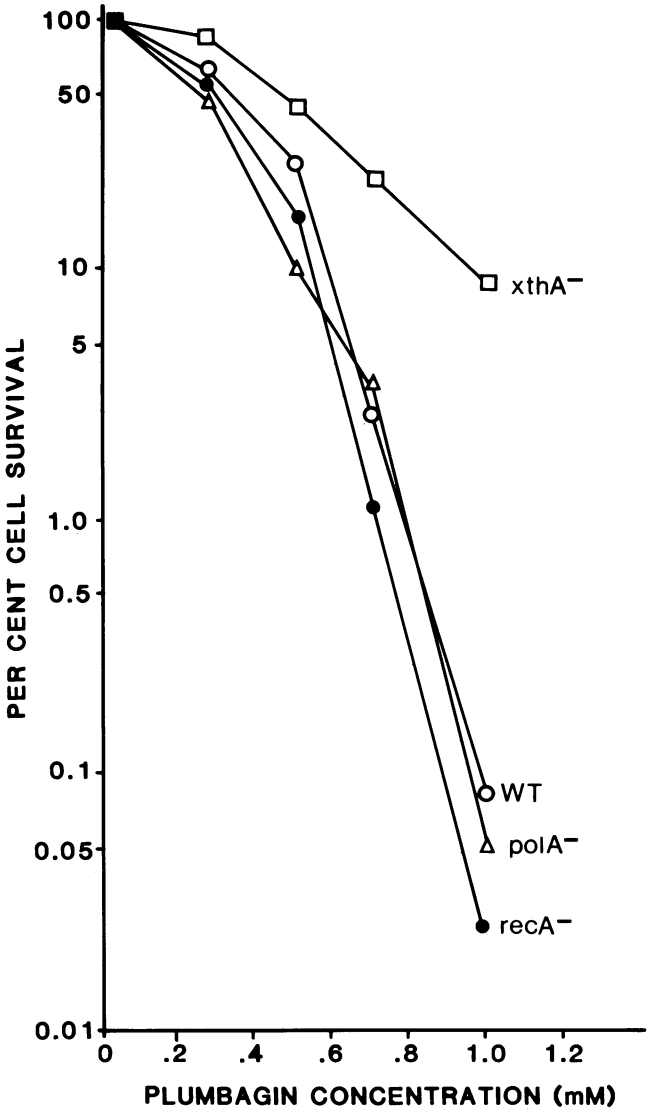

FIG. 1. Lethal effects of plumbagin on wild-type and DNA repair-deficient mutants of *E. coli*. Exponential cells grown in M9+Gs (37°C) were collected by centrifugation, suspended in M9+G without supplements, and incubated at room temperature for 40 min before plumbagin was added to the concentration indicated. Cells were further incubated with shaking at 37°C for 1 h before dilution and plating onto M9+Gs plates. Symbols for strains: AB1157 wild type (○); AB1157 *recA*⁻ (●); JW164 (*polA1*) (△); BW9091 (*xth-1*) (□).

Plumbagin does not induce the SOS response. To confirm the preliminary conclusion that plumbagin does not induce the SOS response, we conducted two additional experiments. First, we examined whether incubation with plumbagin resulted in prophage induction in a lambda lysogen, a known SOS response (39). Concentrations of plumbagin up to 0.5 mM failed to induce lambda prophage, whereas mitomycin C, a known SOS inducer (19), readily induced prophage (Table 4). Second, we looked for β-

TABLE 2. Induced mutation frequency by plumbagin in exponential- and stationary-phase cells[a]

Cell treatment (concn)	Cell density at time of treatment (per ml)	$Trp^- \rightarrow Trp^+$ revertants per 10^8 survivors
None	1.5×10^8	0.86
PLM (0.25 mM)	1.5×10^8	2.30
PLM (0.50 mM)	1.5×10^8	6.10
MMS (17.7 mM)	1.5×10^8	544.00
None	4.0×10^9	3.07
PLM (0.25 mM)	4.0×10^9	3.00
PLM (0.50 mM)	4.0×10^9	2.59
MMS (17.7 mM)	4.0×10^9	1,080.10

[a] Strain AQ634 cells were grown overnight at 37°C in M9+Gs. A portion of the overnight culture was refreshed in the same medium. When cell density reached 10^8 cells per ml, the refreshed culture, together with the overnight culture (2.7×10^9 cells per ml), was counted, centrifuged, resuspended in M9+G (without supplemented nutrients), and allowed to stand at room temperature for 40 min. Plumbagin (PLM) or methyl methanesulfonate (MMS) was then added to cells at the concentration indicated, and the cells were incubated at 37°C for 1 h. Cells were then plated directly (for mutation assay) or diluted and plated (for survival assay) on M9+Gs containing 0.05 μg of tryptophan per ml (growth-limiting concentration). Colonies were counted after 2 days of incubation at 37°C. Each value represents the average of three plates.

galactosidase activity in a *dinD*::Mu d(*lac* Ap) fusion strain in which activity can be induced by a variety of SOS-inducing agents, including mitomycin C (22). Incubation of the *dinD*::*lacZ* fusion strain with plumbagin did not result in an increase in β-galactosidase production (Fig. 3).

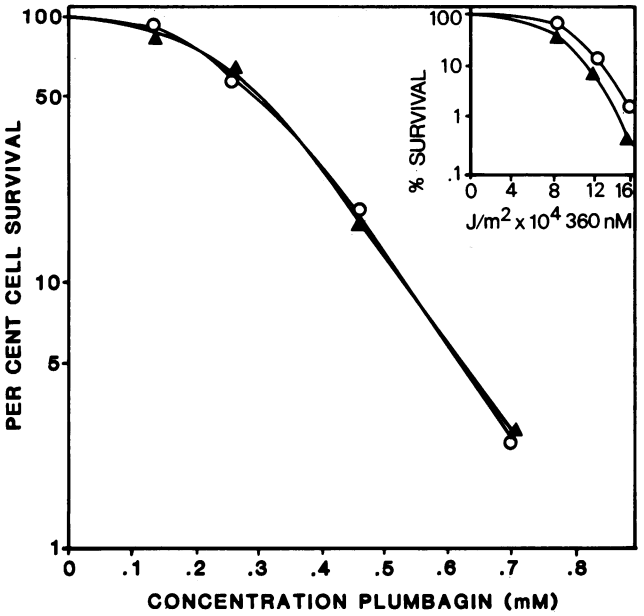

FIG. 2. Effects of hypertonic media on the survival of plumbagin-treated cells. Strain AB1157 cells in mid-log phase were pelleted by centrifugation, resuspended in M9+G without supplements, and allowed to stand at room temperature for 40 min before the addition of plumbagin to the concentrations indicated. Cells were shaken at 37°C for 1 h and then diluted and plated onto normal M9+Gs (○) and M9+Gs with 200 mM NaCl (▲). As a positive control, a sample of AB1157 cells in M9+G was irradiated with near-UV light (360 nm) at the fluences indicated and was then diluted and plated onto normal and high-salt M9+Gs plates (18).

TABLE 3. Effects of plumbagin pretreatment on cell survival after subsequent treatment with far-UV irradiation or high concentration of plumbagin[a]

Pretreatment	Damaging agent	Total no. of cells exposed	No. of survivors	% Survival
None	Plumbagin	4.78×10^8	2.53×10^7	5.2
Plumbagin	Plumbagin	3.61×10^8	6.01×10^7	16.6
None	Far-UV	1.0×10^4	2.79×10^2	2.8
Plumbagin	Far-UV	7.6×10^3	2.10×10^2	2.8

[a] An overnight culture of strain AB1157 was refreshed and exposed to plumbagin (0.075 mM) for 1 h. Cells were centrifuged, suspended in M9+Gs or L broth, and then exposed to plumbagin (2 mM) at 37°C for 2 h or to far-UV irradiation at 54 J/m² as described in the text.

Inactivation of λ phage by exposure to riboflavin/light in the presence of oxygen. Riboflavin in solution is photoreduced upon irradiation with light and reoxidized by exposure to air or O_2 gas (29). We took advantage of this redox cycling characteristic, which generates O_2^- radicals, to damage phage λ. Phage λ was effectively inactivated by exposure to riboflavin/light in a manner dependent upon time of exposure (Fig. 4). Inactivation was also directly related to the intensity of light (data not shown).

Protection from riboflavin-induced damage by catalase, SOD, and anoxia. The results of the experiments shown in Table 5, using oxygen radical scavengers (SOD and catalase) or limiting the level of available oxygen, strongly implicated activated oxygen as being the causative agent of riboflavin/light-induced λ inactivation. SOD, which scavenges O_2^- radicals, provided some protection against inactivation, as did catalase. SOD and catalase together provided greater protection than the additive protection expected, suggesting that the two enzymes protect synergistically. This synergistic protection implies that $OH^\cdot$ radicals might be the main causative agent of damage, as suggested previously (4).

Riboflavin/light treatment induces single-strand breaks in DNA. When supercoiled plasmid DNA was subjected to riboflavin/light treatment as described above, covalently closed circular DNA species were first converted to open circular and then to linear form (Fig. 5). The degree of the conversion to open circular and linear species was proportional to the time of exposure. The results indicate that single-strand breaks were generated by riboflavin/light treatment.

Reactivation of riboflavin/light-damaged λ phage and H_2O_2-damaged λ phage by cells pretreated with plumbagin or H_2O_2. Because pretreatment of cells with the O_2^--generating com-

TABLE 4. Induction of λ prophage in strain AQ550 lysogens as a function of exposure to plumbagin or mitomycin C[a]

Cell treatment (concn)	No. of plaques
Control	84.5
Mitomycin C (1 μg/ml)	>500
Plumbagin (0.25 mM)	87
Plumbagin (0.50 mM)	91

[a] Strain AQ550, a λ *c*I857 *S7* lysogen, was grown overnight. Cells were refreshed to 10^8 cells per ml, and the culture was divided into four equal samples. One sample served as a control, and the others were treated as shown. Cells were shaken at 30°C for 1 h, pelleted, suspended in L broth with 10 mM $MgSO_4$, and incubated at 30°C for 2 h. Bacteriophage titer was determined as described elsewhere (31). Each value represents the average of three plates.

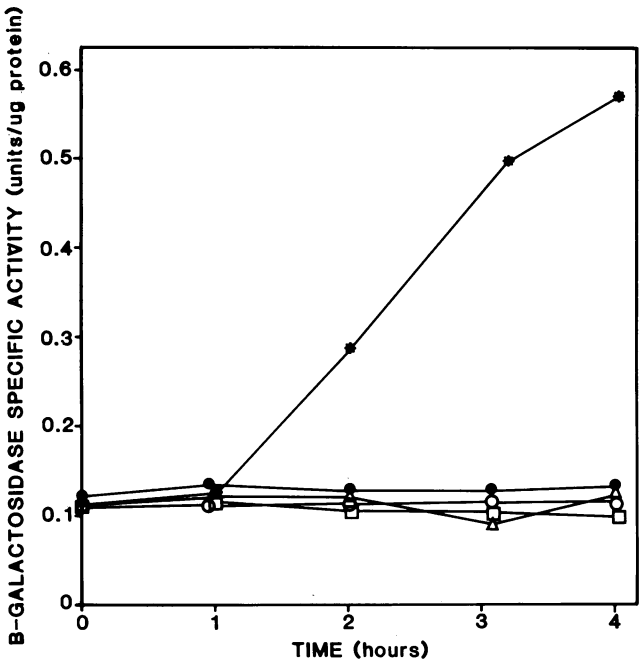

FIG. 3. β-Galactosidase induction in a *dinD*::Mu d(*lac* Ap) fusion strain exposed to plumbagin and mitomycin C. An overnight culture of GW1040 was diluted, grown to 10^8 cells per ml, and then divided into five equal samples of 5 ml each. Plumbagin or mitomycin C was added as follows: control (no plumbagin) (○), 0.05 mM plumbagin (●), 0.2 mM plumbagin (△), 0.5 mM plumbagin (□), and mitomycin C (500 μg/ml) (*). At the times indicated, 0.75-ml samples were withdrawn and assayed for total protein and β-galactosidase activity.

pound plumbagin elicited resistance to subsequent exposure to plumbagin (Table 3), and because riboflavin/light effectively inactivated phage (Fig. 4 and Table 5), we examined whether cells pretreated with plumbagin could reactivate riboflavin/light-damaged λ phage more efficiently than could untreated cells (so-called Weigle reactivation). Riboflavin/light-inactivated λ phage were plated on pretreated or untreated cells. The result (Fig. 4) shows that riboflavin/light-treated phage had a higher plating efficiency on pretreated cells than on untreated cells. The level of reactivation did not significantly improve when the conditions of pretreatment (i.e., the length of time and the concentration of plumbagin) were varied (for example, see Table 6). Although the level of reactivation was not as great as for Weigle reactivation of UV-damaged phage in UV-irradiated cells (41), the level was reproducible, and a range of 50 to 100% increase in viable phage was consistently observed.

There was no significant difference between the reactivation capability of *recA*$^+$ and *recA*$^-$ cells which had been pretreated with plumbagin (Table 6). These results suggest that this reactivation by plumbagin-treated cells is distinct from the reactivation of UV-damaged phage by SOS-induced cells, which is completely dependent on *recA*$^+$ (39).

The results of the reactivation experiments also indicate that riboflavin/light-damaged phage could not be reactivated by H_2O_2-damaged cells (Fig. 4). The reactivation of riboflavin/light-damaged phage did not increase when several different pretreatment concentrations of H_2O_2 (up to 250 mM) were examined (data not shown). Conversely, H_2O_2-inactivated phage could not be reactivated by plumbagin-pretreated cells (Fig. 6).

Assay for single-strand breaks in DNA induced by cell exposure to plumbagin or H_2O_2. Based upon our earlier findings that plumbagin-pretreated cells could reactivate riboflavin/light-damaged λ phage and that exposure of plasmid DNA to riboflavin/light produced numerous single-strand breaks, we suspected that plumbagin might be producing such breaks in the chromosomal DNA. However, when we assayed for single-strand breaks induced during incubation with plumbagin or H_2O_2, using alkaline sucrose gradient centrifugation, we found that whereas H_2O_2 produced numerous breaks, plumbagin produced few if any (Fig. 7). The concentrations of plumbagin and H_2O_2 used in this experiment produced approximately equal amounts of killing.

DISCUSSION

It is unlikely that the observed lethal effects of plumbagin result from a decrease in the flow of electrons through the electron transport chain, since agents that are known to inhibit this flow of electrons are not necessarily lethal (35). Nor do the lethal effects of plumbagin appear to arise from membrane damage (Fig. 2). Because plumbagin is somewhat mutagenic, and because cells pretreated with plumbagin can reactivate oxidatively damaged λ phage, it is likely that the toxicity of plumbagin is, at least in part, a function of DNA damage. The nature of the DNA damage being produced by exposure to plumbagin is yet unclear. Figure 7 indicates that plumbagin does not cause single-strand breaks to any large extent. Furthermore, Fig. 5 clearly indicates that DNA exposed to riboflavin/light is subject to numerous strand

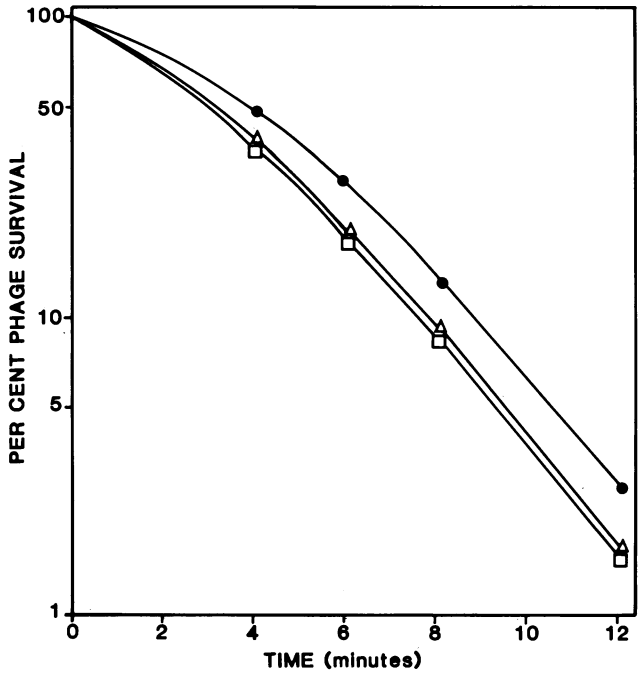

FIG. 4. Survival of riboflavin/light-damaged phage λ in plumbagin-pretreated, H_2O_2-pretreated, and untreated cells. Phage λ *vir* were exposed to riboflavin/white light as described in the text for the times indicated. The phage were then used to infect AB1157 cells that had been left untreated (□) or were pretreated with H_2O_2 (60 μM with 30 μM $CuSO_4$) at 37°C for 40 min (△) or with plumbagin (0.5 mM) at 37°C for 60 min (●). 100% = 4,730 PFU.

TABLE 5. Effects of SOD, catalase, and anoxia on riboflavin/light-induced inactivation of λ *vir*[a]

Scavenger added (concn)	PFU of λ per 100 μl	% Survival
Control (no riboflavin)	4,409	100.0
None	960	21.8
Catalase (1,600 U/ml)	1,573	35.7
SOD (120 U/ml)	1,904	43.2
Catalase (1,600 U/ml) + SOD (120 U/ml)	3,414	79.5
Anoxia (N_2 gas)	3,520	79.8

[a] Inactivation of λ *vir* by exposure to riboflavin/light was performed as described in the text. SOD or catalase was added to the solution just before the addition of riboflavin. N_2 gas was bubbled through the riboflavin solution for 10 min before the addition of phage and then continuously during light exposure.

TABLE 6. Reactivation of riboflavin/light-damaged λ *vir* in *recA*$^+$ and *recA*$^-$ cells pretreated with plumbagin[a]

Treatment of cells (concn)	Treatment of phage	Strain	PFU of phage per 100 μl	% Survival
None	None	*recA*$^+$	1,163	100
		recA$^-$	1,179	100
None	Riboflavin/light	*recA*$^+$	23	1.9
		recA$^-$	21	1.8
Plumbagin (0.3 mM)	Riboflavin/light	*recA*$^+$	40.5	3.4
		recA$^-$	42	3.5
Plumbagin (0.6 mM)	Riboflavin/light	*recA*$^+$	47	4.0
		recA$^-$	42	3.5

[a] Strain AB1157 *recA*$^+$ and AB1157 *recA*$^-$ cells were diluted to 4×10^8 cells per ml before addition of plumbagin. Phage λ *vir* were treated with riboflavin/light before being plated on *recA*$^+$ and *RecA*$^-$ cells. Each value represents the average of three plates.

breaks. Perhaps accordingly, plumbagin-treated cells have only moderately enhanced ability to reactivate λ phage treated with riboflavin/light. These results suggest that single-strand breaks may not be the primary lesion caused by plumbagin and that they are not the target of plumbagin-induced repair.

While we do not yet know the nature of the damage produced upon exposure to plumbagin, several lines of evidence indicate that this damage is different from that caused by H_2O_2 and that the cellular responses to plumbagin and H_2O_2 are distinct. (i) H_2O_2 induces numerous single-strand breaks, but plumbagin does not (Fig. 7). (ii) DNA repair-deficient mutants (*polA*, *recA*, and *xth*) exhibit sensitivity to H_2O_2 but not to plumbagin killing (1, 10; Fig. 1). In fact, *xth-1* cells are less sensitive to killing by plumbagin than are wild-type cells. This is curious in light of evidence that a point mutation in the *xth* locus destroys all of the four known functions of its gene product, exonuclease III (30, 42). We are investigating the possibility that *xth-1* exhibits resistance to plumbagin as a result of hyperrecombination (44). (iii) H_2O_2-damaged phage are reactivated by cells exposed to H_2O_2, but not by cells exposed to plumbagin, although plumbagin-treated, but not H_2O_2-treated, cells reactivate phage inactivated by riboflavin/light. (iv) Plumbagin strongly induces MnSOD, but not catalase (4, 10; unpublished data). (v) H_2O_2 and quinones such as plumbagin give rise to different classes of alarmones in *Salmonella typhimurium* (3, 24).

Our failure to demonstrate plumbagin-induced SOS is interesting given that paraquat, another potent inducer of MnSOD (15), was reported to cause SOS induction (5).

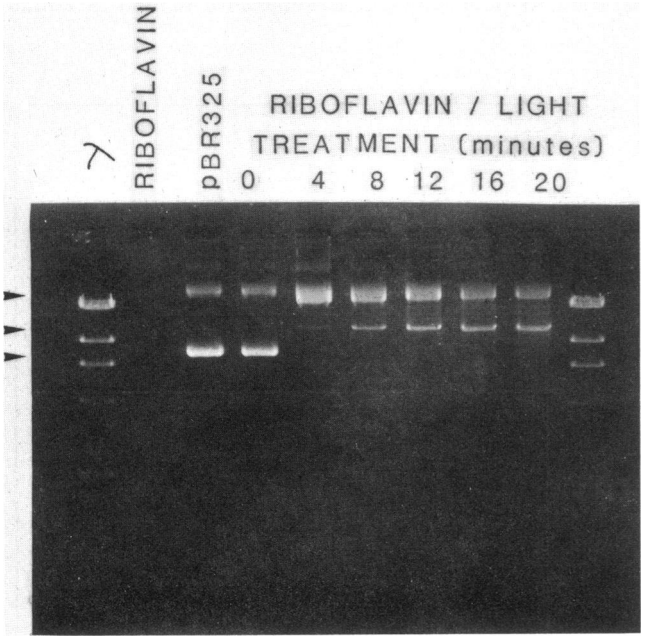

FIG. 5. Agarose gel electrophoretic analysis of plasmid DNA exposed to riboflavin/light treatment. A 200-μl sample of dimeric pBR325 (50 μg/ml) was exposed to white light with and without riboflavin at a final concentration of 3×10^{-4} M. Samples (25 μl) were removed at the times indicated, added to 5 μl of blue dye (0.06% bromphenol blue–0.01 M EDTA–33% glycerol), and chilled in the dark until all samples were collected. All samples were loaded onto a 1% agarose gel (0.04 M Tris acetate–0.1 mM EDTA, pH 7.8), electrophoresed for 2 h, and then stained with 0.5 μg of ethidium bromide per ml. From the top, arrows indicate the mobilities of open circular (nicked), linear, and closed circular (supercoiled) plasmid DNAs, respectively.

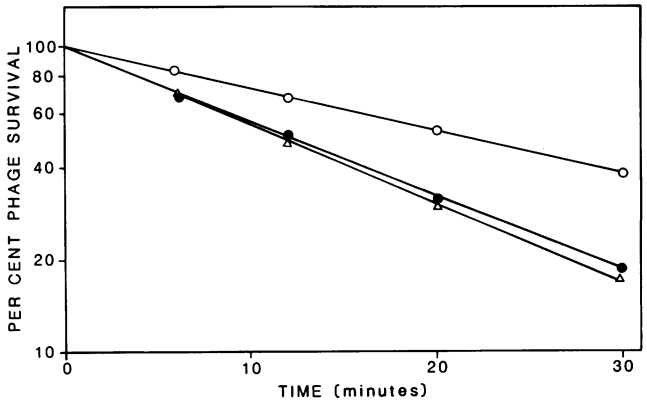

FIG. 6. Survival of H_2O_2-damaged phage λ in H_2O_2-pretreated, plumbagin-pretreated, and untreated cells. Phage λ *vir* were damaged by a 20-min treatment with H_2O_2 (2 mM with 30 μM $CuSO_4$) and then plated on AB1157 cells that were left untreated (△), pretreated with 60 μM H_2O_2 for 1 h (○), or pretreated with 0.5 mM plumbagin for 1 h (●). 100% = 5,126 PFU.

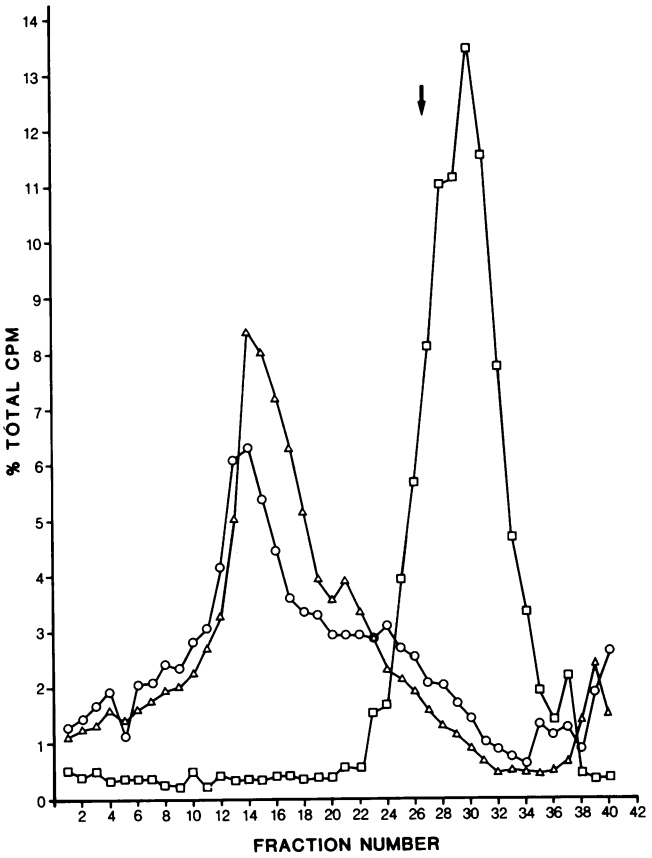

FIG. 7. Alkaline sucrose gradient profiles of DNA from strain AQ624 cells. Early exponential-phase cells in minimal medium were labeled by a 90-min incubation with [^{3}H]thymine (10 μCi; 10 μg/ml). Portions of the labeled culture were then untreated (△), treated with 10 mM H_2O_2 for 1 h (□), or treated with 0.5 mM plumbagin for 1 h (○). Labeled cells were collected by centrifugation and then suspended in 200 μl of lysis buffer for 30 min at room temperature before being loaded on a 5 to 20% alkaline sucrose gradient. A 10-μl sample of ^{14}C-labeled lambda phage was loaded just before centrifugation to allow correct alignment of gradient profiles. The arrow indicates the position of the λ ^{14}C-DNA peak. Centrifugation was in an SW 50.1 rotor at 30,000 rpm (105,000 × *g*) for 2 h at 20°C. Total counts: 24,144 cpm for untreated, 16,651 cpm for H_2O_2-treated, and 34,497 cpm for plumbagin-treated cells.

These observations suggest that paraquat and plumbagin may not share a simple, common mode of toxicity.

Several intracellular chemical reactions have been demonstrated or proposed that suggest the potential for interconversion of active-oxygen species. For example, the presence of SOD, by virtue of its enzymatic activity, implies the generation of H_2O_2 when O_2^- is present. Furthermore, evidence has been presented that in the presence of certain metal ions, the Haber-Weiss reaction results in the formation of $OH^·$ from O_2^- and H_2O_2 (12–14). This might be construed to imply that intracellular damage caused by the introduction of one of these active-oxygen species may be similar or identical to that caused by another. In light of evidence from the present work, as well as evidence from other sources cited above, however, it is now quite clear that damage from active states of oxygen should not be viewed as a single class of events. Rather, different oxidative agents produce a heterologous group of specific types of damage which, in turn, appear to induce specific repair systems.

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