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Photoactivated DNA Nicking, Enzyme Inactivation, and Bacterial Inhibition by Sesquiterpenoid Phytoalexins from Cotton

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When irradiated by the sun or by cool-white fluorescent lamps (300–700 nm), the phytoalexins 2,7-dihydroxycadalene (DHC) and lacinilene C induced single-strand breaks in plasmid pBR322 DNA. UV radiation of wavelengths 239 nm and 300 nm (near the absorbance maxima of DHC) was more effective than radiation of wavelengths 400, 500, and 600 nm in activating DHC to nick DNA. Oxygen was required for full DNA-nicking. Scavengers of reactive oxygen species and of free radicals inhibited the nicking of plasmid by DHC plus radiation. Single-strand breaking of double-stranded DNA by DHC plus radiation was neither nucleotide sequence specific nor base specific as revealed in a DNA sequencing gel. Some preferential cleavage near guanine residues was observed when photoactivated DHC acted on single-stranded DNA. Catalytic activities of deoxyribonuclease I and malate dehydrogenase were greatly reduced after incubation with DHC plus radiation. In the dark, 0.1 mM DHC only partially inhibited multiplication of *Xanthomonas campestris* pv. *malvacearum*, but when irradiated, this concentration of DHC was bactericidal. Radiation alone was only bacteriostatic. It was concluded that DHC is a photosensitizer.

The sesquiterpenoid phytoalexins 2,7-dihydroxycadalene (DHC), lacinilene C (LC), and lacinilene C 7-methyl ether (LCME) accumulate in leaves of upland cotton (*Gossypium hirsutum* L.) after inoculation with incompatible races of *Xanthomonas campestris* pv. *malvacearum* (Smith 1901) Dye 1978b (Essenberg *et al.* 1982; Essenberg *et al.* 1985). Of the three phytoalexins, DHC is the most inhibitory toward growth of this pathogen. LC can be formed from DHC spontaneously by air oxidation (Essenberg *et al.* 1982). After inoculation of cotton leaves, yellow-green fluorescence that is spectrally similar to the fluorescence of LC develops in the mesophyll cells closest to colonies of *X. c.* pv. *malvacearum* (Essenberg *et al.* 1985). Chemical analysis of cells isolated from inoculated leaves and separated by fluorescence-activated sorting showed that DHC, LC, and LCME are localized in the fluorescent cells (Pierce and Essenberg 1987). Local concentrations of the phytoalexins in these cells have been estimated by quantitative extraction and fluorescence microscopy. At the time that bacterial multiplication stopped in leaves of highly resistant cotton lines, DHC, LC, and LCME reached concentrations that, when tested *in vitro*, completely inhibited growth of *X. c.* pv. *malvacearum* (Essenberg *et al.* 1985).

These studies provided evidence that the phytoalexins contribute to bacterial blight resistance of cotton by inhibiting growth of the pathogen. However, nothing was known about the mechanism of that growth inhibition. Because DHC, LC, and LCME are susceptible to photooxidation, previous *in vitro* bioassays of their antibacterial activities were performed in the dark. However, 300–700 nm radiation was recently shown to be required for inactivation by DHC of the icosohedral virus, cauliflower mosaic virus (CaMV) (Sun *et al.* 1988). This inactivation requires the presence of coat protein and appears to be due to the cross-linking of viral DNA and coat protein. Because solar radiation is present in the environment in which these phytoalexins are produced and play their role in disease resistance, it seemed important to study further how near-UV and visible radiation affect the phytoalexins' biological activities. We report here that under 300–700 nm radiation, DHC induces single-strand breaks in DNA, inactivates enzymes, and has enhanced antibacterial activity. LC also is photoactivated to nick DNA.

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MATERIALS AND METHODS

DHC, LC, and plasmid DNA. DHC and LC were isolated from inoculated cotton plants as previously described (Morgham *et al.* 1988). Chemically synthesized DHC (estimated purity 98%) was a gift of R. D. Stipanovic. DHC and LC were stored and handled as previously described (Sun *et al.* 1988). The molar absorptivity of DHC at 313 nm was determined as previously reported for other wavelengths (Essenberg *et al.* 1982). *Escherichia coli* HB101 (pBR322) was cultured overnight in 1 liter of Luria-Bertani broth (Maniatis *et al.* 1982) in a 2-liter flask on a reciprocal shaker operating at 200 strokes per minute at 37° C. Plasmid pBR322 DNA was isolated by the alkaline lysis method (Maniatis *et al.* 1982). Plasmid DNA was purified by NACS-37 chromatography, following the instructions of the manufacturer, Bethesda Research Laboratories. The preparation, concentrated by ultrafiltration with a Centricon 30 membrane filter (Amicon) to 0.2 μg of DNA per microliter and stored at 4° C, contained both closed and open circular forms of the plasmid, and the proportion of the latter increased with storage time.

End-labeled DNA. A double-stranded *Xho*I-*Pvu*II DNA fragment from CaMV DNA-containing plasmid pCa6

(Lebeurier *et al.* 1982) was labeled at its *Xho*I 3′ end by using reverse transcriptase and α^{32}P-dCTP (New England Nuclear) (Smith and Calvo 1980). The resulting 162-bp fragment encompassed nucleotides 1643 (labeled end) to 3990 in the sequence of CaMV (Franck *et al.* 1980) with nucleotides from 1685 through 3870 deleted, and was purified by nondenaturing PAGE (Maxam and Gilbert 1980). A 3′ end-labeled single-stranded DNA fragment (CaMV nucleotides 1927 [labeled end] to 2152) was obtained from pUM130 (Melcher *et al.* 1986) by digestion with *Bam*HI followed by end-labeling and purified by denaturing gel electrophoresis.

Strains of *X. c.* pv. *malvacearum*. The race 1 strain was a spontaneous streptomycin-resistant mutant obtained by L. A. Brinkerhoff from a California field isolate of race 1, which was given to him by W. C. Schnathorst. Strain EMB7, obtained from strain RS4, a very DHC-sensitive mutant of our Oklahoma race 3 field isolate (Essenberg *et al.* 1982), was a spontaneous mutant selected for resistance to light on eosin-methylene blue agar (EMB agar from Difco). Stock cultures were stored in nutrient broth (Difco) containing 16% glycerol at −70° C.

Treatment of DNA with phytoalexins plus radiation. Plasmid pBR322 DNA, 0.2 μg in 1 μl of TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 7.2), was transferred into 0.5-ml conical polypropylene tubes containing sufficient dry phytoalexin to yield 0.7 mM DHC or 1.7 mM LC solutions. Molar ratios of phytoalexin to DNA base pairs were 2.2 for DHC and 5.2 for LC. The tubes were closed, laid on their sides, and suspended 24 cm below the fluorescent lamps in a Conviron E15 plant growth chamber, equipped with cool-white fluorescent and 60-watt incandescent lamps, for 3 hr. Photosynthetic photon flux density at this location, determined with a LI-COR quantum sensor, was 6.3×10^2 μmol/m^2 sec, and air temperature was 30° C. Nonirradiated control tubes were wrapped in aluminum foil and placed in the same location. Where specified, the temperature of the liquid in the tubes was measured with an Omega thermocouple probe inserted through a small hole in the cap. In one experiment, tubes were exposed to solar radiation rather than to 300–700 nm radiation from fluorescent and incandescent lamps. At the end of all radiation treatments, tubes were inspected visually to check that the 1 or 2 μl of water had not evaporated. Where specified, the double-stranded DNA in treated samples was denatured by immersion in boiling water for 1 min or by the addition of 1 μl of 1 N KOH before the addition of loading buffer. TE buffer (15 μl) and 5 μl of Type IV loading buffer (Maniatis *et al.* 1982) were added to each tube of treated plasmid DNA before electrophoresis in 0.8% agarose gels in E buffer (Loening 1969) at 4.1 V/cm for 90 min. Panatomic X (Kodak) negatives of EtdBr-stained gels were scanned with a Helena Laboratories Auto Scanner, using its visible wavelength lamp and 525-nm filter. Relative amounts of DNA of different forms were estimated by comparing their peak heights. Each of the plasmid irradiation experiments was performed two or three times.

End-labeled CaMV DNA fragments (5–20 ng in 3.5–7 μl of water) were subjected to sequencing reactions (Maxam and Gilbert 1980) or to 3-hr exposure to 0.7 mM DHC plus radiation as described above. Solutions of double-stranded DNA (0.1 μg) or single-stranded DNA (0.05 μg) in 35 μl of water were divided as follows: 3.5 μl each for Cyt-specific and Gua-specific reactions, 7 μl each for Cyt plus Thy-specific and Gua plus Ade-specific reactions, and 5 μl for DHC plus radiation and radiation alone. The products were analyzed on 8% polyacrylamide sequencing gels (Maxam and Gilbert 1980). These experiments with end-labeled DNA were performed three times with double-stranded DNA and twice with single-stranded DNA.

Irradiation with specific wavelengths. A piece of Styrofoam was cut to fit the cuvette holder of an Aminco-Bowman fluorescence spectrophotometer. An indentation was made that would secure the lower portion of a cut-off 0.5-ml conical polypropylene tube, containing 2 μl of plasmid DNA solution, with or without DHC, facing the light source. The cuvette chamber was maintained at 15° C with a circulating water bath during exposure of samples to monochromatic radiation from the instrument's xenon lamp.

Emission spectrum of cool-white fluorescent lamps. A Perkin-Elmer model 650-40 fluorescence spectrophotometer was employed. Its xenon lamp was not turned on; instead, the sample chamber door was opened to admit radiation from fluorescent lamps. A flat, front-surface mirror placed in the sample chamber reflected the lamp radiation through the slit to the emission monochrometer, which was varied from 210–800 nm. Spectra were also recorded with a Schott UG-1 or an Ansco UV-absorbing filter covering the slit to control for stray light effects.

Effects of oxygen removal and of reactive-oxygen and free-radical scavengers on DNA cleavage. One μl aliquots of aqueous scavenger solutions were introduced into the 0.5-ml conical tubes containing 1 μl of plasmid DNA solution, with or without DHC. Where specified, the oxygen concentration was decreased by flushing the sample-containing conical tubes with argon in an argon-filled chamber for 2 min before irradiation. The samples were irradiated for 3 hr in the growth chamber and subjected to electrophoresis as described above.

Treatment of enzymes with DHC plus radiation. Bovine pancreatic deoxyribonuclease I (deoxyribonucleate 5′-oligonucleotidohydrolase E.C.3.1.4.5 [DNase I]) from Worthington, 0.11 ng in 1 μl of 10 mM Tris·HCl, pH 7.6, containing 5 mM $MgCl_2$ and 0.10 mg per milliliter of bovine serum albumin, with or without 0.7 mM DHC, was irradiated in the growth chamber for 3 hr as above. The remaining enzymic activity was assayed by the addition, under low laboratory light intensity, of 7.5 μl of water and 1.5 μl of pBR322 plasmid DNA (0.15 μl) in TE buffer. After incubation for 4 min at room temperature, the enzymic reaction was stopped by immersing the tubes in boiling water for 1 min. The samples were then electrophoresed in 1.1% agarose gels and analyzed as above.

Bovine heart malate dehydrogenase (L-malate: NAD^+ oxidoreductase E.C.1.1.1.37 [MDH]) from Sigma, 62.5 ng in 1 μl of 0.10 M potassium phosphate, pH 7.5, with or without 0.025 mM DHC, was irradiated in the growth chamber for 1.5 hr. Remaining enzymic activity was assayed by diluting the sample to 1.0 ml with the same phosphate buffer containing 0.2 mM NADH and 0.05 mM oxaloacetic acid, monitoring the reaction by continuous observation of absorbance at 340 nm. One unit of enzyme activity caused the oxidation of 1 μmole of NADH per minute at 25° C, assuming a molar absorptivity of 6.22×10^3 M^{-1} cm^{-1} for NADH.

Antibacterial activity of DHC and radiation. Twenty microliters of a logarithmically growing bacterial culture in Difco nutrient broth (2×10^7 colony-forming units [cfu] per milliliter) was introduced aseptically into a sterile 1.5-ml conical tube containing dry DHC and vortex-mixed to dissolve the DHC. For each treatment, 10 replicate tubes were prepared, two of which were used for each time point. The tubes, some wrapped in aluminum foil as dark controls, were incubated in a jacketed water bath, with the temperature in the 25–33° C range, and rotated at 180 rpm during irradiation in the growth chamber as above for 2, 4, 6, or 8 hr. Concentrations of viable bacteria were determined by diluting the entire contents of a culture tube appropriately in sterile, saturated calcium carbonate solution (Essenberg *et al.* 1979) and plating on Difco nutrient agar. Variability of this method was estimated by transforming the duplicate determinations to log (cfu/ml) and calculating a pooled estimate of variance of the logarithms.

RESULTS

Induction of single-strand breaks in plasmid DNA by phytoalexins plus radiation. Our preparation of pBR322 DNA contained both closed circular and open circular forms of the plasmid (Fig. 1, lane 5). Incubation of the plasmid with phytoalexins DHC or LC in the dark (lanes 1 and 9) or exposure of plasmid to 300–700 nm radiation in the absence of phytoalexin (lane 7) did not cause noticeable conversion of closed to open circles. However, exposure of plasmid DNA to 300–700 nm radiation in the presence of DHC (lane 3) or LC (lane 11) led to conversion of closed to open circles, indicating induction of single-strand breaks in the DNA. Solar radiation plus DHC also converted closed to open circles, and the conversion was time-dependent (Fig. 2). An increase in the double-stranded linear form was occasionally observed (Fig. 2). Sometimes DNA products remained at the origin (Fig. 1, lane 11), possibly due to cross-linking.

Heat denaturation, which did not affect the electrophoretic mobility of closed circles, converted open circles to faster-migrating single-stranded linear and circular forms, which comigrated (Fig. 1, compare lanes 5 and 6). Pretreatment of the plasmid with DHC or LC in the dark did not alter the pattern of migration of heat-denatured plasmid (compare lanes 2 and 10 with lane 6). Irradiation alone of plasmid DNA resulted in the production of some smaller DNA fragments revealed after heat denaturation (lane 8). However, irradiation of plasmid DNA in the presence of DHC or LC, followed by heat denaturation, revealed extensive conversion of closed circles to small fragments (lanes 4 and 12). The fragments resulting from DHC plus radiation (lane 4) were, on average, smaller than those from LC plus irradiation (lane 12). Thus, most of the undenatured open circular forms in lanes 3 and 11 that resulted from irradiation in the presence of DHC or LC contained more than one single-strand break, but were held in the open circular form by base pairing.

Heat may have made a minor contribution to the observed nicking. The temperature of 1 and 2 μl volumes rose gradually from 25° C to 39° C and 42° C, respectively, during 3 hr of irradiation in the growth chamber. Incubation of DNA and DHC in darkness at 40° C and 50° C for 2 hr resulted in 10 and 14% conversions of closed to open circles,

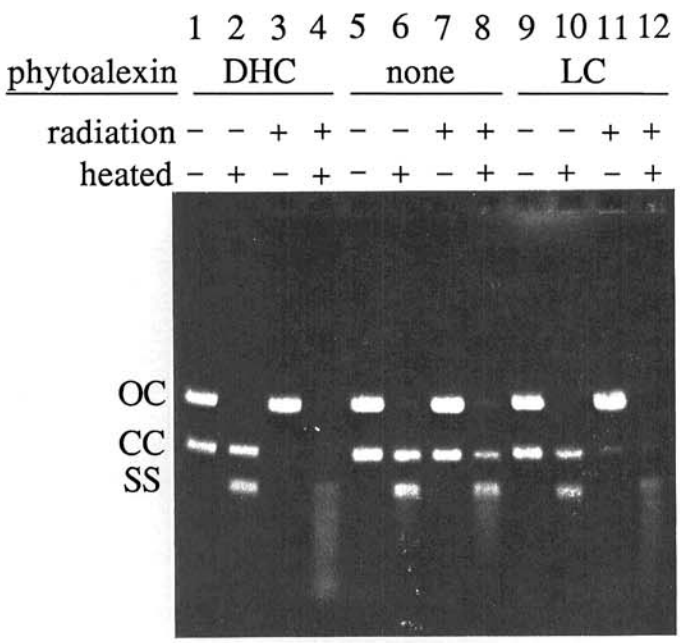

Fig. 1. Phytoalexin- and 300–700 nm radiation-dependence of DNA nicking. Duplicate samples of plasmid pBR322 DNA (0.2 μg in 1 μl of TE buffer [10 mM Tris·HCl, 1 mM EDTA; pH 7.2]) were treated with 0.7 mM 2,7-dihydroxycadalene (DHC) or 1.7 mM lacinilene C (LC) for 3 hr in the dark or under growth chamber radiation as described in text. For each treatment, a duplicate sample was heat-denatured before agarose gel electrophoresis. Forms of plasmid DNA are abbreviated: CC, closed circular; OC, open circular; SS, single-stranded (both linear and circular). The double-stranded linear form (L) was not resolved from OC in this gel.

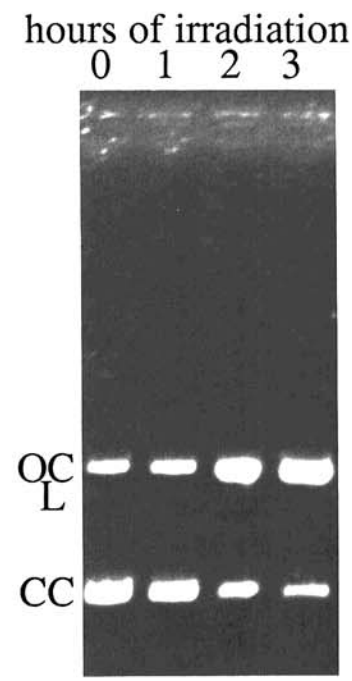

Fig. 2. Time-dependence of DNA nicking by solar radiation plus DHC. Plasmid pBR322 DNA (0.2 μg in 1μl of TE buffer) was treated for the indicated times with 0.7 mM DHC and solar radiation before agarose gel electrophoresis.

respectively. Conversions in the absence of DHC were 0 and 3% at 40° C and 50° C, respectively. However, after 2 hr of irradiation of a DNA-DHC mixture in the growth chamber without additional heating, a 33% conversion of closed to open circles was found. Only at 70° C was there as much loss of closed circles in the dark, and unlike the radiation-induced nicking, the DNA degradation at 70° C was not DHC dependent and produced, in addition to open circles, greater amounts of a high molecular weight form that may have been cross-linked multimers. Therefore, most of the DNA nicking shown in Figure 1, lanes 3, 4, 11, and 12, can be ascribed to the effects of phytoalexin plus radiation, rather than phytoalexin plus heat.

Wavelength specificity for photoactivated DNA nicking. To test further whether the enhanced DNA nicking was due to photoactivation of DHC, mixtures of DHC and plasmid DNA were exposed to monochromatic radiation (239, 300 nm) near the absorbance maxima of DHC (237, 292-304 [broad peak], and 333 nm [Essenberg *et al.* 1982]). Closed circles were converted to open circles (Table 1). Radiation of wavelengths not absorbed by DHC (400, 500, and 600 nm) gave substantially less conversion (Table 1), even though the xenon lamp's photon output was one to three orders of magnitude greater at those wavelengths than at 239 and 300 nm. The cool-white fluorescent and incandescent lamps, used as source of radiation for all experiments except those of Figure 2 and Table 1, emit principally visible radiation that is not absorbed by DHC or DNA. However, cool-white fluorescent lamps do exhibit emission maxima in the near UV range at 313, 334, and 365 nm (Mohan *et al.* 1980). It was of interest to know whether these lamps emit wavelengths that could be absorbed by and thus might photoactivate DHC or DNA or proteins. We confirmed the published emission spectrum of cool-white fluorescent lamps (Mohan *et al.* 1980) and verified that they emit no radiation between 210 nm and the 313 nm mercury vapor emission band by scanning the lamps' emission spectrum with a fluorescence spectrophotometer (data not shown). An apparent emission of uniform, weak intensity over the range of 210–350 nm was shown to be a stray light effect of the more intense visible wavelengths, because it was unaffected by an Ansco UV-absorbing filter and was eliminated by a Schott UG1 filter, which absorbs most visible radiation (Schott Optical Glass, Inc., Duryea, PA). DHC absorbs 313 nm radiation with a molar absorptivity of 4×10^3 M^{-1} cm^{-1}.

Table 1. Wavelength specificity for photoactivated DNA nicking[a]

Wavelength (nm)	Relative photon flux from xenon lamp[b]	Percent of CC nicked[c]			
		Experiment 1		Experiment 2	
		No DHC	0.7 mM DHC	No DHC	0.7 mM DHC
none	0.000	0	0	0	ND
239	0.001	28	48	20	40
300	0.012	0	41	23	60
400	0.16	ND	ND	2	0
500	0.45	ND	ND	0	9
600	1.00	ND	ND	8	17

[a] pBR322 plasmid DNA (0.4 μg in 2 μl of TE buffer) was exposed for 1 hr to monochromatic radiation at 15° C in a spectrofluorometer as described in the text. ND = not determined.
[b] Computed from Figure 3A of Parker and Rees (1960).
[c] Decrease in intensity of the CC band relative to that of untreated plasmid.

Inhibition of DNA cleavage by removal of oxygen and by scavengers of reactive-oxygen species and free radicals. Because photoactivated degradations often involve molecular oxygen (Spikes 1977; Towers 1980), the effect of oxygen depletion was tested. Argon-flushed samples retained 20% and 15% of the initial closed circles after 3-hr exposure of DNA to 300–700 nm radiation plus synthetic or plant-derived DHC, respectively. The double-stranded linear form, which was initially 6% of the total DNA, only increased to 10%. In contrast, the corresponding air-equilibrated samples exhibited complete disappearance of closed circles, and the linear form increased to 28% and 23% of the total DNA after exposure to synthetic or plant-derived DHC, respectively.

All of the tested scavengers of reactive-oxygen species and free radicals partially protected closed circular plasmid from nicking by DHC plus radiation (Fig. 3). The closed circle bands in scavenger-treated samples were intermediate in intensity between those of untreated DNA (lanes 1 and 14) and of DNA treated with DHC plus radiation without scavengers (lane 11). Fewer low molecular weight DNA fragments were generated by DHC plus radiation when scavengers were present than in their absence.

Lack of base or sequence specificity in DNA cleavage. DHC-plus-radiation-induced strand breakage of a 3′ end-

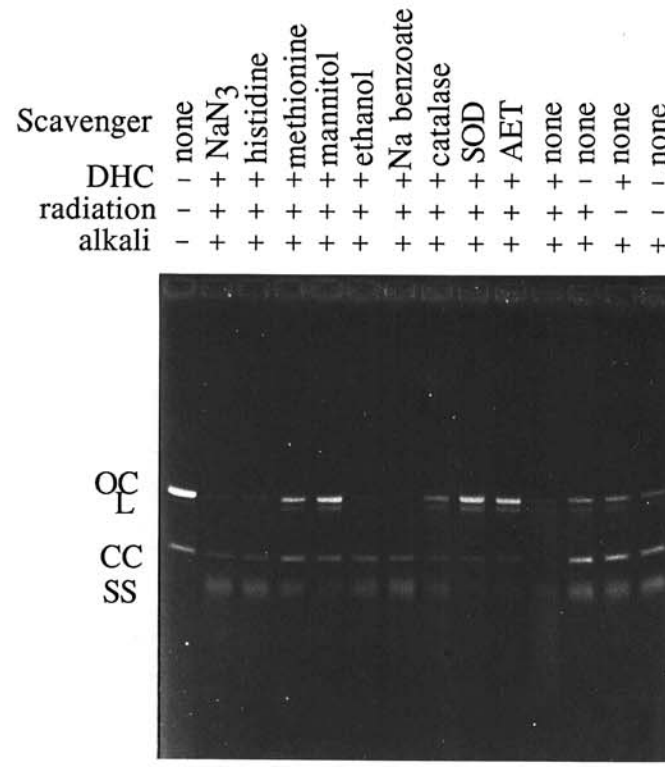

Fig. 3. Effects of scavengers of reactive-oxygen species and of free radicals on the nicking of plasmid DNA by DHC plus 300–700 nm radiation. Plasmid pBR322 DNA (0.2 μg in 1 μl of TE buffer) was treated with radiation as in Figure 1 in the presence of 0.7 mM DHC and reactive-oxygen scavengers as indicated. The concentrations of the scavengers were: 12.3 mM NaN_3, 67 mM L-histidine, 24.4 mM L-methionine, 0.22 M D-mannitol, 6 mM ethanol, 12.1 mM S-2-aminoethylisothiouronium bromide hydrobromide (AET) (Sigma), 69.4 mM sodium benzoate, 31 units per milliliter of superoxide dismutase (SOD) (Sigma), and 185 units per milliliter of catalase (Sigma). All samples except that in lane 1 were subjected to alkali denaturation before electrophoresis. Denaturation of OC was incomplete in some samples.

labeled double-stranded DNA fragment produced bands of approximately equal intensity at each nucleotide position of a nucleotide sequencing gel (Fig. 4A, lane + +). As expected, no bands were detected in the lane containing the untreated fragment. Thus, each nucleotide position of the labeled strand was equally susceptible to cleavage by DHC plus radiation. The products obtained from the corresponding reactions with a single-stranded DNA fragment labeled at its 3′ end are shown in Figure 4B (lane + +). Some of the regularly spaced bands on the autoradiograph were of significantly higher intensity than other bands. The positions of darker bands did not correlate with areas of possible secondary structure. The darker bands appeared at positions consistent with cleavage at guanylate residues and one to three positions in the 5′ direction from guanylate residues.

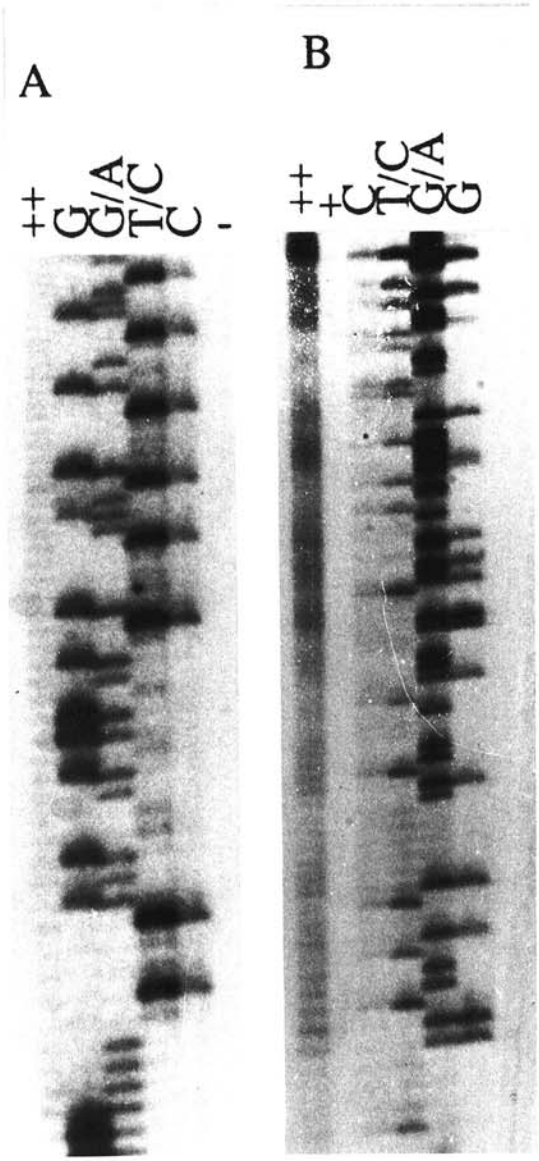

Fig. 4. DHC and 300–700 nm radiation-induced cleavage of 3′-end-labeled double-stranded **(A)** and single-stranded **(B)** CaMV DNA fragments analyzed on 8% polyacrylamide nucleotide-sequencing gels. Solutions of double-stranded DNA (2.8 ng per microliter) or single-stranded DNA (1.4 ng per microliter) were subjected to one of the following treatments: G, G/A, T/C, C Maxam and Gilbert sequencing reactions; + +, 0.7 mM DHC and radiation as in Figure 1 for 3 hr; +, radiation only for 3 hr; −, no treatment.

Enzyme inactivation by DHC plus radiation. To test whether DHC plus radiation can cause modification of molecules other than DNA, two enzymes were subjected to the treatment. Active DNase I cleaves intact plasmid DNA (Fig. 5, lane 2) to small fragments (lane 3). Preincubation of DNase I with radiation alone for 3 hr (lane 5) had no apparent effect on this activity. When the enzyme was preincubated with DHC plus radiation, the activity toward plasmid DNA was greatly reduced (lane 6). When the enzyme was preincubated for 3 hr with DHC in the dark, DNase I activity was slightly reduced; 5% of closed circular DNA remained after 4-min incubation of the plasmid with treated DNase I (lane 4). However, the addition of DHC only during the 4-min incubation gave the same degree of interference, 5% of closed circles remaining (lane 1). The similar patterns in lanes 1 and 4 suggest that the interference by DHC in the dark may have been through interaction with the substrate DNA, perhaps by intercalation into the double helix, rather than through interaction with the enzyme.

Inactivation of MDH by DHC plus radiation was also observed (Table 2). Preincubation of MDH with DHC plus

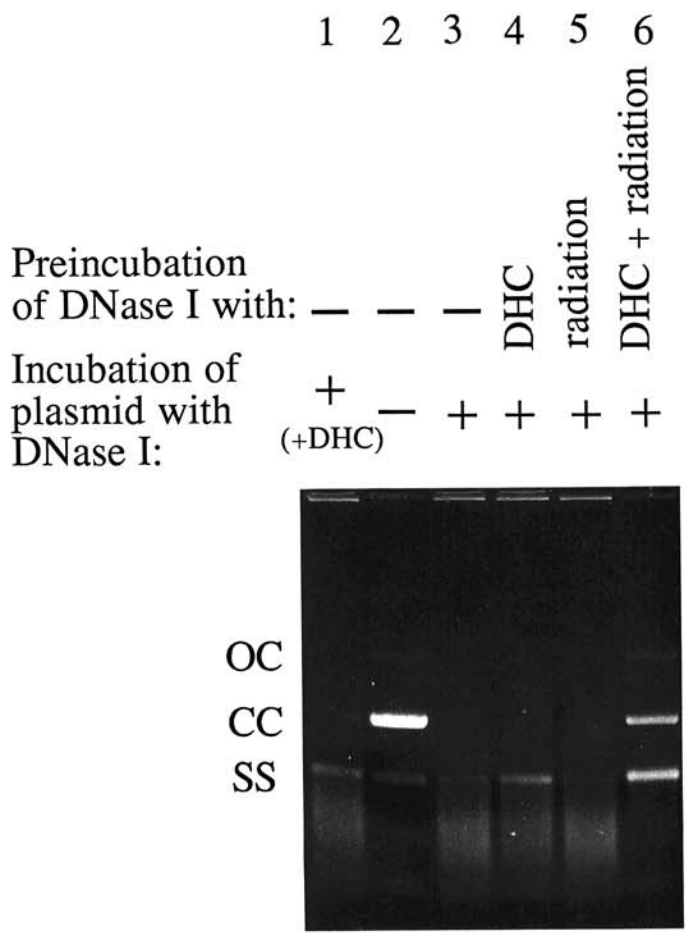

Fig. 5. Effects of DHC and 300–700 nm radiation on plasmid-cleaving activity of DNase I. DNase I (0.11 ng per microliter) was preincubated for 3 hr with 0.7 mM DHC and/or radiation as indicated. Enzymic activity of treated and untreated DNase I was assayed by 4-min incubation with pBR322 plasmid DNA. DHC (0.7 nmole) was added to the previously untreated DNase I of lane 1 at the beginning of the 4-min assay. The enzymic reaction was stopped and the DNA denatured by heating the samples at 100° C for 1 min before agarose gel electrophoresis. DNA untreated except for heat-denaturation was included (lane 2) for comparison.

radiation resulted in loss of about half of the enzymic activity; whereas preincubation with either DHC or radiation alone had nonsignificant effects on enzymic activity. MDH assayed in the presence of 1.0 μM DHC had 97 $\pm$ 5% the activity of MDH alone. Thus the effect of preincubation with DHC plus radiation was inactivation resulting from simultaneous exposure to DHC and radiation, rather than inhibition by the 0.025 μM DHC remaining after the 1,000-fold dilution that was made for the MDH assay.

Effect of radiation on the antibacterial activity of DHC. In the dark, 0.1 mM DHC partially inhibited growth of *X. c.* pv. *malvacearum* race 1 (Fig. 6A), and 0.05 mM and 0.1 mM DHC only weakly inhibited strain EMB7 (Fig. 6B). Radiation alone partially inhibited growth of race 1 (Fig. 6A) and was bacteriostatic to strain EMB7 (Fig. 6B). Radiation plus 0.05 mM DHC was not significantly more inhibitory to EMB7 than radiation alone. However, radiation plus 0.1 mM DHC was bactericidal to both strains, indicating that radiation significantly enhanced the antibacterial activity of this phytoalexin.

DISCUSSION

Photoactivation of DHC, DNA or protein? A major finding of this and related studies (Sun 1987; Sun *et al.* 1988) is that only in the presence of radiation does the phytoalexin DHC become a potent agent *in vitro* in the degradation of DNA and the inactivation of enzymes and CaMV. We conclude from our observations that these reactions are a consequence of the photoactivation of DHC. Although small DNA-nicking effects of DHC at elevated temperatures without radiation or of radiation alone were noted, considerably enhanced nicking of DNA occurred when it and DHC were exposed to radiation of wavelengths absorbed by DHC. The difference between Figures 1 and 2 in the extent of conversion of closed to open circular form of the plasmid may be due to the different wavelength distributions of cool-white fluorescent lamp emission (Mohan *et al.* 1980) and of solar radiation (Seliger and McElroy 1965). The possibility that DNA, rather than DHC, absorbed the photons that promoted the DHC-dependent DNA single-strand breakage is unlikely, in view of the photoactivating effect of cool-white fluorescent lamps (Fig. 1), which emit no UV radiation of a wavelength shorter than the 313-nm mercury vapor emission band. Radiation of this wavelength is absorbed well by DHC (molar absorptivity = 4×10^3 M^{-1}

Table 2. Inactivation of MDH by DHC plus 300–700 nm radiation[a]

Treatment	MDH activity (% of dark control)	
	Experiment 1	Experiment 2
Dark control	100 a	100 a
Radiation	91 a	96 a
0.025 mM DHC	88 a	96 a
0.025 mM DHC + radiation	41 b	53 b

[a] Malate dehydrogenase (MDH, 62.5 ng/μl) was preincubated for 1.5 hr with 0.025 mM DHC and/or radiation as indicated and then diluted and assayed for enzymic activity as described in the text. The dark controls exhibited 0.025 units/ml of MDH activity in experiment 1 and 0.040 units/ml in experiment 2. Values are means of five and six replicates for experiments 1 and 2, respectively. Values followed by the same letter within the same experiment are not significantly different at $P = 0.01$ by Duncan's multiple-range test.

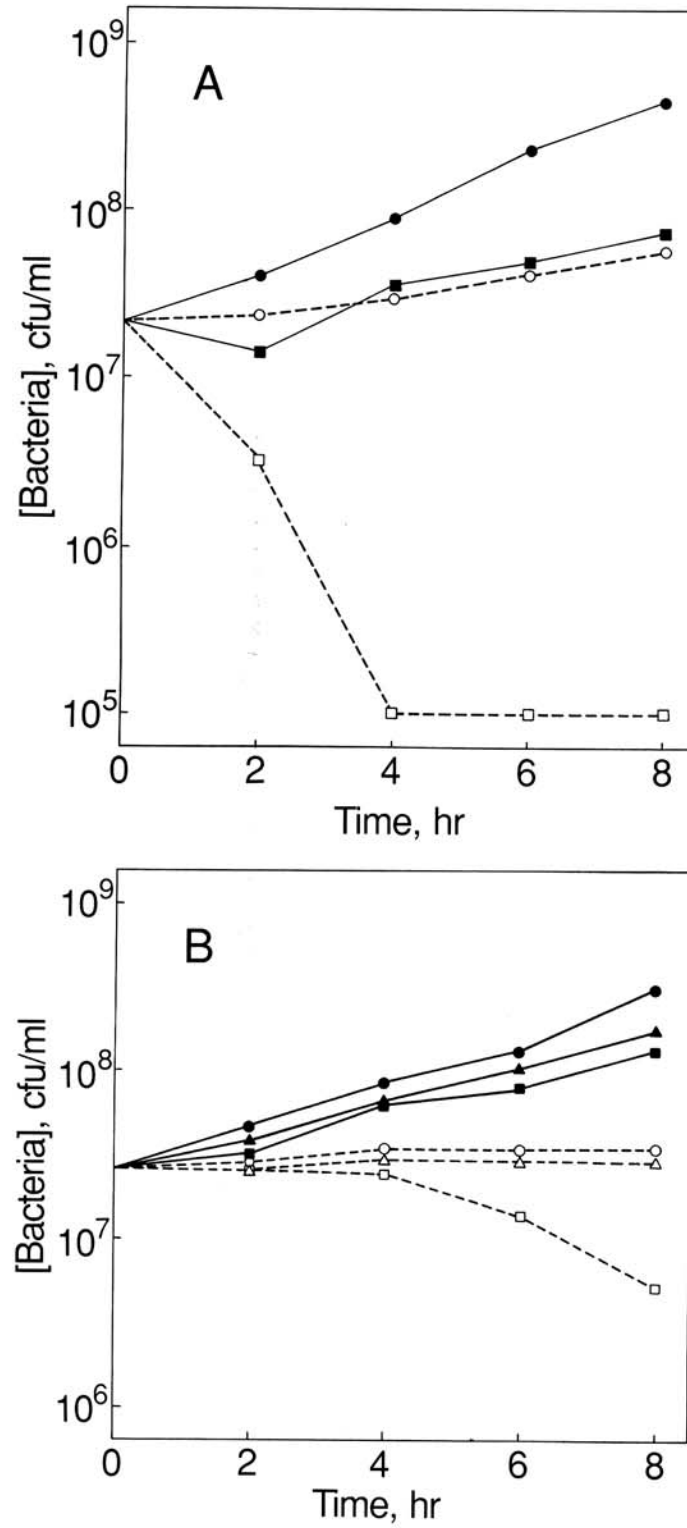

Fig. 6. Effects of DHC and 300–700 nm radiation on multiplication of race 1 **(A)** and strain EMB7 **(B)** of *X. c.* pv. *malvacearum* in liquid culture. Closed symbols, dark; open symbols, irradiated. •, ○, no DHC; ▲, △, 0.05 mM DHC; ■, □, 0.10 mM DHC. Each point is the average of duplicate determinations. The pooled estimates of standard deviation of log (cfu/ml) for **A** and **B** were SD = 0.078 and 0.047, respectively, which correspond to factors of 1.20 and 1.11 for values of cfu/ml in **A** and **B**, respectively.

cm^{-1}), but not by nucleic acids at neutral pH (Beaven *et al.* 1955). Similarly, the radiation-promoted inactivation of enzymes (Fig. 5 and Table 2) was probably due to photoactivation of DHC rather than of protein, because at neutral pH tryptophan absorbs 313 nm radiation only very weakly (molar absorptivity $< 20\ M^{-1}\ cm^{-1}$), and the other common amino acids are transparent to this wavelength (Wetlaufer 1962). Not excluded by our observations, however, are the possibilities that the formation of complexes with DHC shifted the absorbance profiles of DNA and/or protein to include wavelengths emitted by the lamps.

DNA breaks induced by gossypol. Gossypol, a potential male antifertility drug with the same cadalane carbon skeleton as DHC and LC and also produced by cotton, promoted formation of oxygen radicals in rat liver microsomes and in human sperm (de Peyster *et al.* 1984) and caused DNA strand breaks in human leukocytes (Chen *et al.* 1986). Gossypol-dose-dependent degradation of plasmid DNA *in vitro* was observed in the presence of excess 2-mercaptoethanol, oxygen, and Fe(III) or Co(II) (Srivastava and Padmanaban 1987). However, unlike the observations reported here, the reactions observed in the gossypol studies probably resulted from radiation-independent initiation of free-radical reactions. In the presence of Fe(III) and oxygen, DHC also promoted radiation-independent degradation of DNA (Sun 1987).

Low specificity of DNA strand breakage. Photoactivated DHC caused single-strand breaks in double-stranded DNA (Fig. 2). Supercoiled circular plasmid was converted primarily to the relaxed open circular form. The smaller amounts of linear double-stranded DNA sometimes observed (Fig. 2) probably resulted from the chance proximity of a single-strand break near a break, either preexisting or newly formed, in the opposite strand. Supercoiled circular, relaxed circular, double-stranded linear, and single-stranded linear DNAs were all substrates for nicking by photoactivated DHC. The heterogeneous distribution of lower molecular weight single strands in samples of plasmid DNA nicked by photoactivated DHC and denatured before electrophoresis (Fig. 1) demonstrated that nicks occurred at random distances from one another. At the nucleotide level of resolution, the nicking of double-stranded DNA occurred with similar frequencies at all nucleotides (Fig. 4A). The low base specificity observed for the nicking of double-stranded DNA by photoactivated DHC is consistent with a role for the highly reactive and nonselective hydroxyl radical (Kawanishi *et al.* 1986).

With single-stranded DNA, on the other hand, the observed preference for cleavage in the vicinity of guanylate residues (Fig. 4B) is reminiscent of the guanine specificity of certain other DNA-damaging agents (Kawanishi *et al.* 1986; Cadet *et al.* 1983; Cullis *et al.* 1987), some of which act by producing singlet oxygen that reacts preferentially with guanines (Kawanishi *et al.* 1986). Singlet oxygen seems to play a role in DNA strand breakage by the photodynamic dye rose bengal (Nieuwint *et al.* 1985). Evidence suggests that the produced guanine radicals induce cleavage of deoxyriboses and thus cleavage of the deoxyribose-phosphate backbone in their vicinity (Cullis *et al.* 1987). The failure to observe any nucleotide specificity in photoactivated DHC nicking of double-stranded DNA may be due to a lesser importance of singlet oxygen in nicking double-stranded DNA, in which guanines are interior to the double helix, than in breaking single-stranded DNA. Alternatively, the presence of guanines on both strands may obscure a preference for guanines.

Involvement of oxygen and free radicals in DNA nicking. The inhibitory effects of scavengers of reactive-oxygen species and free radicals (Fig. 3) and of oxygen removal on DNA strand cleavage by photoactivated DHC indicate roles for oxygen and free radicals in this cleavage. The photoactivated DHC-mediated nicking of DNA that occurred after flushing with argon may have been due to oxygen-independent free-radical reactions similar to those seen with some photosensitizers (Spikes 1977), or to remaining traces of oxygen. The scavengers used in this study are not specific enough to identify the reactive species involved in DNA cleavage. Azide, histidine, methionine, mannitol, ethanol, and benzoate react rapidly with both the hydroxyl radical and singlet oxygen (Elstner 1982; Singh 1981). Hydrogen peroxide, which is dismutated by catalase, is a potential source of hydroxyl radicals through the Fenton reaction (Cohen 1985). SOD catalyzes dismutation of superoxide, but also reacts rapidly with the hydroxyl radical and singlet oxygen (Singh 1981). AET is a radioprotectant thought to scavenge hydroxyl and hydroperoxyl radicals (Shapira *et al.* 1957) and other free radicals (Bakker *et al.* 1983).

More than one reactive-oxygen species are known to be involved in the photodynamic action of other compounds such as the fungal toxin cercosporin (Daub and Hangarter 1983). Spontaneous dismutation of superoxide can generate hydrogen peroxide, and traces of iron or copper ions can then catalyze formation of the hydroxyl radical through the Fenton reaction (Cohen 1985) and lead to DNA cleavage (Que *et al.* 1980; Brawn and Fridovich 1981). Imlay *et al.* (1988) have recently shown that the DNA-cleaving products of the Fenton reaction include, in addition to the free hydroxyl radical, a species that is not scavenged by ethanol or mannitol.

Enzyme inactivation. The characteristics of the DHC-mediated inhibition of MDH (Table 2) and DNase I (Fig. 5) are consistent with enzyme inactivation by photodynamic action. Photoactivation of isoflavonoid phytoalexins to inactivate glucose-6-phosphate dehydrogenase in an *in vitro* assay system has been reported (Bakker *et al.* 1983). Hydroxyl radical-generating systems have been observed to damage proteins (Davies 1987). The reactivity of singlet oxygen with histidine and methionine (Singh 1981) suggests that it may also damage proteins.

Photoactivated antibacterial activity and plant disease resistance. The more toxic effect of DHC toward *X. c.* pv. *malvacearum* under radiation (Fig. 6) is not surprising in view of the radiation-dependent *in vitro* activities of DHC. The nicking of DNA, the inactivation of enzymes, and the cross-linking of protein with DNA (Sun *et al.* 1988) could all contribute to the enhanced toxicity of DHC under radiation. The inhibition of bacterial growth by DHC in the dark may be due to radiation-independent initiation of free radicals in the presence of Fe(III) or Co(II), or to mechanisms other than those described here. Similar photoactivation of antimicrobial activity has been recently shown for thiophene phytoalexins of *Tagetes erecta* (Kourany *et al.* 1988).

The DNA-nicking activity of the phytoalexin DHC suggests a role for phytoalexins in the induction of some plant pathogenic functions. DNA-damaging agents

mitomycin C, nalidixic acid, and UV radiation induce pectin lyase and the bacteriocin carotovoricin in the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* (Zink *et al.* 1985), suggesting that their synthesis is controlled by the SOS response to damaged DNA. The *recA* product, which when activated by DNA damage is known to induce SOS functions in *Escherichia coli* (Walker 1984), was shown by transpositional inactivation to be required for pectin lyase and carotovoricin induction in *E. c.* subsp. *carotovora* after inoculation into potato tuber tissue and by mitomycin C *in vitro* (Zink *et al.* 1985). DNA-damaging agents that may induce bacterial pectin lyases have been found in plants (Tsuyumu *et al.* 1985). If photoactivated phytoalexins damage DNA *in planta*, then phytoalexins may induce pathogens to express pectin lyase and other pathogenicity-related genes via the SOS mechanism. In *E. coli*, the SOS response includes increased mutagenesis due to the enhancement of error-prone DNA repair (Walker 1984). The activity of photoactivated DHC and LC in nicking DNA raises the possibility that these phytoalexins may, through induction of the SOS response, also function as mutagens toward *X. c.* pv. *malvacearum in planta*.

The diversity of photoactivated reactions of DHC suggests that where it accumulates it may serve to protect the plant from virtually any invading microorganism. It is not surprising that the plant cells in which DHC and LC have been found to accumulate are dead or dying (Pierce and Essenberg 1987). They are the mesophyll cells closest to bacterial colonies in leaves of resistant plants (Essenberg *et al.* 1979). We do not yet know whether these hypersensitively necrotic cells are killed by phytoalexin accumulation or whether, killed by earlier events, they serve the plant as storage sites for these generally toxic defense compounds.

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