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Photosensitization of an Actinophage by Heteroanthracenes.* (31277)

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Several heteroanthracenes have been reported to photosensitize bacteriophages; for example, methylene blue(1), toluidine blue (2), and acridine(3). This is not surprising inasmuch as heteroanthracenes are known to participate in photo-oxidation of both proteins(4,5) and nucleic acids(6,7). However, the heteroanthracenes eosin and neutral red, and triphenyl methanes such as fuchsin and crystal violet have been reported as having no photodynamic action on bacteriophage(8). The present report is concerned with dyes which photoinactivate an actinophage for *Streptomyces venezuelae*. Of the 8 dyes found to be effective, 7 were heteroanthracenes. The conditions for photodynamic killing by different dyes were not the same.

Materials and methods. Actinophage MSP2 was propagated and enumerated on *Streptomyces venezuelae*(9); actinophage MSP2 was purified and concentrated by a combination of chromatography and differential centrifugation(10). The phage and dyes were prepared and diluted in 0.15 M NaCl, pH 7, unless otherwise indicated. Mixtures of phage and dye, 1 mm deep, were illuminated by 2 photoflood bulbs (General Electric BBA) placed 12 inches above the samples.

Results. Of the 27 diverse substances tested for power to inactivate actinophage MSP2, only crystal violet, hydroxylamine and methylene blue were markedly viricidal at the tested concentrations (Table I). Because acriflavine and methylene blue were known to kill bacteriophage photodynamically, 16 of

TABLE I. Effect of Diverse Chemicals on Actinophage MSP2.

Treatment	Conc (μg/ml)	Surviving phage
Acriflavine	75	1.5×10^9
2-amino,3-phenyl butanoic acid	500	1.7×10^9
Anthrone	75	1.7×10^9
Benzopurpurin	50	1.6×10^9
Brilliant cresyl blue	50	6.0×10^8
Brilliant vital red	50	1.3×10^9
Bromthymol blue	75	2.8×10^9
Carmine	75	2.0×10^9
Chlorophyll	75	2.8×10^9
Cholic acid	500	3.5×10^9
Colchicine	500	1.8×10^9
Crystal violet	500	9.5×10^6
Dianil blue 2R	50	1.4×10^9
Diphenylamine	500	2.9×10^9
Eosin Y	250	7.3×10^8
Fumaric acid	500	2.7×10^9
Hydroxylamine	500	1.2×10^6
Janus green B	250	6.5×10^8
Linoleic acid	500	2.1×10^9
2-mercaptobenzthiazole	500	2.6×10^9
Methylene blue	50	$<10^5$
Orcinol	500	2.7×10^9
Pronase	500	1.7×10^9
Quinine	500	2.9×10^9
Riboflavin	500	2.6×10^9
Safranin O	50	1.2×10^9
Thionin	50	8.0×10^8
None (control)	—	2.7×10^9

Chemicals were suspended or dissolved in 0.15 M NaCl and adjusted to pH 7 with 1 N NaOH or 1 N HCl. Phage was added and the mixture was incubated at 30°C for 30 min.

Acriflavine, Janus green B, Safranin O: Allied Chemical Corp., Nat. Analine Div., New York. Bromthymol blue: J. T. Baker Co., Phillipsburg, N. J. Cholic acid, fumaric acid, linoleic acid, pronase, riboflavin: Calbiochem, Los Angeles, Cal. Diphenylamine: Eastman Organic Chemicals, Rochester, N. Y. Anthrone, crystal violet, hydroxylamine hydrochloride, orcinol, quinine: Fisher Co., Minneapolis, Minn. Benzopurpurin 4B, brilliant cresyl blue, brilliant vital red, carmine, dianil blue 2R, methylene blue, thionin: Hartman-Leddon Corp., Philadelphia, Pa. Eosin Y: Matheson, Coleman and Bell, Cincinnati, Ohio. 2-amino,3-phenyl butanoic acid, chlorophyll, colchicine: Nutritional Biochemicals Corp., Cleveland, Ohio. 2-mercaptobenzthiazole: Sharp & Dohme, Rahway, N. J.

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TABLE II. Light Activated Viricidal Dyes.*

Dye	Conc (μg/ml)	Surviving phage	
		Dark	Light
Acriflavine	75	1.5×10^9	$<10^5$ *
Anthrone	75	1.7×10^9	1.1×10^9
Benzopurpurin	50	1.8×10^9	1.8×10^9
Brilliant vital red	50	1.8×10^9	1.5×10^9
Bromthymol blue	75	2.9×10^9	1.3×10^9
Carmine	75	2.1×10^9	1.5×10^9
Chlorophyll	75	1.8×10^9	1.1×10^9
Crystal violet	75	8.0×10^8	2.6×10^7*
Dianil blue 2R	75	2.6×10^9	7.5×10^8
Eosin Y	75	2.0×10^9	5.0×10^7*
Hydroxylamine	1	8.0×10^6	8.0×10^6
Janus green B	75	6.7×10^8	3.0×10^7*
Methylene blue	1	2.2×10^9	$<10^5$ *
Riboflavin	75	1.6×10^9	6.0×10^7*
Safranin O	75	1.2×10^9	4.0×10^6*
Thionin	50	1.9×10^9	3.0×10^7*
None (control)	—	2.4×10^9	1.2×10^9

* Photosensitivity dyes.

The reaction mixtures were incubated at 30°C for 15 min. The light source was 2 photoflood bulbs (BBA) placed 12 inches above the specimens.

the compounds examined previously were tested for photosensitizing ability. Acriflavine, crystal violet, eosin, Janus green, methylene blue, riboflavin, safranin and thionin, at appropriate concentrations, inactivated actinophage when exposed to bright light (Table II). The most active photosensitizing dye was methylene blue; all other dyes had to be used at 10- to 100-fold greater concentrations to achieve comparable photoinactivation. The range of crystal violet concentrations that photodynamically killed, without killing in the dark, was very narrow (Table III). The extent of light-enhanced inactivation of actinophage was time-dependent; in general, phage killing proceeded exponentially with time (Table IV). Actinophage particles mixed with riboflavin and kept chilled while exposed to bright light were not appreciably harmed. Chilling also protected phage from photodynamic action of crystal violet, eosin and safranin. Results with Janus green were not conclusive, but chilling seemed to favor phage survival. Phage mixed with acriflavine, methylene blue or thionin was photoinactivated equally at 5°C and 30°C.

Variability between experiments was encountered, particularly with eosin, Janus green and riboflavin. A recognized variable in the experiments was the period between mixing the phage and dye, and exposure to light. Incubation of the phage-dye mixture for 1 hour prior to illumination did not affect the extent of photodynamic killing nor did it improve the reproducibility of the assays. The reaction between the phage and dye in the dark was, in general, reversible. Dilution of the reaction mixture prior to illumination resulted in far less kill than illumination of the undiluted phage-dye mixtures. Janus green was an exception; dilution prior to light exposure did not reduce photoinactivation of the actinophage.

The extent of photodynamic inactivation of actinophages was markedly affected by the reducing compounds mercaptoethanol and sodium hydrosulfite. Reduced acriflavine did not photosensitize the phage, whereas reduced riboflavin and eosin were more potent photoinactivating agents. The viricidal action of methylene blue was prevented by sodium hy-

TABLE III. Dye Concentration Needed for Photosensitization of Actinophage.

Dye	Conc (μg/ml)	Surviving phage	
		Dark	Light
Acriflavine	75	5.0×10^8	$<10^5$
	15	1.3×10^9	7.1×10^5
	2.5	2.6×10^9	1.4×10^8
Crystal violet	500	9.5×10^6	$<10^5$
	100	3.5×10^7	4.5×10^6
	25	2.2×10^9	7.3×10^8
Eosin Y*	250	3.3×10^8	$<10^5$
	100	2.0×10^9	5.0×10^6
	25	3.2×10^9	6.0×10^8
Janus green B	250	6.7×10^8	2.6×10^7
	100	8.7×10^8	3.8×10^7
	50	2.0×10^9	8.5×10^8
Methylene blue	50	$<10^5$	$<10^5$
	5	6.0×10^6	$<10^5$
	1	2.2×10^9	$<10^5$
Riboflavin*	250	9.6×10^8	3.2×10^6
	75	1.3×10^9	1.8×10^8
	25	9.9×10^8	6.1×10^7
Safranin O	75	1.5×10^9	1.8×10^6
	15	1.9×10^9	3.0×10^7
	2.5	2.3×10^9	7.8×10^8
Thionin	75	6.0×10^8	1.0×10^6
	25	1.8×10^9	1.1×10^6
	2.5	2.3×10^9	3.7×10^8
Control	—	2.0×10^9	1.8×10^9

* Plus 1 mg mercaptoethanol/ml.

Phage and dye were mixed and incubated at 30°C for 15 min with or without exposure to bright light.

TABLE IV. Effect of Time of Light Exposure on Photodynamic Inactivation of Actinophage.

Dye	Conc (μg/ml)	Surviving phage, Exposure in min 0	1	5	15
Acriflavine	50	1.2×10^9	4.8×10^8	2.4×10^6	$<10^5$
Crystal violet	75	2.0×10^9	1.7×10^9	5.6×10^8	7.0×10^6
Eosin Y*	75	2.0×10^9	1.3×10^9	7.0×10^8	4.1×10^7
Janus green B	75	9.4×10^8	6.2×10^8	3.2×10^8	8.1×10^7
Methylene blue	1	1.0×10^9	2.0×10^7	$<10^5$	$<10^5$
Riboflavin*	50	1.3×10^9	4.9×10^8	3.1×10^8	1.6×10^7
Safranin O	75	1.4×10^9	2.0×10^8	1.6×10^7	4.0×10^6
Thionin	50	2.5×10^9	7.5×10^8	2.4×10^7	1.2×10^6
None (control)	—	2.4×10^9	2.3×10^9	2.3×10^9	2.1×10^9

* Plus 1 mg mercaptoethanol/ml.

Phage and dye were mixed and incubated at 30°C. The mixtures were exposed to bright light for 1 to 15 min.

drosulfite but not by mercaptoethanol (Table V).

Air was required for photoinactivation of the actinophages by acriflavine, crystal violet, methylene blue and riboflavin. Evacuation of the reaction vessel did not drastically reduce the photosensitization of phages by eosin and safranin. Phage neutralization was greatest when the reaction mixture was prepared in 0.005 M NaCl and decreased with increasing salt concentrations. Addition of bovine plasma albumin to a concentration of 1 mg/ml did not enhance nor attenuate the photoinactivation of phage.

Mixtures of photosensitizing dyes were tested; the extent of phage neutralization by acriflavine and riboflavin was intermediate between that of the two agents singly. The mixtures of acriflavine and crystal violet, acriflavine and methylene blue, riboflavin and crystal violet or riboflavin and methylene blue killed more phage than either dye alone. Crystal violet in combination with methylene blue behaved like the former dye alone (Table VI).

TABLE V. Effect of Mercaptoethanol and Sodium Hydrosulfite on Photodynamic Killing of Actinophage MSP2.

Dye	Conc (μg/ml)	log Pd/Pl*: Control	Mercapto-ethanol	Hydro-sulfite
Acriflavine	50	3.1	.2	.7
Crystal violet	75	1.8	1.7	1.9
Eosin Y	75	.6	1.4	1.6
Methylene blue	1	3.1	3.1	.4
Riboflavin	50	.7	2.4	2.3
Safranin	50	1.1	.4	.6
None	—	0	0	0

* $\log \text{Pd/Pl} = \log_{10} \frac{\text{phage titer of dark control}}{\text{phage titer after light exposure}}$.

Phage and dyes were mixed; mercaptoethanol or sodium hydrosulfite was added when indicated to a final concentration of 1 mg/ml or 100 μg/ml respectively; the reaction mixtures were incubated in the dark or exposed to bright light for 15 min and then assayed for viable phage.

Discussion. All of the tested heteroanthracenes, whether containing sulfur, oxygen or nitrogen, were photosensitizing agents. The substituted, homocyclic anthracenes did not photoinactivate actinophage MSP2. Crystal violet was the only photodynamic triphenylmethane dye found, and none of the tested dis-azo dyes were light-activated viricides. As is generally true for photochemical reactions, phage-inactivation proceeded exponentially with time. Moreover, photochemical reactions are relatively insensitive to temperature changes; this was true for photosensitization of acriflavine and methylene blue, but not for crystal violet, eosin and riboflavin. Apparently, additional factors influence photoinactivation by these latter 3 dyes. The dyes were not irreversibly bound to the phage material, at least in the dark. It is not known whether the light reaction fixed dye to the phage or merely served as a catalyst for photoinactivation.

Several lines of evidence suggest that acriflavine, crystal violet, methylene blue and riboflavin act differently. The viricidal potency of riboflavin was increased by mercaptoethanol whereas that of acriflavine was abolished and that of crystal violet or methyl-

TABLE VI. Photosensitization of Actinophage by Combinations of Dyes.

Dye #1	Conc (μg/ml)	Dye #2	Conc (μg/ml)	Plaque forming units/ml Dark	Light
Acriflavine	25	—	—	1.2×10^9	8.4×10^5
Crystal violet	50	—	—	1.3×10^9	7.0×10^7
Methylene blue	1	—	—	1.1×10^9	2.8×10^6
Riboflavin*	25	—	—	1.2×10^9	4.8×10^6
Acriflavine	25	Crystal violet	50	1.3×10^9	4.6×10^5
"	25	Methylene blue	1	1.1×10^9	2.4×10^5
"	25	Riboflavin	25	1.3×10^9	1.2×10^6
Crystal violet	50	Methylene blue	1	1.1×10^9	5.7×10^7
" "	50	Riboflavin*	25	1.2×10^9	1.6×10^6
Methylene blue	1	" *	25	1.1×10^9	5.3×10^5

* Plus 1 mg mereaptoethanol/ml.

Phage and dye combinations were mixed; one set was exposed to bright light for 15 min; the other set was incubated in the dark.

ene blue was unaltered. Photoinactivation by crystal violet and riboflavin was temperature-dependent but that by acriflavin or methylene blue was not. Moreover, the dyes in combination do not compete for binding sites, but generally have additive effects. Experiments using acriflavine and crystal violet solutions as light filters indicated that the apparent exceptions, that is, acriflavine and riboflavin, or methylene blue and crystal violet, are probably due to the overlapping light absorption spectra. The vulnerable locus seems to be the phage deoxyribonucleic acid because added protein did not protect the phage particles. Moreover, cursory experiments indicate that photoinactivated phage is still antigenic and that plaque-forming capacity is lost more rapidly than the power to kill host cells.

Summary. The heteroanthracenes acriflavine, eosin, Janus green, methylene blue, riboflavin, safranin and thionin inactivated actinophage MSP2 when exposed to bright light. The extent of photoinactivation was determined by dye concentration, duration of exposure to light, ionic strength of the milieu, and availability of air. In general, combinations of photosensitizing dyes had an additive effect, indicating different mechanisms of action. The data suggest, however, that all of the dyes were damaging the phage deoxyribonucleic acid.

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