EFFECT OF SODIUM AZIDE ON RADIATION DAMAGE AND PHOTOREACTIVATION

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During investigations on the role of bacterial pigments in ultraviolet irradiation damage and in photoreactivation, several agents were tested as suppressors of pigmentation effects. *Chromobacterium violaceum* is extremely radiosensitive in that it shows very high inactivation by ultraviolet and also high photorecovery with white light. Sodium azide depresses both of these effects significantly. Previously, Wyss *et al.* (1948) demonstrated an increase in the mutation rate of *Micrococcus pyogenes* var. *aureus*, grown in the presence of subinhibitory concentrations of this compound, and Clark *et al.* (1950) were able to stimulate recombination in *Escherichia coli*, strain K-12, under the same conditions. On the other hand, Bacq and Herve (1951) report a protective effect of azide and other respiratory poisons against X-ray damage in mice. A correlation of these effects with the known inhibitory action of azide on various iron-porphyrin enzymes appears evident, and an investigation of its applicability to other systems seemed to warrant testing on a broader basis.

MATERIALS AND METHODS

The organisms used in this study were *Chromobacterium violaceum*, ATCC strain 7461; *Escherichia coli*, strain B/r; *Micrococcus pyogenes* var. *aureus*, (University of Texas Department of Bacteriology stock strain), unless indicated otherwise. All liquid cultures were grown in nutrient broth, and nutrient agar served as plating medium for assay of cultures by colony counts.

In all cases aliquots were removed from log-phase cultures, centrifuged, washed in 0.85 per cent saline, and resuspended in saline solution for ultraviolet irradiation. Irradiation was performed at a distance of 50 cm from a Hanovia mercury vapor lamp (model C2770), operating at 120 milliamperes with 90 per cent of its spectrum at wavelengths below 2600 A. The emitted radiation was stabilized by turning the lamp on at least 20 minutes before use. The actual output under these conditions was determined to be about 18 ergs per mm^2 per second. Exposure times varied with the species employed. In all cases 20 ml samples were irradiated in large open petri dishes (d = 14 cm) and received constant gentle agitation during irradiation.

For photoreactivation five ml samples in screw-cap tubes were placed for one hour in a constant-temperature water bath (25 C for *C. violaceum*, 37 C for the other species) at a distance of 24 cm from the light source, a Sylvania 200 watt Floodlite. A filter of 1.6 cm of 0.05 M $CuCl_2 \cdot 2H_2O$ was interposed between the light and the samples for absorption of infrared rays.

Two experimental approaches were chosen: in one case ("growth experiments"), subinhibitory concentrations of azide were added to the medium in which the test cultures were grown. In the other type ("exposure experiments"), varying concentrations of azide were added to saline suspensions of the organisms at different times before or after irradiation.

The azide preparation was a one per cent stock solution of sodium azide in a phosphate-free citrate-sodium hydroxide buffer at pH 7; the solution was sterilized by filtration. Preliminary testing showed this buffer to be without detectable effect on the phenomena described below.

RESULTS

Growth experiments were slightly handicapped by the limited concentrations of azide at which the gram negative forms, *C. violaceum* and *E. coli*, could grow in primary cultures. In the case of the former organism, a concentration of 10 ppm (approximately 0.23 millimoles) was found to be the highest concentration permitting growth. In cultures grown under these conditions, ultraviolet radiation survival was increased fivefold and the degree of photoreactivation (Kelner, 1951) was reduced by 70 per cent. The degree of photoreactivation was chosen as a measure of photo-

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reactivation since it expresses the reactivated fraction rather than a survivor ratio.

In *E. coli*, grown in 10 ppm azide broth, ultraviolet radiation survival was doubled and the degree of photoreactivation reduced to slightly over 50 per cent. Cultures were obtained also after extended incubation in the presence of 100 ppm of the compound. However, in this case we may have dealt with an adapted population possessing an altered metabolism so that a comparison with the parent culture would be invalid (Grunberg-Manago, 1950). This assumption could be verified in part since a culture, adapted to 200 ppm of azide by repeated transfers in the presence of increasing concentrations, did not show more resistance to inactivation by ultraviolet radiation or a higher incidence of streptomycin-resistant cells than a parallel culture grown in the absence of azide.

TABLE 1

Streptomycin resistance of Micrococcus pyogenes cultures grown in azide after exposure to irradiated broth

ULTRAVIOLET EXPOSURE OF BROTH	EXPOSURE OF CELLS TO BROTH	STREPTOMYCIN-RESISTANT MUTANTS PER MILLION CELLS*	
		Azide grown culture	Control culture
min	*min*		
60	0†	212	73
Control	0†	132	84
60	90	289	95
Control	90	148	82
60	300	4,880	108
Control	300	144	71

* Streptomycin level: 3.0 units per ml of nutrient agar.

† Bacteria were inoculated into the appropriate broth and sampled immediately so that exposure to the broth before plating was less than 3 minutes.

Cultures of *M. pyogenes* var. *aureus*, grown in the presence of 0.03 per cent azide, showed up to fivefold increases in ultraviolet radiation survival while the degree of photoreactivation was reduced to one-third. In partial repetition of the work of Wyss *et al.* previously cited (1948), cultures grown in the presence of azide were employed in testing for induced mutations to streptomycin resistance after irradiation with ultraviolet radiation. A majority of the ten strains tested, when grown in nutrient broth, showed an increase in the incidence of streptomycin-resistant cells following direct irradiation with ultraviolet radiation or exposure to ultraviolet radiation treated broth; however, some of the strains did not respond to either of these treatments. This difference in behavior between strains was interpreted in terms of catalase production. When grown in the presence of azide, mutation incidence in the latter strains increased significantly, presumably due to the poisoning of catalase by azide. This "mutagenic" effect was observed when the strains were grown in irradiated substrate, as well as when they were irradiated directly. At the same time, the incidence of "spontaneous" mutants in azide grown cultures was higher than in parallel cultures grown without azide (tables 1 and 2).

TABLE 2

Ultraviolet (UV) survival and streptomycin resistance in azide grown cultures of Micrococcus pyogenes after direct ultraviolet irradiation

UV EXPOSURE OF CELLS	UV SURVIVAL		STREPTOMYCIN-RESISTANT MUTANTS PER MILLION CELLS	
	Azide grown culture	Control culture	Azide grown culture	Control culture
sec				
0	—	—	152	54.8
35	1.58×10^{-3}	3.25×10^{-4}	357	ca 100

Exposure experiments. The concentrations of azide used in these experiments were generally higher than those tolerated by the test organisms in the growth experiments but were chosen so as to give a minimum of killing by the compound under the conditions of the experiments.

When 0.015 per cent azide was added to saline suspensions of *C. violaceum* as little as 30 seconds prior to the start of ultraviolet irradiation, the number of ultraviolet radiation survivors increased up to twelvefold. Concentrations of the compound up to 0.15 per cent gave slightly erratic results, but in all cases increases in ultraviolet radiation survival were evident. With 0.03 per cent azide the degree of photoreactivation was reduced to one-eighth of its value in the absence of the compound (table 3). Concentrations above 0.05 per cent did not produce any further reduction, and actually increased the degree of photoreactivation in some cases. When resting

cells in saline were exposed to 0.03 per cent azide at 5 C for 60 minutes prior to ultraviolet irradiation, the number of ultraviolet radiation survivors increased to twenty times that of the same culture without exposure to the compound. When an aliquot of these cells was subjected then to treatment with white light, the degree of photoreactivation was almost ten times less than that of the untreated culture (table 4).

The addition of azide to irradiated bacterial suspensions immediately after ultraviolet radiation and before exposure to white light did not affect the number of ultraviolet radiation survivors, but reduced the degree of photoreactivation several times (table 5). Treatment of azide with ultraviolet radiation and white light (singly and combined) prior to its addition to the cells did not affect its protective action against radiations of either type. These latter findings seem to rule out the possibility of azide action via intermediates of its photochemical decomposition, such as "active" N_2 (Bonnemay, 1942); such compounds have a very short half-life.

In some cases it was observed that the number of viable cells in nonultraviolet irradiated controls decreased up to one-third upon exposure to white light in the presence of azide. This phenomenon was not investigated in detail, but since it never quantitatively approached the reduction in degree of photoreactivation in the same experiment, it would not influence the results obtained to any appreciable extent.

In *E. coli*, the presence of 0.02 to 0.03 per cent azide during irradiation increased ultraviolet radiation survival to three times that of the sample irradiated without azide. With the experimental arrangement used, the degree of photoreactivation of this organism was consistently small, but

TABLE 3

Ultraviolet (UV) survival and photoreactivation (PHTR) in the presence of azide

SPECIES	UV EXPOSURE OF CELLS	CONCENTRATION OF AZIDE	UV SURVIVAL	DEGREE OF PHTR
	sec	%		
Escherichia coli	45	0	3.78×10^{-4}	6.73×10^{-4}
	45	0.03	1.05×10^{-3}	1.60×10^{-4}
Micrococus pyogenes	35	0	1.31×10^{-4}	3.80×10^{-3}
	35	0.03	3.80×10^{-4}	6.60×10^{-4}
Chromobacterium violaceum	30	0	3.53×10^{-6}	5.15×10^{-2}
	30	0.03	8.25×10^{-6}	6.92×10^{-3}

$$\text{Degree of PHTR} = \frac{\text{light survivors} - \text{dark survivors}}{\text{nonirradiated dark control}} \text{ (}cf\text{ Kelner, 1951).}$$

TABLE 4

Ultraviolet (UV) survival and photoreactivation (PHTR) after pre-irradiation exposure to azide

SPECIES	UV EXPOSURE OF CELLS	CONCENTRATION OF AZIDE	UV SURVIVAL	DEGREE OF PHTR
	sec	%		
Escherichia coli	50	0	1.48×10^{-4}	1.60×10^{-4}
	50	0.03	2.15×10^{-4}	2.10×10^{-6}
Micrococcus pyogenes	35	0	3.67×10^{-4}	6.08×10^{-2}
	35	0.05	1.04×10^{-3}	3.26×10^{-2}
Chromobacterium violaceum	30	0	2.27×10^{-6}	1.56×10^{-2}
	30	0.03	5.39×10^{-5}	1.92×10^{-3}

Pre-irradiation exposure for 60 minutes at 5 C.

normal photoreactivation was reduced further to about one-fourth of its value after irradiation of cultures in the presence of azide (table 3). Pre-ultraviolet irradiation exposure to azide resulted in doubled ultraviolet radiation survival and almost complete elimination of the effects of photoreactivation (table 4).

Ultraviolet radiation survival in suspensions of *M. pyogenes* var. *aureus* was increased three to six times by irradiation in the presence of azide concentrations between 0.03 and 0.05 per cent. The decrease in the degree of photoreactivation was of the same order (table 3). Pre-irradiation exposure to 0.05 per cent azide for one hour increased ultraviolet radiation survival threefold and halved the degree of photoreactivation (table 4). Exposure to azide immediately following ultraviolet irradiation (other conditions identical with pre-irradiation treatment) did not seem to affect ultraviolet radiation survival, but the degree of photoreactivation was reduced up to a hundredfold (table 5).

TABLE 5

Ultraviolet (UV) survival and photoreactivation (PHTR) after post-irradiation exposure to azide

SPECIES	UV EXPOSURE OF CELLS	CONCENTRATION OF AZIDE	UV SURVIVAL	DEGREE OF PHTR
	sec	%		
Micrococcus pyogenes	35	0	1.36×10^{-6}	3.03×10^{-2}
	35	0.05	2.00×10^{-6}	3.42×10^{-4}
Chromobacterium violaceum	30	0	1.40×10^{-7}	2.25×10^{-3}
	30	0.03	—*	9.03×10^{-4}

* In this experiment the number of survivors of azide treatment was reduced so much that no colonies were formed on these plates.

Post-irradiation exposure for 60 minutes at 5 C.

DISCUSSION

From the results it appears that azide reduces to some extent the sensitivity of bacterial cells to two distinct types of radiation, ultraviolet radiation and radiations of longer wavelength contained in the visible spectrum of white light. On the basis of the recognized action of azide as a respiratory poison it may be suggested that, in azide treated and azide grown cells, some component(s) of the enzyme systems involved may constitute primary sites of ultraviolet radiation action. These are blocked temporarily or inhibited in their function so that they are not available to mediate in the chain of reactions leading from radiation event to cell inactivation. As type reaction of this sort we may consider the well established catalase-azide complex although the participation of another (or several other) heavy-metal enzyme systems is by no means excluded. Evidence exists that cytochromes and cytochrome oxidase are involved in radiation damage and protection (Haas *et al.*, 1952; King *et al.*, 1952; Mefferd and Matney, 1952; for the implication of cytochrome oxidase in the effects of CO treatment in connection with ultraviolet radiation and X irradiation) and are sensitive to azide poisoning (Stannard and Horecker, 1948; Stoppani, 1949; Slawson and Snyder, 1952). Tissières (1950) also reports an effect of azide on succinic dehydrogenase in *Aerobacter aerogenes*. To our knowledge the latter is the only recent report of azide action on a nonheavy-metal enzyme.

Regardless of the details of the photoreactivating process it appears likely that here also the ultimate effect is accomplished following a series of chemical events, one or several of which may be connected with reactions catalyzed by heavy-metal enzymes. If, as seems probable, the responsible factor for photoreactivation is a chromophore group, its direct inhibition by azide would be possible if it were a labile structure of porphyrin-like configuration. In this connection, Slawson and Snyder (1952) were able to show the effect of azide on such pigments in the yeast genus *Candida*. The particular role played by *violacein*, the pyrrol-type pigment of *C. violaceum* (Tobie, 1936), and its possible interaction with azide are still under investigation. An auxiliary

respiratory function has been ascribed to this pigment by Friedheim (1932).

The increase in the incidence of streptomycin-resistant cells after growth in or exposure to azide seems to be well accounted for by the mechanism postulated by Wyss *et al.* (1948) and referred to above, and would constitute a specialized case of enzyme inhibition by azide.

The observed action of azide in the three different systems studied has some implications which may lead to a better understanding of the processes involved in mutation, ultraviolet killing, and photoreactivation. The observation that azide increased ultraviolet radiation survival and increased the incidence of mutations in the same experiment seems to be good evidence that the relationship between the inactivating and mutagenic actions of ultraviolet radiation is far from simple, and that the two effects are not merely quantitatively different aspects of one phenomenon as was occasionally suggested in early investigations of the actions of radiations. Following photoreversal studies of ultraviolet radiation effects, Newcombe and Whitehead (1951) had reached a similar conclusion, namely, that at least a part of the ultraviolet killing in *E. coli* was not due to mutagen poisons but to some other agency.

The inhibiting action of azide on ultraviolet inactivation and on its reversal by white light is probably the first instance in which a compound is shown to act on these two processes in an apparently analogous manner. Photoreactivation thus may not constitute a simple reversal of inactivation but a photochemical process (and perhaps additional dark reactions; *cf* Dulbecco, 1950) in its own right. Compounds of the same type may play a catalytic role in several processes, i.e., perhaps pigments are primarily involved in photoreactivation whereas enzymes are the corresponding agents in ultraviolet inactivation and mutagenesis. The two latter processes may, in addition, be highly complex since they seem only partly photoreversible. Inhibition by azide may extend to all these agents, provided they are of a similar chemical nature (porphyrin-type). Further work on the quantitative aspects of the azide effect may elucidate the interrelationships between the processes in question.

SUMMARY

Growth in suitable concentrations of sodium azide, and even the mere presence of this compound during ultraviolet irradiation, reduces the sensitivity of three bacterial species to the lethal action of ultraviolet radiation. Simultaneously, the degree of photoreactivation is reduced. Exposure of resting cells to azide prior to irradiation produces essentially the same effects, whereas exposure following ultraviolet irradiation does not affect ultraviolet radiation survival, but is effective in reducing photoreactivation. Moreover, growth in the presence of sodium azide increases the incidence of "spontaneous" and ultraviolet radiation induced mutants for streptomycin resistance in "catalase-rich" strains of *Micrococcus pyogenes* var. *aureus*.

The blocking or temporary inactivation of heavy-metal enzymes or porphyrin-type pigments by azide is suggested as the mechanism responsible for these effects, and possible relationships between ultraviolet inactivation, mutagenesis, and photoreactivation are discussed.

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