THE ULTRAVIOLET PHOTOCHEMISTRY AND PHOTOBIOLOGY OF VEGETATIVE CELLS AND SPORES OF *BACILLUS MEGATERIUM*

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ABSTRACT The ultraviolet (UV) photochemistry and photobiology of spores and vegetative cells of *Bacillus megaterium* have been studied. The response of vegetative cells of *B. megaterium* appears qualitatively similar to those of *Escherichia coli*, *Micrococcus radiodurans*, and *Bacillus subtilis* with respect to photoproduct formation and repair mechanisms. UV irradiation, however, does not produce cyclobutane-type thymine dimers in the DNA of spores, although other thymine photoproducts are produced. The photoproducts do not disappear after photoreactivation, but they are eliminated from the DNA by a dark-repair mechanism different from that found for dimers in vegetative cells. Irradiations performed at three wavelengths produce the same amounts of spore photoproduct and give the same survival curves. Variation of the sporulation medium before irradiation results in comparable alterations in the rate of spore photoproduct production and in survival.

INTRODUCTION

Ultraviolet (UV) radiation forms specific photoproducts in DNA. Pyrimidine dimers of the cyclobutane type are a special class of DNA photoproduct that have been investigated in vivo and in vitro (1–3). These studies on DNA as well as those on model systems such as dinucleotides (4), synthetic polydeoxynucleotides (5), and frozen solutions of thymine (6) showed that thymine dimers are formed between adjacent thymines in polynucleotides. At large doses of UV, more than 50% of the biological damage to transforming DNA irradiated in vitro is attributable to dimers (7). Further evidence that thymine-containing dimers are important in the lethal action of UV irradiation in bacterial cells is the observation that dimers are excised from the DNA of radiation-resistant strains of *Escherichia coli* but they are not excised in radiation-sensitive strains of the same bacterium (8, 9). Exposure of UV-irradiated DNA in vitro to long-wavelength UV in the presence of a photoreactivating enzyme results in the disappearance of thymine dimers from the DNA (reviewed in reference (10)) by monomerization of the dimers (11). Monomerization of dimer appears to occur during direct photoreactivation in vivo (12).

Thymine dimers of the normal cyclobutane type are not formed, however, in the

DNA of UV-irradiated bacterial spores (13, 14). The absence of thymine dimers in spore DNA after irradiation may partially explain its extreme resistance to UV. However, such irradiation does produce unidentified thymine photoproducts in amounts involving up to 40% of the thymines in the spores' DNA. Our measurements (13) showed that the conversion of 50,000 thymine residues per spore had little effect on survival. This fact would suggest that the spore photoproducts are not involved in the mechanism leading to spore death. On the other hand, if the spore photoproducts are involved in spore death, then a repair mechanism can be postulated.

In this paper we report the comparative study of UV irradiation on survival of and photoproduct formation in spores and vegetative cells of *Bacillus megaterium*. We present evidence that the spore photoproducts are involved in the mechanism of spore death, and report a possible repair mechanism in differentiating spores. The repair mechanism is different from the excision repair of vegetative cells. Portions of this work have been reported previously (15–17).

MATERIALS AND METHODS

Spores of *B. megaterium* (strain QMB1551, kindly supplied by Dr. Hillel S. Levinson) were produced on a liver extract medium as described previously (13). Labeled spores were produced in this medium containing 50 mM methyl ^{3}H-thymidine (New England Nuclear Co.) of specific activity 6.7 c/mmole. In some experiments, $MnSO_4$ (100 μg/ml) was added to the liver extract medium. As shown below, the addition of $MnSO_4$ to the sporulation medium increased the resistance of the spores when they were subsequently irradiated in water.

M9 medium (18) with 1% glucose and supplemented with 0.5% yeast extract was used for germination and vegetative growth unless stated otherwise. This medium was inoculated with a suspension of heat-shocked (60°C, 15 min) spores (final concentration, 10^7 spores per ml). Under these conditions, germination was completed in 5 min; vegetative cells appeared at 60 min; and cell division began at 90 min. The doubling time after the first division was 20 min. Vegetative cells were produced by allowing a spore inoculum to grow for 2 to 2.5 hr, at which time the cells were still in exponential growth. Tritium-labeled vegetative cells were produced by adding 30 μc/ml methyl ^{3}H-thymidine to the medium prior to growth of the spore inoculum. For studies on colony formation, spores or vegetative cells were plated on the same medium used for growth, supplemented with 20 g of agar per liter, or on nutrient agar (Difco).

Irradiations were carried out as previously described (13). Spores at a concentration of 2×10^7 per ml were irradiated in distilled water. Vegetative cells were irradiated after dilution of the exponential culture 20-fold with M9 minus glucose. The diluted cell suspension had a concentration of 8×10^6 cells per ml. Incident radiation intensities varied with the wavelength of irradiation and ranged between 10^3 and 10^4 ergs per mm^2 per min. Intensities were determined with a photocell calibrated against a thermopile. Normally 1- to 2-ml stirred samples were irradiated in 1-cm path-length cuvettes and the incident intensities corrected for absorption and scattering by the method of Morowitz (19).

Photoreactivation or photoprotection was effected by light of wavelength 4050 A filtered through a Mylar film. The use of Mylar film eliminated any scattered light from the monochromator of wavelengths less than 3100 A.

In studies of photoproduct formation, spores and vegetative cells were labeled with ^{3}H-

thymidine before irradiation. The irradiated samples were hydrolyzed for 30–60 min in 88% or 97% formic acid at 175°C. Photoproducts were separated by paper chromatography with a butanol:acetic acid:water (80:12:30 by volume) solvent system (20). Chromatograms were analyzed for radioactivity by using a scintillation counter as previously described (13). The chromatograms contained 20,000–170,000 cpm. In most experiments, acid-soluble material was removed prior to hydrolysis by washing the pellets of spores or cells with water, extracting the pellets with cold 5% trichloroacetic acid (TCA), and then washing the TCA-insoluble pellets with ether or ethanol. In unirradiated and irradiated spores treated in this manner, 5–7% of the total radioactivity was found in compounds other than thymine. However, these compounds migrated near the origin ($R_f < 0.2$), so that radioactivity in the regions of photoproducts from irradiated spores was less than 0.2% of the total radioactivity on the chromatogram. Phenol extraction of DNA prior to hydrolysis did not result in a significant difference in the amounts of spore photoproducts recovered as compared with unextracted spores, but phenol extraction did eliminate the 5–7% of the radioactive non-thymine material in unirradiated spores.

In experiments studying excision or repair of photoproducts, ^{3}H-thymine–labeled spores or vegetative cells that had been irradiated were inoculated into growth medium and allowed to grow for various periods of time before isolation of photoproducts. For vegetative cells, the TCA-insoluble material was treated as above, whereas the TCA-soluble material was evaporated to dryness to remove the TCA before hydrolysis and chromatography. When irradiated spores were allowed to grow, it was necessary to examine the growth medium as well as the TCA-soluble and -insoluble fractions for photoproducts. Hydrolysates of the usual growth medium interfered with the chromatographic separation of photoproducts from thymine. Therefore spores were germinated, and the resulting cells grown for various lengths of time in a minimal medium (containing (per ml) glucose 50 μg, $MnSO_4$ 1 μg, L-alanine 50 μg, and potassium phosphate buffer pH 7.5 140 μg). At these low concentrations, it was possible to dry, hydrolyze, and chromatograph 0.5-ml samples with no effect on the chromatographic separation of photoproducts from thymine.

RESULTS

Survival

The relative sensitivities of spores and vegetative cells of *B. megaterium* to UV irradiation (2650 A) are shown in Fig. 1. These survival curves are typical of those for cells thought to have a repair mechanism (reviewed in reference (21)). The final slopes of the curves have a ratio of 9.8, whereas the intercepts of these slopes with the ordinate have a ratio of 10. Exposure of UV-irradiated vegetative cells to photoreactivating light (Fig. 2) resulted in the recovery of all but 10–20% colony-forming ability, yielding a photoreactivable sector of 0.53. Exposure of cells to light (4050 A) before ultraviolet irradiation did not alter the survival curves, indicating that this wavelength does not produce photoprotection in *B. megaterium* vegetative cells. The photoreactivation observed in Fig. 2 was thus probably direct photoreactivation and not photoprotection. Irradiation with 4050 A results in photoreactivation but no photoprotection in cells of *E. coli* (22).

UV-irradiated bacterial spores were exposed to photoreactivating light (*a*) immediately after irradiation, (*b*) after the spores had germinated (10-min develop-

ment), and (*c*) after the spores had developed into vegetative cells (60-min development). In no case was there an effect of photoreactivating light on survival.

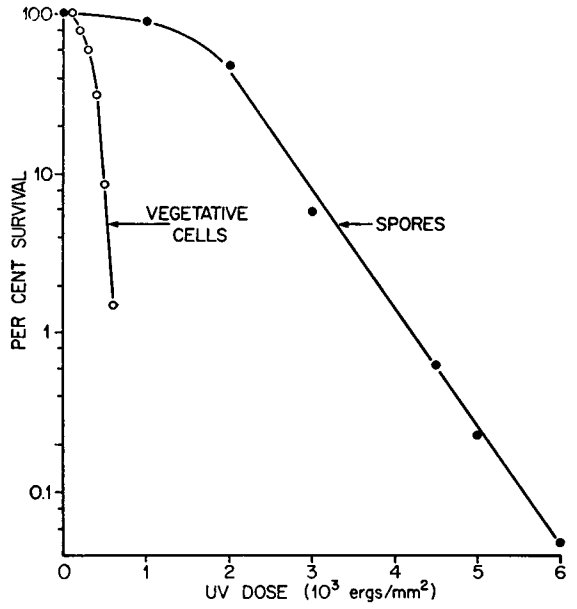

FIGURE 1 Survival of *Bacillus megaterium* vegetative cells and spores to 2650 A radiation. Cells were irradiated in dilute yeast extract medium. Spores were produced in medium without added $MnSO_4$ and irradiated in water. Samples were plated on yeast extract agar medium.

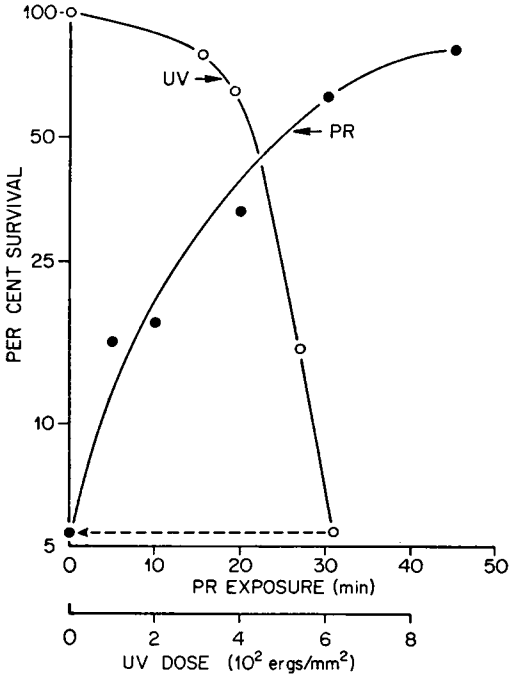

FIGURE 2 Survival and photoreactivation (PR) of *Bacillus megaterium* vegetative cells. Irradiation and plating conditions were the same as in Fig. 1. Wavelength of inactivating light was 2650 A and of photoreactivating light, 4050 A. The dose rate of the photoreactivating light was 7800 ergs per mm^2 per min.

Thymine Photoproducts in Spores and Vegetative Cells

As previously reported, thymine dimers ($\widehat{UT}$, uracil-thymine heterodimer formed by deamination, during hydrolysis, of the cytosine-thymine dimer; $\widehat{TT}$, thymine-thymine homodimer) were produced in vegetative cells of *B. megaterium* in amounts comparable to those found in vegetative cells of other organisms (13). However, thymine dimers were not found in spores of this organism, but three unidentified photoproducts (*a*, *b*, and *c*, Fig. 3) were produced (13). Dose-response curves at

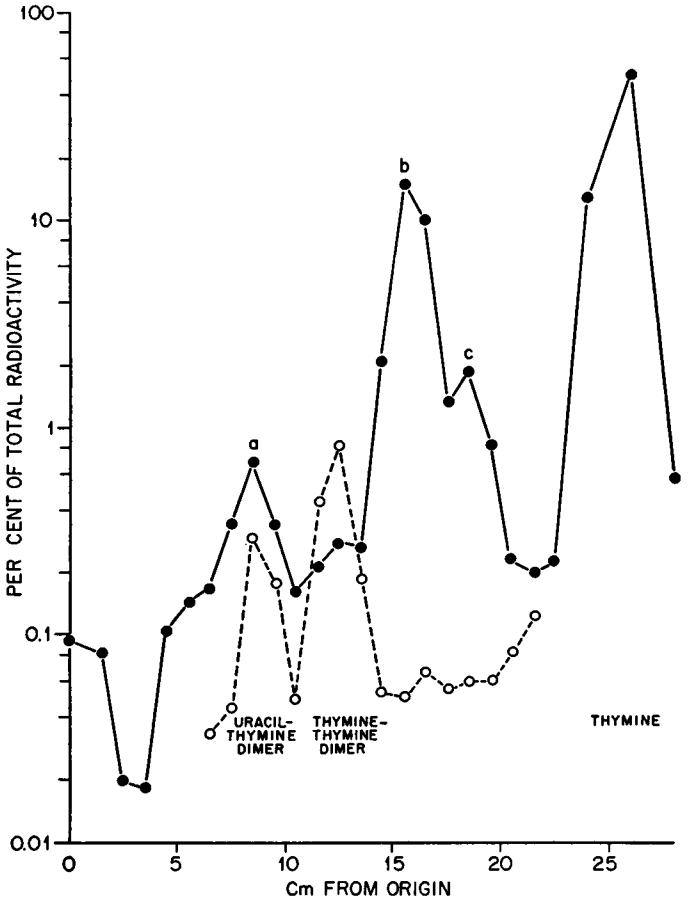

FIGURE 3 Graph of chromatograms of hydrolyzed DNA from *Bacillus megaterium* spores (solid lines) and hydrolyzed *B. megaterium* vegetative cells (broken lines) irradiated at 2650 A. Spores and cells were labeled with tritiated thymidine. Spores were irradiated with 5×10^4 ergs/mm^2; vegetative cells, 2×10^3 ergs/mm^2. R_f's of photoproducts: *a* at 0.20, *b* at 0.38, *c* at 0.44, uracil-thymine dimer at 0.21, thymine-thymine dimer at 0.29, and thymine at 0.61. The small amount of radioactivity at the origin was also present in unirradiated samples (13).

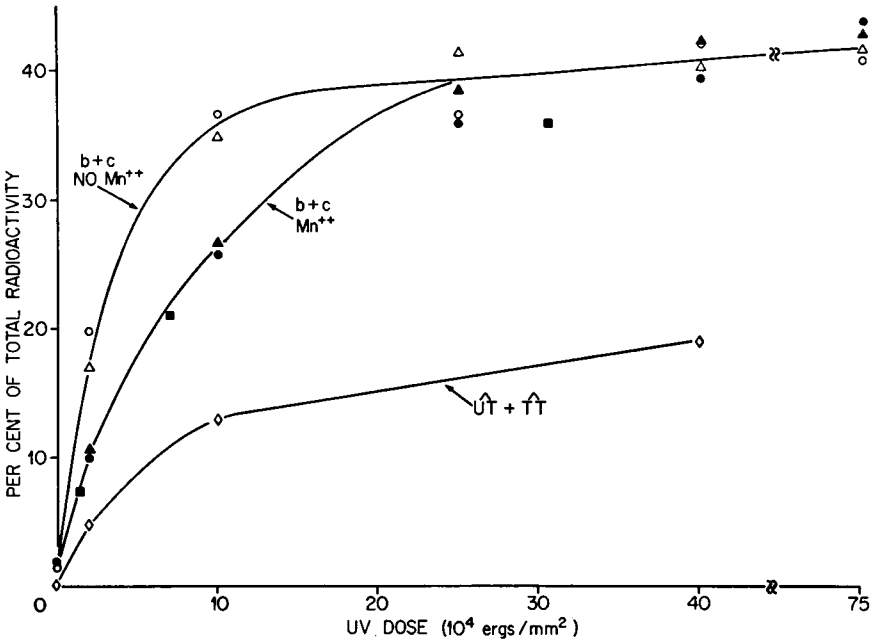

FIGURE 4 Dose-response curves for the production of thymine dimers and spore photoproducts *b* and *c*. Conditions of irradiation and identification of photoproducts as given in section on Materials and Methods. Wavelengths of irradiations: ■ 2378 A; ▲, △ 2650 A; ●, ○ 2804 A; open symbols are for spores produced without added manganese, closed symbols for spores produced with added manganese. ◇ $\widehat{UT}$ plus $\widehat{TT}$ dimers in *B. megaterium* vegetative cells irradiated with wavelength 2650 A.

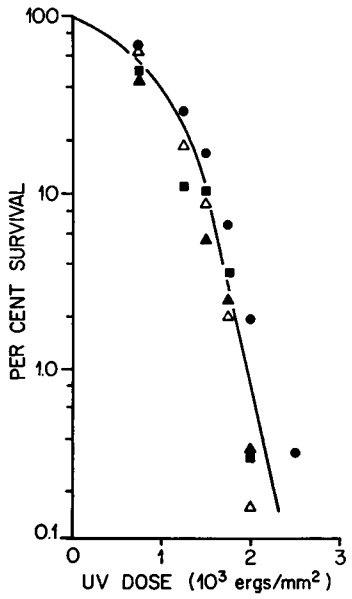

FIGURE 5 Survival of spores produced without added manganese in the sporulation medium. Irradiated with a wavelength of 2650 A on the day of harvest, ▲; 2650 A 8 days after harvest, △; 2378 A 1 day after harvest, ●; and 2804 A 1 day after harvest, ■.

different wavelengths for the two major spore photoproducts (*b* and *c*) are shown in Fig. 4 together with a dose-response curve for the production of thymine dimers in vegetative cells of *B. megaterium*.

In spores the major photoproducts are produced to a much greater extent than are pyrimidine dimers in vegetative cells. Saturation of photoproduct formation in spores was reached when 40% of the spores' thymines were involved, as compared with 18% at 2650 A in vegetative cells. These results are in fair agreement with the finding of 30% reported by Smith and Yoshikawa for *B. subtilis* spores irradiated at 2537 A (14). Unlike the production of thymine dimers in *E. coli*, the spore photoproducts were produced in equal amounts and at equal rates by equivalent doses of any of three wavelengths: 2378 A, 2650 A, or 2804 A (Fig. 4). In addition, short-wavelength irradiation (2×10^5 ergs/mm^2 at 2378 A) failed to alter the amount of photoproducts (*b* and *c*) produced by a previous saturating dose of long-wavelength (2804 A) irradiation. Long-wavelength irradiation alone converted 32.5% of the thymines into spore photoproduct, whereas long-wavelength followed by short-wavelength irradiation yielded 32.4%.

Because spores responded to three wavelengths in an identical manner, we determined whether or not spore survival curves were the same at these wavelengths. Fig. 5 shows that the loss of the spore's ability to form colonies is, within experimental error, the same at these three wavelengths.

Fate of Thymine Photoproducts in Vegetative Cells and Spores

When vegative cells of *B. megaterium*, labeled with tritiated thymidine, were allowed to grow in nonradioactive medium for 30 min following irradiation, the thymine dimers disappeared from the DNA (acid-insoluble material) (Fig. 6) and appeared in the acid-soluble material of the cells (Table I). These results are similar

TABLE I

EXCISION AND MONOMERIZATION OF THYMINE-CONTAINING DIMERS ($\widehat{UT}$ + $\widehat{TT}$) IN *BACILLUS MEGATERIUM* VEGETATIVE CELLS

Labeled vegetative cells were produced and irradiated as described in section on Materials and Methods. Samples were irradiated with 600 ergs/mm^2 at 2650 A. Photoreactivation was carried out with 2.6×10^5 ergs/mm^2 at 4050 A through Mylar. Photoreactivation time was 34 min. Irradiated cells were grown for 35 min. Thymine dimers were isolated as described in section on Materials and Methods.

	Total ^{3}H	$\widehat{UT}$ and $\widehat{TT}$				Total
		Acid-insoluble		Acid-soluble		
	cpm	*cpm*	%	*cpm*	%	%
UV, no growth, no photoreactivation	139×10^3	459	0.33	52	0.04	0.37
UV, growth	169×10^3	204	0.12	375	0.22	0.34
UV + photoreactivation	153×10^3	52	0.03	70	0.05	0.08

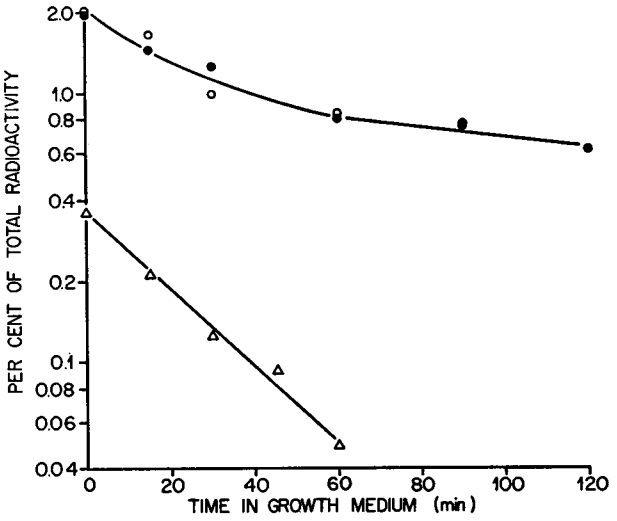

FIGURE 6 Disappearance of photoproducts from spores and excision of thymine dimers from vegetative cells. Trichloroacetic acid–insoluble photoproducts remaining in spores germinated in complete medium, ●; total photoproducts remaining in samples of spores and medium, spores germinated in minimal medium, ○; TCA-insoluble thymine dimers remain- in vegetative cells of *B. megaterium*, △.

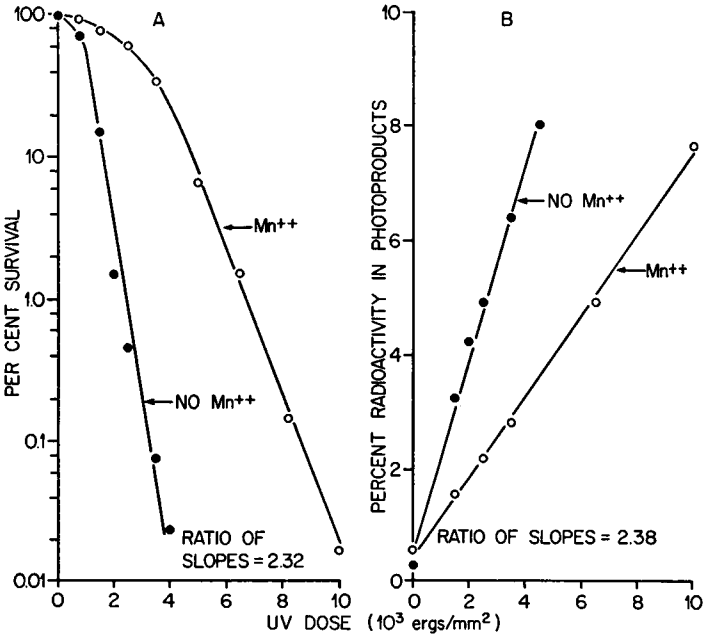

FIGURE 7 A. Survival curves of spores produced with added manganese (○) and without added manganese (●) in the sporulation medium. B. Production of photoproducts $b + c$ in spores grown with manganese (○) and without manganese (●).

to those found with resistant strains of *Escherichia coli*, *Micrococcus radiodurans*, and *Bacillus subtilis* (8, 9, 23–25). When prelabeled vegetative cells were exposed to photoreactivating light immediately after UV irradiation, a substantial decrease in the total numbers of dimers occurred (Table I). This result is expected when direct enzymic photoreactivation occurs.

When labeled spores were irradiated and allowed to differentiate into vegetative cells, more than 60% of the photoproducts vanished from the acid-insoluble material by 120 min (Fig. 6), but did not appear in the acid-soluble fraction. The spore photoproducts might have been rapidly excreted into the medium as are thymine dimers excised from the DNA of UV-irradiated *M. radiodurans* and *B. subtilis* (23–25). This possibility was eliminated by following the disappearance of the spore photoproducts during differentiation in a minimal glucose-alanine-salts medium (see section on Materials and Methods). In this experiment the cells were not centrifuged from the medium before hydrolysis. Rather, the total sample was evaporated to dryness, hydrolyzed, and chromatographed. The results, in Fig. 6, show that the spore photoproducts again disappear.

Biological Effect of the Spore Photoproducts

^{3}H-thymidine–labeled spores were produced in the presence and absence of $MnSO_4$. After thorough washing, the spores were suspended in water and irradiated with various doses of UV. Samples were taken both for plating on nutrient agar and for hydrolysis and determination of photoproducts. The results shown in Fig. 7 A indicate that spores grown in the absence of added manganese are 2.32 times as sensitive as spores grown in the presence of manganese as determined from the slopes of the survival curves. This result is mirrored by the kinetics of production of photoproducts *b* and *c* (Fig. 7 B), 2.38 times as many photoproducts being produced in sensitive spores as in the more resistant spores grown in the presence of manganese. The presence of manganese in the sporulation medium altered only the initial rate of photoproduct production and not the extent (Fig. 4).

DISCUSSION

Vegetative cells of *B. megaterium* appear to be able to cope with thymine photoproducts in their DNA by photoreactivation and an excision resynthesis mechanism qualitatively similar to the excision mechanism in *M. radiodurans* and *B. subtilis* (23–25) and the repair mechanisms in *E. coli* (8, 9). In spores of *B. megaterium*, UV lesions involving different photoproducts are produced in the DNA. These photoproducts disappear during development of the spores into vegetative cells (Fig. 6). Their disappearance may reflect a repair mechanism, since the spore photoproducts appear to be involved in the reactions leading to spore death (Fig. 7).

The mechanism is different from that found in vegetative cells, however, since the spore photoproducts disappear from the DNA and do not appear in the medium or the acid-soluble fractions as do cyclobutane-type thymine dimers excised from vegetative cells. This disappearance probably involves conversion of the photoproduct back to normal thymine residues.

The lack of photoreactivation of spores by exposure to long-wavelength light immediately after UV irradiation is not surprising, since intact spores exhibit very little enzymatic activity. The absence of photoreactivation after the spores had developed for 60 min suggests either that the photoreactivating enzyme had not been produced at this stage of development (or is produced only when needed) or that the spore photoproducts are not substrates for the photoreactivating enzyme. The latter hypothesis is supported by an experiment kindly performed by Dr. Jane K. Setlow in which DNA extracted from irradiated spores failed to compete with irradiated transforming DNA for the photoreactivating enzyme. DNA and synthetic polymers containing pyrimidine dimers compete very effectively with irradiated transforming DNA for this enzyme (reviewed in reference (26)).

The cyclobutane ring of pyrimidine dimers formed in DNA by high doses of 2800 A radiations can be broken by short-wavelength UV radiation, e.g. <2400 A, with the production of normal pyrimidines (3). UV quanta of all wavelengths break, as well as make, thymine-containing dimers. Thus, after large doses of irradiation, a photosteady state results in which the number of dimers formed depends in part on the wavelength of irradiation (1, 2). Typically, DNA of vegetative cells (1) and DNA in vitro (2) contain approximately 2% and 20% of the thymine residues as thymine-containing dimers following high doses of 2400 A and 2800 A, respectively. Intermediate conversions are obtained with intermediate wavelengths. In no case are all adjacent thymine residues converted to dimers.

The large amounts of spore photoproducts and the failure of 2378 A irradiation following 2800 A to alter the amount of photoproducts observed suggest that the photosteady state for the production of these photoproducts, if such a state exists, is markedly different from that of the thymine dimers. In fact, the maximum level of production of these photoproducts (40% of the total thymine) is very near the level expected for the frequency of adjacent thymine sequences in this organism. This fact would suggest that photoproducts *b* and/or *c* might be dimers of thymine, although different from the thymine dimer found in vegetative cells. Results from the irradiation of dried polydeoxynucleotides (17) and from the irradiation of DNA solutions at 77°K (R. O. Rahn, personal communication) suggest, but only suggest, that the spore photoproducts *b* and/or *c* are indeed dimers of thymine, although different from the thymine-containing cyclobutane dimers found in vegetative cells.

Thymine dimers are not formed readily in aqueous thymine solutions, but dimers are formed in frozen thymine solutions (6) and in thymine dinucleotides (4).

These facts suggest that the relative orientations of the pyrimidine residues are important for dimer formation. In fact, conditions which alter the orientation of the bases in DNA (i.e., drying or freezing aqueous solutions of DNA to 77°K) drastically reduce the numbers of thymine-containing dimers which are formed (27, 28). Under these conditions (dry or frozen), new thymine-containing photoproducts are produced which have chromatographic properties similar to those of the spore photoproducts (13, 14; R. O. Rahn, personal communication).

These investigations support the hypothesis that DNA in spores is in a form different from that found in vegetative cells. The data tell us nothing about the conformation of DNA within the spore. Recent data (29) suggest that yet a third form of DNA may exist within germinating spores. In this case the conformation is such that the production of both thymine dimers and spore photoproducts occurs at greatly reduced rates, if at all.

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