SOME EFFECTS OF ULTRAVIOLET RADIATION ON SPORULATING CULTURES OF *BACILLUS CEREUS*[1]

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Received for publication April 3, 1957

Zelle and Hollaender (1955) summarized data from several sources which showed that spores of aerobic bacilli are usually about twice as resistant to killing by ultraviolet light as are vegetative cells of the same species. Recently Stuy (1956) showed that spores of *Bacillus cereus* are many times more resistant than their vegetative cells. In seeking an explanation for the discrepancies in these data, we noticed that almost without exception data comparing radiation resistance of spores and their vegetative cells were published prior to the report by Kelner (1949) on photoreactivation. Since only vegetative cells of the sporeforming bacilli are capable of photoreactivation, the differences in sensitivity to ultraviolet light between these cells and their spores would be minimized unless precautions were taken to exclude the photorestoration process. Furthermore, in many of the reports, the vegetative cells and spores were irradiated on the surface of a nutrient medium. Spores rapidly lose their resistance to ultraviolet light when placed in an environment suitable for germination (Mefferd and Wyss, 1951; Stuy, 1956), so it is possible that at the time the spores were irradiated they had actually begun germination, and consequently were more sensitive to destruction.

When the spores of *Bacillus anthracis* were placed in environment favorable for germination, their sensitivity to ultraviolet light began to increase within 5 min and in a relatively short time their sensitivity paralleled that of vegetative cells. Since the change in sensitivity of germinating spores occurs with such dramatic suddenness, it was felt that a system in which these changes occur at a more leisurely pace would be more amenable to experimental manipulations. Accordingly our attention was drawn to the system described by Hardwick and Foster (1952) in which they studied the sporogenesis of washed vegetative cells of various aerobic sporeformers which were removed from the growth medium and shaken in distilled water. This system, which they termed "endotrophic" sporulation, seemed to offer several advantages to a study of changes in radiation resistance during sporulation, among which were: (a) the culture may be followed during sporogenesis in the absence of exogenous nutrients and without cell multiplication; (b) the spores form simultaneously in almost all cells and within a short time interval; and (c) the metabolic changes preceding sporogenesis occur at a slower rate than those of spore germination.

We have, therefore, studied the changes in ultraviolet sensitivity of *B. cereus*, its loss of the ability to be photoreactivated, and development of heat resistance during the complete cycle of endotrophic sporulation. We have found that loss of the ability to be photoreactivated occurs at the time the cells become ultraviolet resistant, and that the resistance to ultraviolet light precedes resistance to heat by as much as 2 hr. These ultraviolet resistant forespores have been shown to be as resistant to radiation as fully mature spores formed by conventional means.

MATERIALS AND METHODS

The laboratory strain of *B. cereus* was first tested because it gave less chain formation than other stock cultures in our collection of the genus *Bacillus*. This strain was extremely sensitive to the white light used in the photoreactivation experiments, so a stable mutant was derived from it by a technique analogous to that used by Witkin (1946) for obtaining *Escherichia coli* strain B/r from *E. coli* strain B. The vegetative cells of this strain, designated OT, showed enhanced resistance to white light and therefore could be used more effectively in photoreactivation studies; however, its spores are equivalent to the parent strain in radiation and heat resistance,

[1] This work was supported in part by the Division of Biology and Medicine, Atomic Energy Commission and by a grant from the American Cancer Society, Inc. on recommendation by the Committee on Growth, National Research Council.

although the sporulating qualities are somewhat lower.

The cultures were prepared by inoculating a glucose-glutamic acid salts medium (GGS) used by Foster and Heiligman (1949) with a standard spore suspension previously pasteurized at 65 C for 20 min. The cultures were incubated at suboptimal temperatures of about 20 C with vigorous aeration until the population reached a density of about 10^8 cells/ml. This treatment insured a practically spore free vegetative culture as shown by the fact that pasteurized aliquots of it revealed less than 100 spores/10^8 vegetative cells. The cultures from GGS medium were washed twice in M/15 phosphate buffer, pH 7.1, and resuspended in distilled water to a concentration of approx 10^6 to 10^7 cells/ml, and shaken at 33 C until sporulation occurred. In the experiments to be described, the age of the culture was taken as the time interval after it was placed in distilled water. By varying the temperature and length of incubation in the GGS medium, the time of the onset of sporulation could be modified.

The endotrophically sporulating cultures were sampled hourly after starting the 33 C incubation period. Dilutions of one aliquot were plated directly to determine the total number of bacteria present. This number was found not to vary significantly during the period of incubation in distilled water necessary for sporulation to occur. One aliquot was diluted 1:10 in the phosphate buffer and 10 ml was added to a petri plate for irradiation. The ultraviolet (UV) source was a General Electric germicidal lamp, 15 watts, which was placed at a distance of 15 in from the surface of the plate during irradiation (240 ergs/mm^2). The bacterial suspension was agitated continuously during the 60 sec irradiation period, and all operations were carried out in a room with yellow light except for the photoreactivated cultures. Spores were defined as the survivors when another aliquot of the culture in a 1:10 dilution of the phosphate buffer was heated at 65 C for 15 min. The plating medium used to determine survivors in all the manipulations was nutrient agar to which 3.0 g of glucose and 1.0 g of Na_2HPO_4 per liter had been added.

Photoreactivation was studied by transferring two aliquots of the UV irradiated bacterial suspension to each of two screw cap pyrex tubes. One of the tubes was wrapped in aluminum foil, and both immersed in a water bath maintained at 14 C. They were then illuminated with a General Electric Photospot flood lamp at a distance of 15 in.

RESULTS

When washed free of the culture medium and shaken in distilled water at 33 C, the strain of *B. cereus* used in this study would usually sporulate between the 6th and 8th hr, although sporulation could be induced to occur more rapidly by suitable modifications of the procedure.

Stuy (1955) reported that *B. cereus* is extremely sensitive to white light and that when UV treated cells of this organism are exposed to photoreactivating wave lengths, many are killed. Our photoreactivation curve is presented in figure 1. Maximum photoreactivation at 14 C occurs after 30 min exposure to white light and further exposure results in a rapid inactivation, even with the selected white light resistant culture, at a rate approximating that of the control cells that had not been subjected to UV. Therefore, 30 min periods of photoreactivation were used routinely in further experiments. A temperature study showed that holding the cells at higher temperatures during the photoreactivation process results in a decreased amount of photoreactivation. It is emphasized that in all experiments UV irradiated controls were shielded with aluminum foil and kept in the water bath along with the UV irradiated and normal unirradiated cells which were exposed to the white light. In no case was

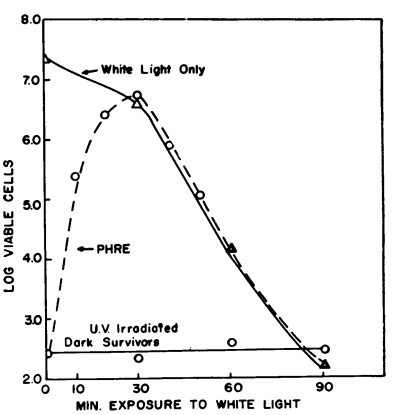

Figure 1. Fifteen-hour culture of *Bacillus cereus* in phosphate buffer. One aliquot was exposed only to the white light, one aliquot was irradiated with ultraviolet (UV) for 60 sec and held in the dark, and the other aliquot (PHRE) was irradiated 60 sec with UV and then exposed to white light for the indicated time.

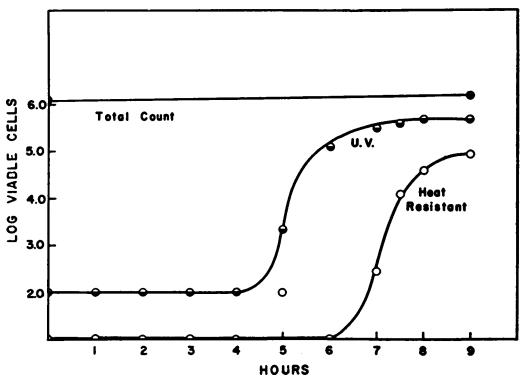

Figure 2. Washed vegetative cells of *Bacillus cereus* incubated at 33 C in distilled water. Aliquots were removed every hour. One sample ultraviolet irradiated 60 sec and the other heated at 65 C for 15 min. Survivors determined by plate counts.

there a detectable change in the viability of this control during the time covered in these experiments. Therefore, it seems that the progressive loss of viability of the UV irradiated cells after 30 min of photoreactivation is due to the white light alone and not to some complex metabolic event such as that postulated by Barner and Cohen (1956) for irradiated *E. coli* in the presence of growth medium. The pronounced lethal effects of white light on the genus *Bacillus* are not unique to this organism. Hill (1956) reported that strong illumination greatly reduced plaque formation in phage infected *E. coli* and was somewhat less strongly lethal to it, and Oppenoorth (1956) reported that sporulation in some strains of brewing yeast is inhibited by exposure in glass containers to sunlight.

Data of figure 2 show that although the total viable population is not appreciably changed over a 9-hr incubation period in distilled water, the heat resistance, i.e., formation of spores, and the sensitivity of the culture to UV is profoundly altered. Use of the suboptimal temperature during growth of the cells resulted in an almost completely asporogenous culture during the first 6 hr of incubation in distilled water, and after that time sporulation began at a rapid rate, reaching a plateau after 9 hr. Exposure of parallel aliquots of these cells to 60 sec UV light resulted in the loss of viability of over 99 per cent of the cells during the first 4 hr. However, beginning at the fifth hr, a rapid loss of sensitivity became apparent, and continued through the seventh hr, at which time heat resistance also began to develop.

Radiation resistance experiments comparing these 5-hr endotrophically sporulating cells (which had acquired no heat resistance and therefore would not be termed "spores") with mature spores produced on agar plates, showed that for the range from 240 to 1200 ergs/mm^2, the survivor curves had essentially identical slopes. Although aliquots exposed to all these levels of UV were subjected to our white light source with exposures ranging from 30 min to 2 hr, no photoreversal of the ultraviolet lethality could be obtained after the cells arrived at the prespore stage characterized by increased ultraviolet resistance.

The data shown in figure 3 were obtained with cells which were held at a temperature of 22 C for 18 hr and then at 15 C for an additional 6 hr. Under these conditions sporulation began after only a 3-hr incubation period at 33 C in distilled water. Here also the rise in UV resistance preceded the formation of heat resistance by an hour. The ability of the irradiated cells to be photoreactivated changed markedly as the incubation in distilled water progressed. The rise in the photoreactivation ability shortly after replacement is a general phenomenon and has been observed to occur in all succeeding experiments. The loss in ability to be photoreactivated occurs at approximately the same time that the cells begin to exhibit enhanced resistance to UV. This loss for the capacity of photoreactivation could conceivably be due to the depletion of a participating intracellular metabolite which is diverted to the more immediate needs of cellular maintenance when the bacilli are placed in distilled water, or it could be a consequence of the rearrangement of the cellular substance prior to sporulation. Experiments designed to discriminate between these possibilities, while not completely conclusive, favored the latter view. In these experiments, known inhibitors of sporulation such as alanine or glucose were added to the replacement cultures and the response to photoreactivation followed for a number of hours. Under these conditions the degree of photoreactivation remained constant. The possibility that these results were due to the conversion of the inhibitors to the postulated metabolite participating in photoreactivation cannot be excluded.

The results obtained from using cells which were

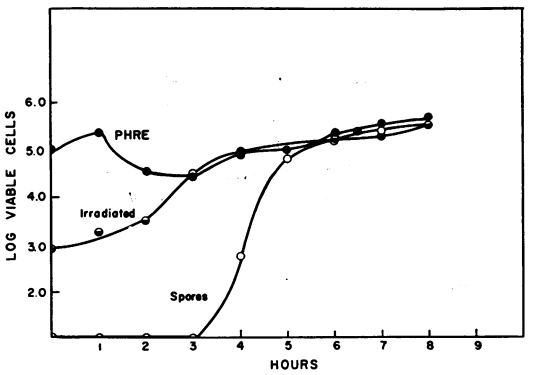

Figure 3. Sporulation of *Bacillus cereus* in distilled water at 33 C after growth at 22 C for 18 hr and 15 C for 6 hr. One aliquot irradiated with ultraviolet light 60 sec, one aliquot ultraviolet irradiated 60 sec and photoreactivated 30 min, one aliquot heated at 65 C for 15 min. Survivors determined by plating. Note the shortening of the time before onset of sporulation.

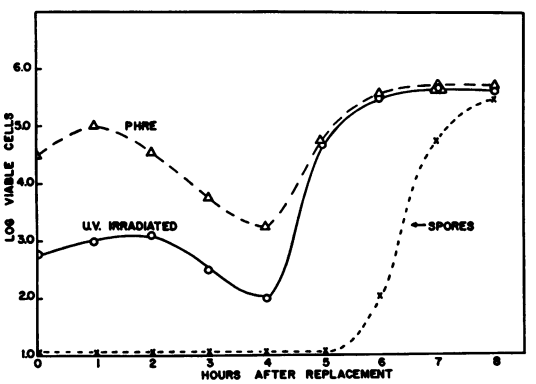

Figure 4. Sporulation of *Bacillus cereus* in distilled water at 33 C after growth at 22 C for 15 hr. Treatment same as in figure 3. Note longer period of time before onset of sporulation.

grown at 22 C for 15 hr before replacement are shown in figure 4. In this experiment, and in those subsequent to it, cytological examinations of the cells were made throughout the period of endotrophic sporulation with a phase bright-contrast microscope. It was observed that at the fifth and sixth hr, forespores were just beginning to become clearly delineated and that almost no typical refractile spores were present. By the seventh hr only a few forespores were still visible, while many typical, brightly refractile endospores could be seen. By the eighth hr practically every cell contained a typical spore. Thus it would seem that ultraviolet resistance is associated with the appearance of the forespore, while further maturation is necessary before heat resistance is significantly raised.

DISCUSSION

Search for the variation in radiation resistance between wild type and mutant strains, between organisms in different phases of the growth curve and even in synchronized cultures during the various stages in the division cycle are complicated by extraneous modifications in the environment. This study with the developing spore appears to restrict changes to those in the organism itself. Chemical differences that have been reported between the spore and the vegetative cell from which it is derived include nucleic acid composition, dipicolinic acid, pyrolle pigments and mineral salts. The modification of the photoresponse of the sporulating organism to both the lethal action of UV and the reversing action of white light suggests that the explanation may be in these chemical changes.

It is well established that in UV treated cells the nucleic acids are probably the primary site of damage. It has been postulated that a relatively higher concentration of RNA in spores as compared to vegetative cells accounts for the greater UV resistance of the former (Curran, 1952). Recent analyses, however, have revealed that the vegetative cells of many bacilli have a significantly higher concentration of both RNA and DNA than their spores (Fitz-James, 1955; Bennett and Williams, 1955; Tinelli, 1955). Therefore, it seems untenable to attribute the high degree of UV resistance of spores to their nucleic acid composition.

Dipicolinic acid has been shown to comprise over 10 per cent of the dry wt of the spores of a variety of the aerobic sporeformers, while the vegetative cells of the same species seem to be entirely devoid of this compound (Powell and Strange, 1953). In view of the fact that dipicolinic acid absorbs strongly in the ultraviolet region of the spectrum, it seems likely that it would afford protection to the genetically susceptible portions of the spore by simply absorbing the exciting light before it could reach this part of the cell. However, quantitative measurements on the appearance of dipicolinic acid during the course of endotrophic sporulation of *B. mycoides* show that

its appearance can be correlated almost precisely with the appearance of the spores (Foster and Perry, 1954). Since UV resistance is gained some time before the spores appear it would seem that this compound which is unique to spores must play some other part in the cellular economy.

Of the compounds implicated in the photoreactivation process, two have been most widely investigated. Berger *et al.* (1953) showed that azide-treated cells were refractory to the photoreactivation process, a fact which suggests the involvement of a heavy-metal enzyme in this phenomenon. More recently Bellamy and Germain (1955) showed that certain bacteria, such as streptococci which are deficient in porphyrin are not capable of photoreactivation. Goucher *et al.* (1956) reported that certain strains of Azotobacter which possess cytochrome may be photoreactivated to a reasonable extent, while one strain which lacked this pigment was completely without response to the photoreactivating wave lengths satisfactory for the other strains. Keilin and Hartree (1947) have shown that spores of *B. subtilis* contain only 5 per cent as much cytochrome as their vegetative cells; the loss of the ability for photoreactivation exhibited by the spores may be due to a deficiency of these pigments.

Kelner (1953) showed that when *E. coli* strain B/r is treated with UV one of the most striking changes exhibited by these cells is the inhibition of the synthesis of DNA, while the synthesis of RNA is relatively unaffected. When these treated cells are then exposed to light of longer wave lengths, the synthesis of DNA is resumed at a rate approximating that of normal cells. In *B. megaterium*, Tinelli (1955) showed that the synthesis of RNA in sporulating cultures suspended in a deficient medium shows a sharp rise during the last 6 hr before sporulation, followed by a decline preceding the appearance of spores. Endotrophically sporulating cultures of *B. cereus* analyzed at different stages show a similar RNA metabolism (Romig and Wyss, *unpublished data*). In this connection it is interesting to note that the degree of photoreactivation shown in figures 3 and 4 exhibits a pattern similar to the RNA synthesis during the course of endotrophic sporulation, so it seems possible that the loss of the ability for photoreactivation exhibited by our sporulating cultures may be correlated with a new nucleic acid balance attained during spore formation.

SUMMARY

By studying cultures of *Bacillus cereus* during the course of endotrophic sporulation, it has been shown that the increased level of resistance exhibited by the spores to the lethal effects of ultraviolet light develops as much as 2 hr before heat resistance is manifested. The ultraviolet resistance of the developing spores was correlated with the appearance of the forespore, whereas the attainment of heat resistance is delayed until development of the mature spore. In the process of spore formation susceptibility to photoreactivation and to the lethal effect of low doses of ultraviolet are lost at about the same time.

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