Actinomycin D Inhibition of UV-Dark Repair in *Bacillus subtilis*[1].

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Dark repair of UV-induced damage may be studied with a specific nuclease from *Micrococcus lysodeikticus* that degrades DNA containing pyrimidine dimers (Strauss, Searashi and Robbins, 1966). The transforming activity of DNA from UV-irradiated cells is drastically reduced by this nuclease, whereas DNA extracted from cells which have recovered from UV damage is not susceptible to inactivation. We have used the enzyme to demonstrate the inhibition of the dark repair process by actinomycin D. Since dark repair is not affected by other inhibitors of RNA or protein synthesis we conclude that actinomycin D probably inhibits by combining with the DNA, making it unavailable as a substrate for one or more of the repair enzymes. The synthesis of new mRNA is not required for the repair process.

Materials and Methods

Cultures of *Bacillus subtilis* *ind*$^+$*thy*$^-$ were used as DNA donors in these experiments. The strain is a derivative of strain 168 *ind*$^-$*thy*$^-$ provided by Dr. Frank Rothman. The overnight cultures used in these experiments were prepared by incuba-

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tion with shaking for 16 hours at 37°C in CHT50 medium (Reiter and Strauss, 1965). Lysates for transformation assay were prepared with lysozyme, pronase and duponol. A crude extract of *M. lysodeikticus* (Strauss, 1962) was added at the time of lysis as previously described (Strauss, Searashi and Robbins, 1966). Transformation was by the protocol of Anagnostopoulos and Spizizen (1961) except that competent, strain 168 *ind*$^-$ cells were frozen and stored in 15% (v/v) glycerol for up to a week before use. The actinomycin was a gift from Merck and Company.

Experimental

During recovery when actinomycin D is present in the medium with UV-irradiated cells, the DNA of the cells remains susceptible to the *M. lysodeikticus* nuclease (Fig. 1). The transforming activity of DNA extracted from UV-irradiated cells incubated with actinomycin is not decreased. The actinomycin D must therefore prevent the removal of the UV lesions that are the substrate for the nuclease. This inhibitory activity of actinomycin does not extend to all repair processes. Although damage produced by treatment of *B. subtilis* with the alkylating agent methyl methanesulfonate (MMS) is repaired (Reiter and Strauss, 1965), this repair is not inhibited by actinomycin. (Table 1).

One might suppose that dark repair requires a series of induced enzymes and that the UV-damaged DNA serves as an inducer. Actinomycin would then be expected to inhibit repair by preventing the formation of the mRNA required for the synthesis of new enzymes (Levinthal, Keynan and Higa, 1962). If this explanation were correct, inhibitors of protein and RNA synthesis in general would be expected to inhibit dark repair. However, neither chloramphenicol, puromycin or 8-azaguanine duplicate the actinomycin effect (Table 2).

Strains that are able to carry out the dark repair of UV-induced damage have been shown to release a portion of their DNA in an acid-soluble form during what

(Table 1) Effect of Actinomycin D on Recovery from UV- and from methyl methanesulfonate-induced Damage

	Control	UV-treated ($N/N_o = 9.8 \times 10^{-2}$)	MMS-treated ($N/N_o = 6.1 \times 10^{-3}$)
Not Incubated	243	2.0	7.6
Incubated 40 min	466	78.5	59.5
Incubated 40 min plus 2 μg/ml Actinomycin D	248	4.0	50.7

Recorded transformants per 0.1 ml after a 1:1000 dilution of the cell lysates.

A late log phase culture of strain ind^+thy^- was harvested, washed and treated. The culture was then incubated for 40 min in CHT50 medium in the absence of added thymidine.

(Table 2) Effect of Inhibitors on Recovery from UV-Induced Damage

	Control	UV-treated ($N/N_o = 0.1$)
Not Incubated	1128	46.1
Incubated 40 min	1060	457.
Plus Actinomycin D 2 μg/ml	-	55.2
Plus Chloramphenicol 20 μg/ml	-	332.
Plus 8 Azaguanine 50 μg/ml	-	542.
Plus Puromycin 50 μg/ml		802.

Recorded transformants per 0.1 ml after a 1:1000 dilution of the cell lysates. Cell suspensions were lysed in the presence of the *M. lysodeikticus* extract.

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is assumed to be the repair period (Boyce and Howard-Flanders, 1964). The release of acid-soluble residues begins immediately upon incubation of the irradiated cells in a medium containing a carbon source; breakdown is inhibited when incubation takes place in buffer (T. Searashi, personal communication). We have found that some breakdown of DNA to acid-soluble products occurs in the presence of actinomycin

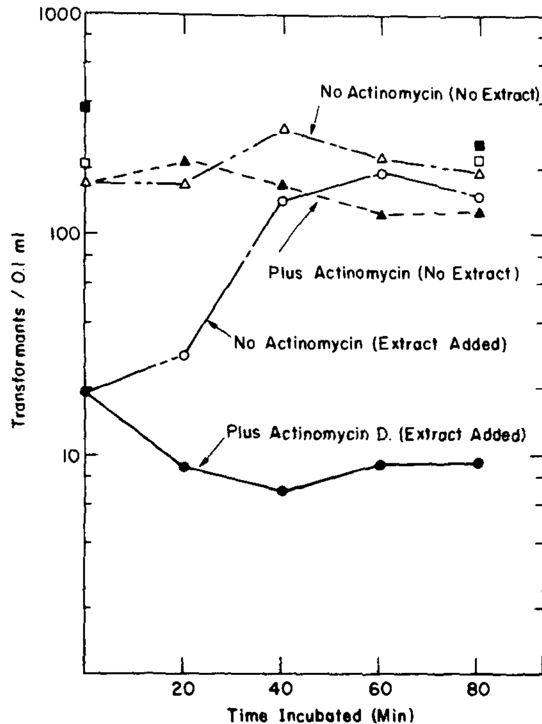

Fig. 1. Effect of actinomycin D on the repair of UV-induced damage. Cells were irradiated to a survival of 1.3 x 10^{-2} and then incubated in growth medium for the indicated times. Circles - cells lysed in the presence of the M. lysodeikticus enzyme; triangles - lysis with no M. lysodeikticus extract. Filled circles or triangles indicate the presence of 2µg/ml of actinomycin D during the recovery period.

(Fig. 2) but that the appearance of the breakdown products is delayed until after a significant portion of the UV-induced lesions has been removed from the DNA (Fig. 1). The data shown in Fig. 1 may be compared with those in Fig. 2 since the cells have been inactivated to approximately the same extent (1.3 x 10^{-2} and 9 x 10^{-3}). Since such a comparison shows that breakdown of DNA in an irradiated population of cells continues long after repair has been completed and that breakdown of DNA occurs in the absence of repair, we conclude that at least a large part of the observed breakdown, while it may be consequent to repair, is not directly involved in the repair process itself.

Although it appeared likely that actinomycin might block dark repair by preventing

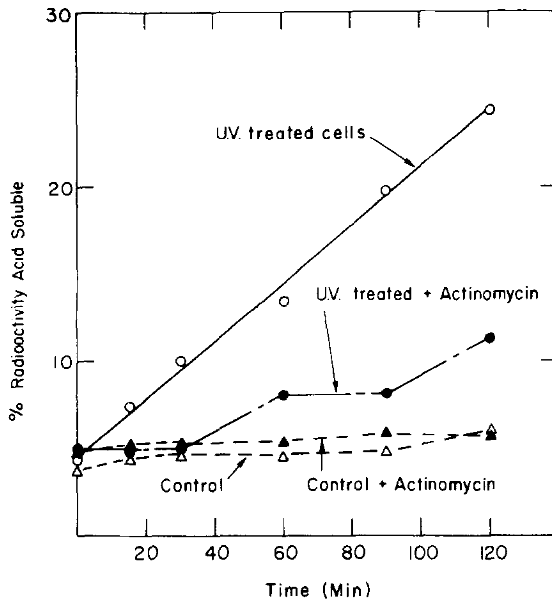

Fig. 2. Appearance of acid-soluble radioactivity after UV irradiation of B. subtilis. An overnight culture of B. subtilis 168 ind$^+$ thy$^-$ was prepared on medium containing H^3-thymidine. The cells were collected washed and irradiated to a survival of 9×10^{-3}. They were then incubated in medium containing thymidine and, where indicated, 2µg/ml of actinomycin. At intervals samples were precipitated with cold 5% trichloracetic acid, the supernatants collected, ether extracted, added to Brays solution and counted in a liquid scintillation counter. Triangles-control, no UV; Circles-UV irradiated. Filled circles or triangles-plus 2µg/ml actinomycin D.

excision, we investigated the possibility that it might also prevent a later step in the repair process, repair synthesis of new DNA. DNA synthesis was measured both by the diphenylamine reaction and by the incorporation of H^3-labeled thymidine into cold acid-insoluble material. We found that 2µg/ml of actinomycin inhibited DNA synthesis in both UV-irradiated and non-irradiated exponentially growing cells. We also found that H^3-labeled thymidine was not incorporated into the DNA of irradiated, resting stage cells when they were incubated in the presence of actinomycin. This indicates the absence of any repair replication (Pettijohn and Hanawalt, 1965). Although actinomycin may obviate the need for repair synthesis by preventing the excision of

thymine dimers, it is possible that it may also inhibit both normal and repair DNA synthesis (Farmer, 1966).

Discussion

We conclude from these experiments, first that the actinomycin D inhibition of dark repair is due to a combination with the DNA which prevents operation of pre-existing repair enzymes rather than to an inhibition of the synthesis of induced repair enzymes, and second, that the massive breakdown of DNA to acid-soluble fragments is not a part of the repair process although it may be associated with it. If the synthesis of induced enzymes were required for repair we would expect chloramphenicol, azaguanine and puromycin to inhibit the process, but these compounds have little or no effect. Chloramphenicol has previously been shown not to inhibit the repair process in *E. coli* (Setlow, 1964). We therefore conclude that actinomycin combines with DNA in the manner of the acridines (Setlow, 1964), inhibiting the operation of the repair enzymes.

We infer from these data that the process of breakdown of DNA from irradiated cells is associated with, but is not a part of, the repair process, because breakdown continues long after the completion of repair, as measured by the loss of susceptibility to the *M. lysodeikticus* nuclease. In addition, breakdown without repair does occur in the presence of actinomycin, albeit after a lag period not observed when irradiated cells are incubated in the presence of inhibitor. It has been shown (Strauss, Reiter, and Searashi, in press) that breakdown does not occur in *uvr⁻* *B. subtilis* unable to repair UV damage. Therefore, there must be some association between the breakdown and repair, but the exact nature of the association is as yet unknown.

Summary

Actinomycin D inhibits dark repair of UV-damage in *Bacillus subtilis*. This inhibition is not due to an inhibition of mRNA or induced enzyme synthesis. Actinomycin D prevents the removal of UV photoproducts from DNA. After a lag, breakdown of

DNA occurs in UV irradiated cells in the presence of actinomycin. It appears likely that there is no direct relationship between the dark repair process and the massive breakdown of DNA to acid-soluble fragments.

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