MECHANISM OF PHOTOINDUCED AND ANTIMYCIN A-INDUCED CAROTENOID SYNTHESIS IN MYCOBACTERIUM MARINUM. EFFECT OF PROFLAVINE*

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A number of non-photosynthetic microorganisms produce carotenoids only when illuminated. Some of the organisms most recently used to study this light-induced carotenoid synthesis are Mycobacterium sp. (Rilling, 1964; Batra and Rilling, 1964), Mycobacterium marinum (Mathews, 1963; Batra and Rilling, 1964) and Fusarium aquaeductuum (Rau, 1967a). Studies with mycobacteria have shown that the light-induced carotenoid synthesis consists of a light reaction which is followed by a series of dark metabolic reactions that eventually lead to substantial carotenogenesis (Rilling, 1964; Batra and Rilling, 1964). The light reaction is independent of temperature and requires oxygen and light; the dark reactions are temperature-dependent and also require oxygen. We have also reported that antimycin A can induce carotenoid synthesis in M. marinum in the absence of light (Batra, 1967). Chloramphenicol and puromycin blocked both the light-induced and the antimycin A-induced carotenoid synthesis. This suggested that the two inhibitors probably act by inhibiting protein synthesis (carotenogenic enzymes).

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In this paper, we report that proflavine (80μg/ml) strongly inhibits both the light-induced and the antimycin A-induced carotenoid synthesis in M. marinum. Inasmuch as proflavine is believed to block the process of transcription, it is probable that a DNA-directed RNA is synthesized when the bacteria are illuminated or when antimycin A is added.

EXPERIMENTAL PROCEDURE

Organism: M. marinum (ATCC 927) was grown strictly in the dark as previously described (Batra, 1967). After harvesting, bacteria were frozen until used.

Induction of Carotenoid Synthesis: Frozen bacteria were thawed, washed twice with 20 times their weight of 0.05 M phosphate buffer, pH 8.0, and finally suspended in the same buffer to obtain a 3.3% suspension. Carotenoid synthesis was induced either with light (20 minutes of 700 foot-candles) at 0° or with 37.5 μM antimycin A. After induction, the bacteria were incubated in the dark at 30° in air with shaking. Proflavine, in aqueous solution, was added during the course of this incubation.

Extraction of Carotenoids: Incubation was terminated with 0.5 ml of 40% trichloracetic acid and the bacteria were extracted twice with acetone-methanol (7:2) and once with ethyl acetate as described previously (Batra, 1967). This procedure resulted in the extraction of both the carotenoids and proflavine. In order to remove proflavine the extracts were mixed with 60 ml of saturated NaCl solution and Skellysolve B. Under these conditions, proflavine remained in the aqueous phase. Absorbance of the organic phase at 450 mμ was used to determine the amount of carotenoids, assuming $E^{1\%}_{1cm} = 2500$ as is the case for most carotenoids (Goodwin, 1955).

RESULTS AND DISCUSSION

Data in Fig. 1 indicate that the addition of proflavine immediately after induction strongly inhibited both the light-induced and the antimycin A-induced carotenoid synthesis in M. marinum. Maximal inhibition of carotenogenesis was achieved with 80 μg/ml proflavine.

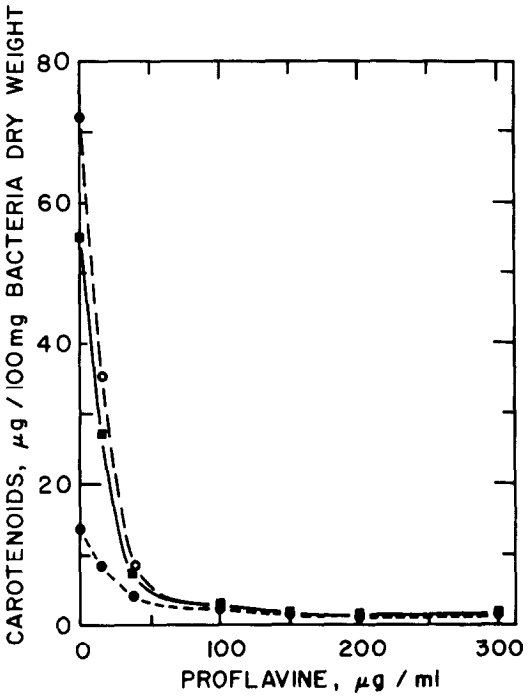

Fig. 1 Effect of proflavine concentration on carotenoid synthesis in M. marinum. Bacteria suspended in 50 mM phosphate (pH 8.0) were induced in the following manner: Light-induction (●)— bacterial suspension was illuminated with 700 foot-candles of light from two cool white fluorescent tubes for 20 minutes in air; antimycin A-induction (■)— ethanolic solution of antimycin A(37.5 μM) was added to 25-ml Erlenmeyer flasks, ethanol was evaporated with N_2 and bacterial suspensions were added; and light plus antimycin A-induction (○)— illuminated bacterial suspensions were added to flasks containing 37.5 μM antimycin A. After illumination and/or antimycin A addition, 50 mM glycerol, 3 mM $(NH_4)_2$ SO_4 and varying amounts of proflavine were added and incubation was started in the dark. After 24 hours of incubation, carotenoids were extracted; the amounts of carotenoids synthesized are shown on the y-axis.

In order to study the kinetics of proflavine inhibition of carotenoid formation, proflavine was added at various times during the course of dark incubation. At the end of the incubation period (40 hours), carotenoids were extracted and the amounts determined. Data are plotted in Fig. 2. When proflavine was added at 0-time, carotenoid synthesis was completely inhibited. However, as the addition was delayed, progressively more and more carotenoids were synthesized. In the light-induced system, proflavine addition had no effect after 4 hours. In the

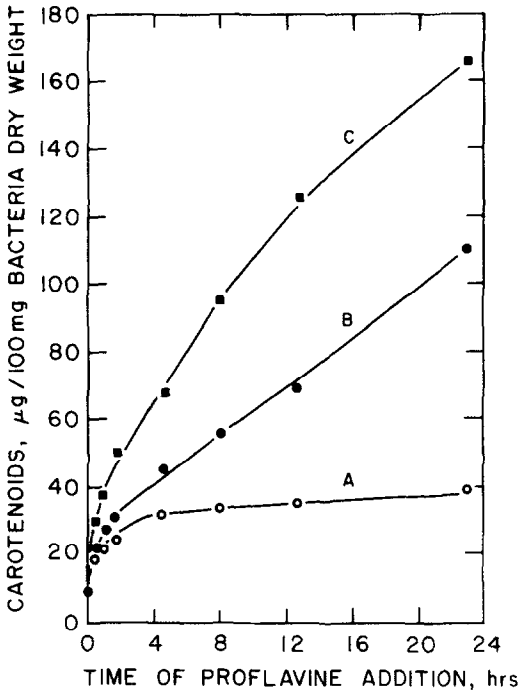

Fig. 2 Kinetics of proflavine inhibition of carotenogenesis in light-induced (A), antimycin A-induced (B), and antimycin A plus light-induced (C) bacteria. Induction was performed according to the procedure described in the legend to Fig. 1. After induction, 50 mM glycerol and 3 mM $(NH_4)_2 SO_4$ were added and incubation was started in the dark. Proflavine (80 μg/ml) was added at designated times during the course of this incubation. Total incubation time was 40 hours; carotenoids synthesized during this time are shown on the y-axis.

antimycin A-induced and antimycin A plus light-induced systems, on the other hand, proflavine addition continued to have an effect for more than 20 hours.

Since the ability of proflavine (and other acridines) to form complexes with DNA (Peacocke and Skerrelt, 1956; Lerman, 1961) and thereby inhibit DNA replication and transcription (Lerman, 1964; Nicholson, 1966) is well established, kinetics of proflavine inhibition of carotenogenesis can be explained in the following manner. In the light-induced system, light either acts by photo-oxidizing (O_2 is required during illumination) a repressor present in the dark-grown bacteria or by producing a photo-oxidized metabolite that can act as an inducer for the synthesis of carotenogenic enzymes. Similarly, antimycin A may perform the same function. However, since the inductive effects of light and antimycin A are additive (Figs. 1 and 2; Batra, 1967), the two must act at

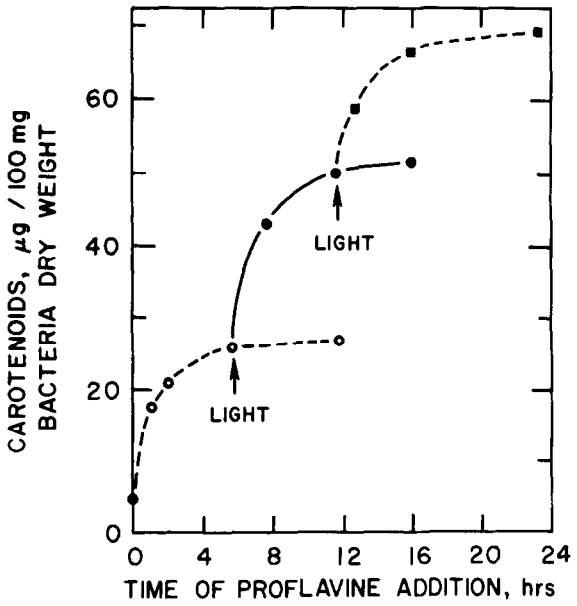

Fig. 3 Effect of proflavine on carotenoid synthesis in periodically illuminated bacteria. Bacterial suspensions in 25-ml Erlenmeyer flasks were illuminated according to the procedure given in the legend to Fig. 1. After the addition of 50 mM glycerol and 3 mM $(NH_4)_2 SO_4$, bacterial suspensions were incubated in the dark for 5 hours. During the course of this incubation, proflavine was added at various times. After 5 hours, all flasks were chilled and flasks which had not received proflavine were illuminated for the second time. All flasks were again incubated in the dark for 5 hours and proflavine was added at various times. At the end of this incubation, all flasks were chilled and flasks to which no proflavine had been added, were illuminated for the third time. Incubation was then started and proflavine was added at various times. Total incubation time was 40 hours; carotenoids synthesized during this time are shown on the y-axis.

different sites. Regardless of whether the induction is with light or with antimycin A, once the system is derepressed bacteria can synthesize DNA-directed RNA so that the synthesis of carotenogenic enzymes can occur. In the light-induced system, proflavine continued to have an effect for 4 hours. Thus, one must assume that there is continuous synthesis of m-RNA up to this time. The reason that the transcription process is turned off at this time is that possibly the repressor substance reaccumulates or that the photo-oxidized metabolite acting as an inducer is degraded. If this is true, then a second illumination at this time should again make the bacteria sensitive to proflavine addition and this indeed happens (Fig. 3). In the antimycin A-induced system, proflavine continued to have an effect for more than 20 hours. This is due to the fact that antimycin A was present throughout the dark incubation and thus it kept the system in the derepressed state so that the synthesis of DNA-directed RNA and the de novo synthesis of carotenogenic enzymes continued to occur. These enzymes subsequently catalyzed the synthesis of carotenoids.

A tentative mechanism of how light and antimycin A may induce carotenoid synthesis in M. marinum is shown in Fig. 4. It should be noted that this mechanism accounts for the additive effects of antimycin A and light.

Rau (1967b) has recently reported that sulfhydryl reagents p-chloromercuribenzoate (PCMB) and p-hydroxymercuribenzoate (PHMB) can substitute for light for carotenoid formation in F. aquaeductuum. He has shown that actidione, a biosynthetic inhibitor of proteins, inhibits both the light-induced and the PCMB (or PHMB)-induced carotenoid formation. Our preliminary work indicates that PCMB (or PHMB) does not induce carotenogenesis in M. marinum and that antimycin A has no effect in F. aquaeductuum. It is probable that although the initial light-mimicing effect by chemicals may be different in the two organisms,

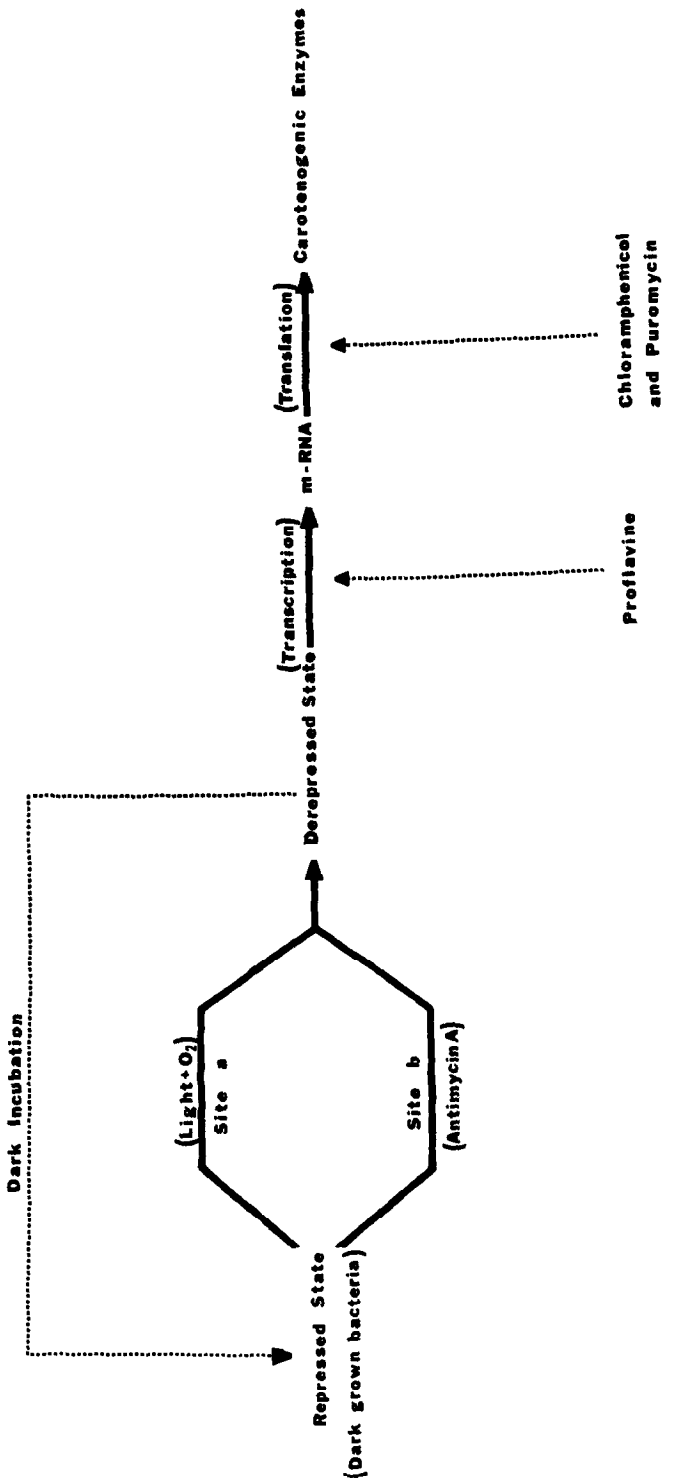

Fig. 4 Proposed mechanism for light-induced and antimycin A-induced carotenoid synthesis in M. marinum.

the basic mechanism as outlined in Fig. 4 for M. marinum may apply equally well to F. aquaeductuum.

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