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**SUMMARY**

*Mycobacterium marinum* produces carotenoids when exposed to light or when antimycin A is added. Structural characteristics of antimycin A in relationship to its induction of carotenoid synthesis were determined in order to make comparisons with the characteristics required for its inhibition of the electron transport system. All four components of the antimycin A complex (A₁, A₂, A₃, and A₄) were equally active. Studies with a number of derivatives and synthetic analogues showed that the substituted dilactone ring and the phenolic hydroxyl group, but not the N-formyl (or an N-carbonyl) group, are indispensable for the induction activity of antimycin A.

Since the N-formyl group (together with the phenolic hydroxyl group and the dilactone ring) is required for the inhibitory activity of antimycin A in the electron transport system, the possibility that antimycin A induces carotenogenesis by interfering in the electron transport system was, therefore, discounted. Additional evidence for the lack of involvement of the electron transport system in the induction of carotenogenesis was provided when it was found that the replacement of the dilactone ring with a long alkyl chain resulted in the total loss of induction activity but not of inhibitory activity. A hypothesis that antimycin A and light induce carotenogenesis by inducing protein synthesis has been evaluated.

Antimycin A is an antibiotic complex produced by a number of species of the genus *Streptomyces* (1). The isolation of this complex was first achieved by Dunshee et al. (2). Since then a number of studies have shown that this complex is remarkably effective in inhibiting the electron transport system of aerobic organisms, particularly yeast, fungi, and animals (1). The site of inhibition is at the cytochrome level (3, 4). Structure-activity relationship studies have shown that the phenolic hydroxyl group, the substituted dilactone ring, and the N-formyl group are indispensable in the inhibition of electron flow (5–7). The work on the chemistry and the mode of action of antimycin A in the animal system has been reviewed by Rieske (1).

Previous studies in this laboratory have shown that the non-photoautotrophic bacterium, *Mycobacterium marinum*, produces carotenoids only when exposed to light (8). This photo-induced carotenoid synthesis consists of two phases (9): (a) a photochemical reaction which, in addition to light, requires O₂ and is temperature-independent; O₂ appears to participate directly in the photochemical reaction rather than as an electron acceptor (10); and (b) a series of dark reactions which are temperature-dependent, require O₂ but not light, and lead eventually to substantial carotenoid production. Further studies have shown that antimycin A is capable of inducing carotenoid synthesis in the absence of light in *M. marinum* (8, 11). Since the induction effects of light and antimycin A were additive, it was concluded that the sites of action of these two agents are apparently different. Two observations suggested that antimycin A induces carotenoid synthesis by a mechanism other than through its effect on the electron transport system (8). (a) Addition of antimycin A did not depress O₂ uptake by the organism; and (b) 2N-heptyl-4-hydroxyquinoline-N-oxide, which is known to block electron flow at the same site as antimycin A (12), was ineffective in induction. It is now believed that antimycin A induces carotenoid synthesis by inducing the synthesis of carotenogenic enzymes. Three observations have provided indirect evidence for this hypothesis. (a) A lag period of 4 hours following induction with antimycin A or light before carotenoids appear would suggest the time needed for the synthesis of enzymes (8); (b) a nitrogen source is required (13); and (c) inhibitors of transcription and translation inhibit carotenoid production (8, 13, 14). Our working hypothesis, therefore, is that antimycin A and light induce carotenoid production by derepressing the genetic sites resulting in the synthesis of mRNA and carotenogenic enzymes. This hypothesis has received further support from a recent investigation of Elson et al. (15), who have reported that antimycin A stimulates protein synthesis and cell division in *Tetrahymena pyriformis*.

Since the induction of carotenoid synthesis by antimycin A...
presents a very unusual and paradoxical effect, we have conducted a structure-induction activity relationship study of antimycin A. In addition to identifying the active molecular components, these studies have provided proof that antimycin A in *M. marinum* induces carotenoid production not by blocking electron flow but by some other mechanism, perhaps by inducing enzyme production.

**EXPERIMENTAL PROCEDURE**

*M. marinum* (ATCC 927) was cultured in a liquid medium in the dark as previously reported (8). Cells were harvested at the end of the log phase of growth and frozen until needed. Frozen bacteria were thawed, washed twice with 50 mM phosphate buffer, pH 8.0, and suspended in the same buffer to obtain a 3.3% suspension.

Unless stated otherwise, antimycin A (or its derivative) was added as an ethanolic solution to a 25-ml Erlenmeyer flask. Ethanol was removed with a stream of Nz, and 3.0 ml of the bacterial suspension was added. When needed, illumination was for 30 min at 0° with 700 footcandles of light from two cool white fluorescent tubes. This dose of light is sufficient for saturation (8). After the addition of 30 mM glycerol and 3 mM (NH₄)₂SO₄, bacterial suspensions were shaken and incubated in the dark at 30° for 24 hours. Incubation was terminated by the addition of 0.2 ml of 20% trichloroacetic acid. Carotenoids were extracted twice with 3 ml of a mixture of acetone and methanol (7:2) and once with 2 ml of ethyl acetate. Extracts were pooled and absorbance was determined at 450 nm. To calculate the amount of carotenoids as µg per 100 mg of bacteria, dry weight, an extinction coefficient, ε₄₅₀, of 2500 was used as an average value (16).

All solvents were redistilled. The chemicals used were the highest quality available. Antimycin A was a generous gift from Dr. Donn Buyske, Ayerst Laboratories, Montreal, Canada. Dr. Buyske also provided A₁, A₂, A₃, and A₄ components of the antimycin A complex. To identify the active substituents on the antimycin A molecule, the following derivatives and synthetic analogues were tested with respect to their ability to stimulate carotenoid production with and without light: antimycin A-O-methyl ether, blastmycin acid, deformylantimycin A hydrochloride, deformyl-N-acetylantimycin A, antimycin A diacetate, antimycin A triacetate, and a number of analogues in which the dilactone ring had been replaced with an alkyl chain. A number of these derivatives and synthetic analogues of antimycin A were kindly supplied by Dr. F. M. Strong, University of Wisconsin; other derivatives were prepared in this laboratory. The procedure or reference for the preparation of the derivatives (or both) is given under “Results and Discussion,” where data obtained from the use of each derivative are discussed.

**RESULTS AND DISCUSSION**

**Activity of Individual Components of Antimycin A Complex—** In all previous studies, the antimycin A complex was used. Since this complex consists of at least four components designated as A₁, A₂, A₃, and A₄ (17, 18), the activity of the individual components in the induction of carotenogenesis in *M. marinum* was determined. Data in Table I show that each component was as active as the antimycin A complex. The optimal concentration in each case was 40 µM. It should also be noted that the induction effects of light and each of the components were additive as is the case with the antimycin A complex and light. Unless stated otherwise, in all subsequent studies the antimycin A complex was used.

Since the antimycin A components are believed to differ structurally in the alkyl substituent of the dilactone ring (1), it would appear that the alkyl chain does not participate directly in the induction activity of antimycin A. A similar conclusion is reached when the inhibitory effects of the individual components of the antimycin A complex on the electron transport system are determined (1).

**Evaluation of Dilactone Ring Substituent—** To determine the importance of the substituted dilactone ring in the induction of carotenoid synthesis, a number of synthetic analogues of antimycin A lacking the substituted dilactone ring were used. These compounds were substituted amidines of 3-formamidosalicylic acid and differed from antimycin A in having an alkyl chain substituting for the dilactone ring structure (7). In each case, complete loss of the inductive activity was observed (Table II). This was true regardless of the length of the alkyl chain or its substituents.

In the inhibition of the electron flow, the substituted dilactone ring is thought to confer a proper degree of lipid solubility to the molecule (7). This conclusion was reached because the com-
Ability of 3-formamidosalicylamides to induce carotenogenesis

Experimental conditions were similar to those indicated in Table I. Concentration of the compounds was 40 pm. Same results were obtained when the concentration was increased to 80 pm.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>R Group</th>
<th>Carotenoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Antimycin A complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Blastmycic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. N-Hexyl-3-formamidosalicylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. N-Octyl-3-formamidosalicylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. N-Decyl-3-formamidosalicylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. N-Dodecyl-3-formamidosalicylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. N-Decaoyl-3-formamidosalicylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. N-Phenyl-3-formamidosalicylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. N-Benzyl-3-formamidosalicylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. N-(β-Phenethyl)-3-formamidosalicylamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. N-(Ethyl-γ-phenylpropanoyl)-3-formamidosalicylamide</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II

pounds in which the dilactone ring had been replaced with a long straight chain alkyl substituent still possessed considerable inhibitory activity (7). In fact, the extent of inhibition by the analogues was proportional to the length of the alkyl chain. In contrast, our studies show that the induction activity is completely lost when the dilactone ring is replaced with a side chain regardless of its length or its substituents. Thus, the conclusion is inescapable that in the induction of carotenogenesis by antimycin A, the substituted dilactone ring may not only confer a proper degree of lipid solubility on the molecule but more importantly may also be involved directly in the interaction of antimycin A with its site of action. The nature of the interaction between antimycin A and its induction site is pure conjecture at this time.

Evaluation of N-Formyl Group—If antimycin A was deformedylated (5), the resulting compound remained considerably active in the induction of carotenogenesis, as was observed by means of deformylantimycin A hydrochloride (Fig. 1) and deformyl-N-acetylantimycin A (Fig. 2). Concentrations needed for maximal induction were approximately 40 pm in both cases, which is also the concentration of antimycin A needed for maximal induction (8). It should be noted that the induction effects of light and the deformylated compounds were additive, as were those of light and antimycin A (8, 13). These results, therefore, show that the N-formyl group (or an N-carbonyl group) is not required for the induction activity of antimycin A. In contrast, however, the N-formyl (or an N-carbonyl) group is required for the inhibitory activity of antimycin A in the electron transport system (5).

Evaluation of Phenolic Hydroxyl Group—To evaluate the role of the phenolic hydroxyl group in the induction of carotenogenesis, the O-methyl ether derivative of antimycin A was prepared and crystallized according to the procedure of van Tamelen et al.
The melting point of the crystals was 109–110°. The purity of the compound was further checked with nuclear magnetic resonance spectroscopy by recording the spectrum on a Varian HA-60-IL spectrometer with tetramethylsilane as the internal standard and CDCl$_3$ as the solvent in each case (Fig. 3). The spectrum of this compound is as expected. The downfield resonance due to the phenol proton has been replaced by an intense singlet at 6.1 $\tau$, attributable to the methoxy protons. More subtle changes between 1 and 3.5 $\tau$ are probably due to the loss of hydrogen bonding effects of the phenol proton which was removed.

The use of the methylated antimycin A for the induction of carotenoid synthesis has provided interesting results. Although the compound was unable to induce carotenoid synthesis in the dark as can antimycin A, it has the ability to stimulate many fold the light-induced carotenoid synthesis (Fig. 4). For example, the addition of 40 $\mu$M antimycin A-O-methyl ether had little effect on induction in the organism not exposed to light, but when it was given together with light, carotenoid production more than tripled. Thus, the methylation of antimycin A leads to its synergistic effect with light.

If the phenolic hydroxyl substituent was replaced with an O-acetyl group (5), the resulting derivatives (antimycin A diacetate and antimycin A triacetate) were only slightly less active than the parent compound (Table III). These results appear contradictory to what was observed with antimycin A-O-methyl ether, but the activity of the acetate derivatives is probably the result of the unstable nature of the O-acetyl linkage in the aqueous system (pH 8.0) used (1).

**Concluding Comments on Relationship of Structure and Activity of Antimycin A**—From the above results, it can be concluded that the phenolic hydroxy group and the substituted dilactone ring, but not the N-formyl group (or an N-carbonyl group), individually and collectively are required for the induction of carotenoid synthesis in the absence of light in *M. marinum*.

When the effects of various derivatives and synthetic analogues of antimycin A on the inhibition of the electron flow are studied, one finds that all three substituents, the phenolic hydroxy group, the substituted dilactone ring, and the N-formyl group (or an N-carbonyl group) are required (5–7). Since the N-formyl group is required for inhibition of the electron flow (5), but not for the induction of carotenoid synthesis, it provides direct proof that antimycin A must induce carotenogenesis by a mechanism other than through its interference in the electron transport system. Supporting evidence for the lack of involvement of the electron transport system in the induction of carotenogenesis has been previously provided (8). (a) Antimycin A addition does not depress O$_2$ uptake by the organism, and (b) 2N'-heptyl-4-hydroxyquinoline-N-oxide, which blocks electron flow at the same site as antimycin A (12), is unable to induce carotenogenesis.

**Further Studies with Antimycin A-O-methyl Ether**—As mentioned, the effect of antimycin A-O-methyl ether in the induction
TABLE III

Ability of antimycin A di- and tri-acetates to induce carotenoid synthesis

Concentration of the compounds was 40 μM. Same results were obtained when the concentration was increased to 80 μM. Other conditions were similar to those described in Table I.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Structure</th>
<th>Carotenoid (μg/100 mg bacteria, dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without light</td>
</tr>
<tr>
<td>1. None</td>
<td></td>
<td>1.9</td>
</tr>
<tr>
<td>2. Antimycin A</td>
<td><img src="image" alt="Structure" /></td>
<td>98.4</td>
</tr>
<tr>
<td>3. Antimycin A diacetate</td>
<td><img src="image" alt="Structure" /></td>
<td>76.8</td>
</tr>
<tr>
<td>4. Antimycin A triacetate</td>
<td><img src="image" alt="Structure" /></td>
<td>70.2</td>
</tr>
</tbody>
</table>

![Graph](image)

**Fig. 5.** Carotenogenesis as a function of the time of illumination in the presence (●) and absence (○) of antimycin A-O-methyl ether, 40 μM. Other conditions were similar to those in Table I.

The synergistic effect was observed when the concentration of the compound was kept constant and the dose of light was varied (Fig. 5). For instance, bacteria induced with 15 min of light produced 20 μg of carotenoid per 100 mg of bacteria, dry weight, whereas the addition of antimycin A-O-methyl ether without light resulted in the production of only 5 μg of carotenoid per 100 mg of bacteria, dry weight. But if both were given, the bacteria produced 60 μg of carotenoid per 100 mg of bacteria, dry weight.

![Graph](image)

**Fig. 6.** Time course of carotenogenesis. (●), antimycin A-O-methyl ether alone (40 μM); (○), 30-min light and then dark incubation; (▲), antimycin A-O-methyl ether (40 μM) and 30-min light followed by dark incubation; (■), incubation in continuous light. Other experimental conditions were similar to those in Table I.

To explain the synergistic effect, some parameters of the effect of antimycin A-O-methyl ether on the induction of carotenogenesis were determined. The time course study of carotenogenesis in the organism which was exposed to light for a short time and then incubated in dark in the presence of antimycin A-O-methyl ether showed a lag period of approximately 4 hours, after which the rate of carotenoid production increased rapidly and then tapered off after about 44 hours (Fig. 6). Similar kinetics was observed in the organism which was dark-incubated in the absence of antimycin A (13). In contrast, when the organism was only exposed to light for a short time and then dark-incubated, the rate of carotenoid production...
approached zero after about 24 hours, approximately 20 hours earlier than the antimycin A-O-methyl ether plus light (initial) system. However, when the organism was incubated in continuous light rather than in the dark, the kinetics of carotenogenesis was similar to that of the antimycin A-O-methyl ether plus light (initial) system (Fig. 6).

The lag period of 4 hours observed following induction with antimycin A-O-methyl ether plus light indicated that certain events occurred preparatory to actual carotenoid production. Perhaps carotenogenic enzymes were synthesized during this period. Use of chloramphenicol has provided evidence for protein synthesis de novo following induction. When chloramphenicol was added immediately after induction, carotenoid synthesis was completely blocked (Fig. 7). If the addition was delayed, progressively more carotenoids were synthesized. When the kinetics of the chloramphenicol inhibition in the light (initial) system was compared with that of the antimycin A-O-methyl ether plus light (initial) system or with that of the antimycin A system (8), it was found that chloramphenicol addition had no effect after 4 hours in the light (initial) system, whereas it had an effect on the latter systems beyond 4 hours of incubation. A similar situation was found when the organism was incubated in continuous light rather than in the dark (Fig. 7). This suggested that in the light (initial) system, protein synthesis had terminated after 4 hours, but not in the antimycin A-O-methyl ether plus light (initial) system or in the antimycin A system, or in the continuous light system. When the organism was given antimycin A-O-methyl ether alone, chloramphenicol addition had no effect (Fig. 7), indicating that protein synthesis had not been initiated.

The synergistic effects observed when the light-exposed organism is dark-incubated in the presence of antimycin A-O-methyl ether can now be explained as follows. The methylation of antimycin A results in its loss of ability to induce protein synthesis, but once light has been given, the methylated antimycin A gives the organism continuous stimulus, resulting in uninterrupted protein synthesis. This leads to much larger carotenoid production than would be expected if the derivative and light were to be given separately.

Elson et al. (15) have recently shown that antimycin A promotes protein synthesis and cell division in T. pyrofomis. In this instance again, electron transport system is not involved. It should be very interesting to relate the structural characteristics of antimycin A with its stimulation of protein synthesis and cell division in T. pyrofomis. Such a study will more firmly establish this paradoxical effect of antimycin A at the level of protein synthesis. Studies are also underway in this laboratory to provide direct evidence for the action of antimycin A at this level.

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REFERENCES

A Study of the Relationship of Structure and Activity of Antimycin A in the Induction of Carotenoid Synthesis in *Mycobacterium marinum*

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