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Inhibition of Carotenoid Synthesis in *Myxococcus fulvus (Myxobacterales)*

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Abstract. 1. The inhibitory effects of CPTA, nicotine, DPA, and San 6706 on carotenogenesis in *Myxococcus fulvus* were investigated.

2. The effects of CPTA, D-nicotine, and L-nicotine were very similar. The action of the drugs wasadditive. The cyclization was inhibited at low doses, the introduction of the hydroxyl group at C-1′ at higher doses. Lycopene accumulated at high drug concentration. The mode of action of the inhibitors is discussed.

3. In a carotenoid mutant of *M. fulvus* a stimulation of the "7,8-dehydrogenase" by CPTA was observed.

4. The specific carotenoid content of bacteria was increased by DPA due to an enhanced formation of phytoene. At low doses of DPA small amounts of an intermediate carotenoid glucoside ester, a 7,8-dihydro derivative, were detected.

5. DPA was taken up by the plasma membrane. Quantitative removal of DPA by washing was not possible.

6. San 6706 specifically and reversibly blocked the desaturation of phytoene.

Key words: Myxobacteria — Carotenoid Glucosides — Carotenoid Synthesis — Nicotine — Diphenylamine — Herbicides.

Myxobacteria are characterized by the presence of monocyclic and sometimes acyclic carotenoids containing glycosyl groups at C-1′ and double bonds at C-3′,4′. The main pigment of *M. fulvus* is myxobacton ester (1′-glucosyloxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-4-one ester) (Kleinig *et al.*, 1970; Reichenbach and Kleinig, 1971) which is a compound of the plasma membrane (Kleinig, 1972). During the investigations on the biosynthesis of these pigments, several substances considered as possible inhibitors have been examined. The aim of the present report is to describe the effects on carotenogenesis and its regulation in *M. fulvus* of four inhibitors, 2-(4-chlorophenylthio) triethylamine hydrochloride (CPTA), nicotine, diphenylamine (DPA), and 4-chloro-5-(dimethylamino)-2-α,α,α(trifluoro-m-tolyl)-3(2H)-pyridazinone (San 6706), which are very promising for further studies on myxobacteria.

Abbreviations Used. CPTA = 2-(4-chlorophenylthio) triethylamine hydrochloride; DPA = diphenylamine; San 6706 = 4-chloro-5-(dimethylamino)-2-α,α,α(trifluoro-m-tolyl(-3(2H)-pyridazinone.

Materials and Methods

M. fulvus strain Mxf2 and a carotenoid mutant *M12* (Reichenbach, unpublished) were cultivated in Casitone liquid medium [Casitone (Difco) 1%, $MgSO_4$ 0.1%, pH 6.8, not adjusted] as 100 ml batches in 500-ml Erlenmeyer flasks at 30°C on a rotary shaker. The cultures were illuminated with 3 fluorescent tubes, because carotenoid synthesis is light-stimulated in this organism.

Cultures were harvested in the logarithmical growth phase. Densities were measured at 623 nm.

Inhibitors were applied in water (nicotine) or in 0.5 ml acetone solution (CPTA, DPA, San 6706) in the appropriate concentrations. D-nicotine was kindly provided by Prof. K. Decker, Freiburg, CPTA by Amchem Products, Reading, England, and San 6706 by Sandoz AG, Basel, Switzerland.

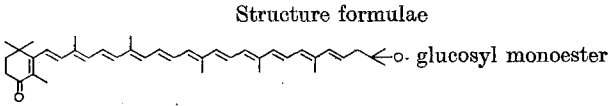
Binding studies with ^{3}H-nicotine (spec. activity 308 mCi/mmol, The Radiochemical Centre. Amersham. England) on the plasma membrane fraction (Kleinig, 1972) and the 100000 g supernatant were performed according to the classical methods of equilibrium dialysis and centrifugation test. Concentration ranges of 0.01—0.0001 µmoles nicotine per 10 mg protein were used. Activities of aliquots of the samples were measured in 10 ml of 90% aqueous Insta Gel (Packard Instruments) in a Tri Carb.

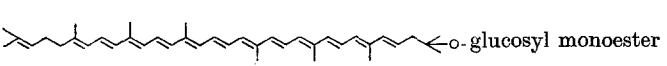
Carotenoid pigments and menaquinone were extracted with acetone and separated on silica gel thin layers with petroleum ether/diethylether/acetone mixtures as reported previously (Kleinig, 1972; Kleinig and Reichenbach, 1973b). For quantitative measurements of carotenoid pigments the ε values of the corresponding carotenes were used, which were calculated from the $E^{1\%}_{1cm}$ values given by Davies (1965): phytone 68000, phytofluene 73000, ζ-carotene 123000, neurosporene 161000, lycopene 185000, γ-carotene 166000. An ε value of 18600 was used for menaquinone (Isler *et al.*, 1958). DPA was determined according to the procedure of Salton and Schmitt (1967)

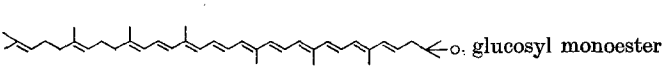
Results and Discussion

When *M. fulvus* was grown under the standard conditions described above, generation time was about 6 h and the specific carotenoid content was about 0.45 (nmoles per mg dry weight) during the log growth phase. The pigment pattern of untreated cultures was 80% myxobacton ester (I), 15% 4-ketotorulene, and 5% minor components including traces of phytoene and phytofluene, carotenes, myxobactin ester, hydroxylated pigments and others (see ref. Reichenbach and Kleinig, 1971).

Structure formulae

(I) 

(II) 

(III) 

The Effect of CPTA and Nicotine

CPTA was found to accumulate lycopene in certain plants and fruits that normally do not accumulate this pigment (Coggins *et al.*, 1970; Yokoyama *et al.*, 1971; Hsu *et al.*, 1972). This effect was interpreted as an inhibition of the cyclases (Hsu *et al.*, 1972; Batra *et al.*, 1973) similar to the effect caused by nicotine (Howes and Batra, 1970).

The effect of CPTA on carotenogenesis in *M. fulvus* is shown in Fig. 1. At low concentrations (0.1—10 μM) the cyclization reaction was gradually inhibited and myxobacton ester (I) was replaced by an acyclic carotenoid glucoside ester (II). The structure of this pigment has been described elsewhere (Kleinig and Reichenbach, 1973a). At higher doses (20—100 μM) a second effect became evident. The carotenoid glucosides decreased rapidly and lycopene accumulated. This means that the introduction of the hydroxyl group at C-1′ of carotenes was inhibited. These two effects of CPTA were nearly identical with the effects caused by nicotine in *M. fulvus* (Kleinig and Reichenbach, 1973b), CPTA, however, acts at a hundredfold lower dose. The action of both drugs was, indeed, additive as can be seen from the log concentration-response curves in Fig. 2. It can not be decided from this graph, however, whether the interaction is functional or competitive, although the latter is more probable, since both reactions, the cyclization and the hydroxylation at C-1′, may

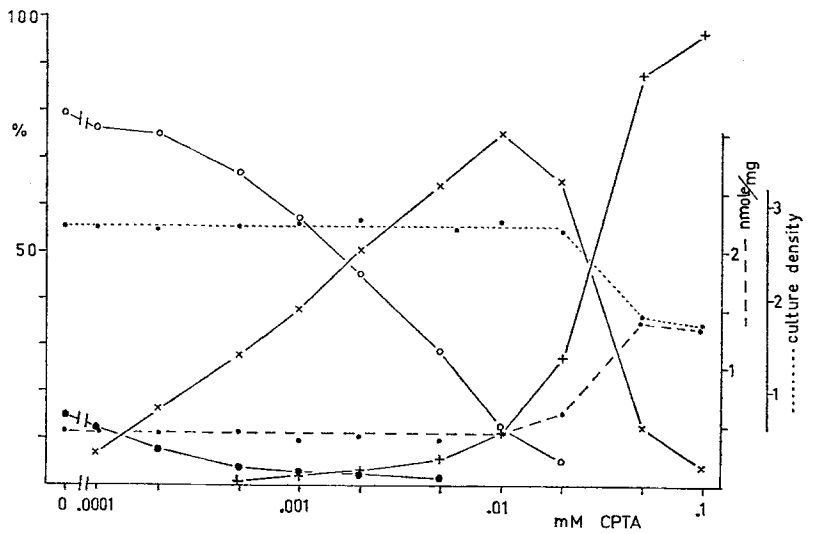


Fig. 1. Effect of CPTA on carotenoid synthesis in *M. fulvus*. Values are given in per cent of total carotenoid and in nmoles total carotenoid per mg dry weight. –o–o– myxobacton ester (I); –×–×– acyclic glucoside ester (II); –•–•– ketotorulene; –+–+– lycopene

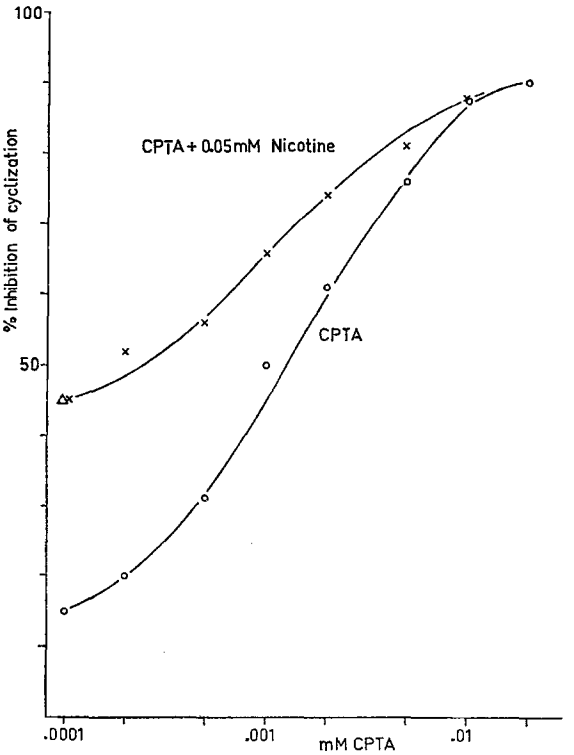


Fig. 2. Log concentration-response curves for CPTA and CPTA in the presence of 0.05 mM nicotine. The triangle gives the value for 0.05 mM nicotine alone. The formation of the acyclic glucoside ester (II) at the expense of myxobacton ester (I) is given

involve protonation at C-2/C-2′ as the first stage (Liaaen-Jensen, 1963; Singh *et al.*, 1973). This could mean that both inhibitors act by interference with this reaction itself and not e.g. by binding to the corresponding enzymes as suggested by Batra *et al.* (1973). The former interpretation is also supported by the fact, that synthetic D-nicotine has the same effects as naturally occurring L-nicotine in *M. fulvus*. Furthermore, preliminary binding studies with ^{3}H-nicotine on isolated plasma membranes and soluble proteins of *M. fulvus* were negative. The action of CPTA at lower doses than nicotine can be explained by the better lipid solubility of CPTA, since both the cyclization and the hydroxylation are supposed to be membrane-associated. Expectedly, although the effects of CPTA were reversible, it was considerably more difficult to remove this drug by washing than nicotine.

Lycopene was the only carotene synthesized at higher doses of CPTA (20—100 μM). Especially, no 3′,4′-didehydrolycopene could be detected.

This could indicate that the hydroxylation step (and the glycosidation) at C-1′ preceeds the introduction of the C-3′,4′-double bond in the biosynthetic pathway leading to myxobacton ester. The occurrence of 4-ketotorulene, however, can not be explained than without the assumption of an additional mechanism. Neurosporene was also not accumulated, although this pigment seems to be the step in the carotene pathway from which the glycoside pathway branches off (see below).

The specific carotenoid content of the bacteria remained constant over a wide range of CPTA concentrations as can be seen from Fig. 1. At higher doses, however, lycopene accumulated. This could be interpreted as a specific stimulation. There is, however, another explanation which seems to be more probable in connection with two further observations. Concentrations of CPTA above 0.1 mM seriously inhibited bacterial growth. Also at lower doses, the cultures never reached the same high densities of untreated cultures at the end of the log growth phase. The same has been observed with nicotine (Kleinig and Reichenbach, 1973b). The second observation was, that *M. fulvus* generally accumulated carotenoids at the end of the log growth phase. Thus, the lycopene accumulation may simply reflect such an end-log situation. The same interpretation might be valid for the carotenoid accumulation in a flavobacterium in the presence of nicotine as observed by McDermott *et al.* (1973) and perhaps in *Blakeslea trispora* in the presence of CPTA as found by Hsu *et al.* (1972).

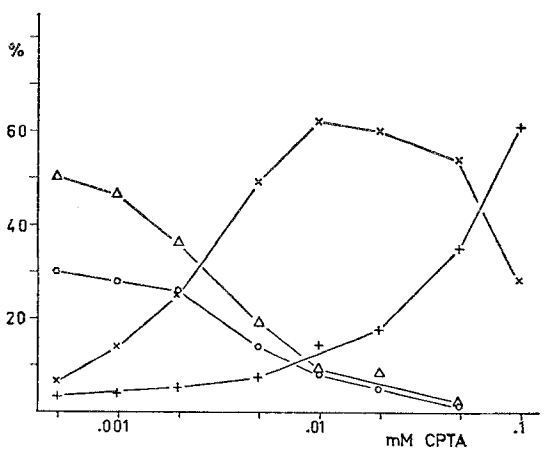


Fig. 3. Effect of CPTA on the carotenoid synthesis in the carotenoid mutant *M12* of *M. fulvus*. Values are given in per cent of total carotenoid. –o–o– myxobacton ester (I); –×–×– acyclic glucoside ester (II); –▵–▵– 7,8-dihydro derivatives (e.g. III); –+–+– lycopene

One further effect of CPTA is shown in Fig. 3. A mutant *M 12* of *M. fulvus* has been isolated (Reichenbach, unpublished) that contains appreciable amounts of 7.8-dihydroglycoside esters besides myxobacton ester. The structure of these pigments will be described elsewhere (Reichenbach *et al.*, unpublished). CPTA caused the same net effects in this mutant as in the wild strain. The acyclic glucoside ester was synthesized at lower doses, lycopene was formed at higher doses of CPTA. Unexpectedly, no 7,8-dihydro-pigments were enriched, although the corresponding dehydrogenation step somehow seems to be defective in this mutant. This indicates a stimulation of the "7,8-dehydrogenase" which can, however, not be explained at the moment. The same CPTA effect might be also responsible for the absence of neurosporene in the wild strain as reported above.

The Effect of DPA

DPA is known to suppress the formation of the more unsaturated carotenoids in many microorganisms and has been found to be very useful for elaboration of possible biosynthetic pathways and for the enrichement of new pigments (e.g. Goodwin and Osman, 1954; Liaaen-Jensen *et al.*, 1971; Davies, 1970; Malhotra *et al.*, 1970a).

The effect of increasing DPA concentrations on carotenoid formation and growth of *M. fulvus* is shown in Fig. 4. In contrast to CPTA and ni-

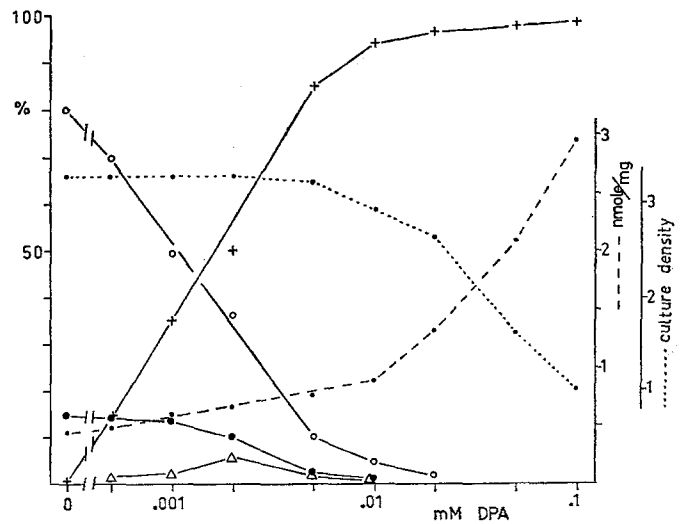


Fig. 4. Effect of DPA on carotenogenesis and growth in *M. fulvus*. Values are given in per cent of total carotenoid and in nmoles total carotenoid per mg dry weight. –o–o– myxobacton ester; –•–•– ketotorulene; –▵–▵– 7,8-dihydro derivative (III); –+–+– phytoene

cotine, DPA caused an increase of the specific carotenoid content already at very low concentration due to an enhanced formation of phytoene. A concomitant gradual inhibition of growth was also observed. Phytofluene and the other carotenes of the Porter-Lincoln series were present only in negligible concentrations. Comparatively small amounts of a less unsaturated glucoside ester, 1′-glucosyloxy-3′,4′-didehydro-1′,2′, 7,8-tetrahydro-ψ,ψ-carotene ester (III), appeared at very low doses of DPA and reached its highest concentration at 0.002 mM DPA (about 6% of total carotenoid). This pigment was also found in the mutant M 12 (Reichenbach *et al.*, unpublished) and is believed to be an intermediate in the myxobacton ester pathway.

The synthesis of isozeaxanthin instead of canthaxanthin in the presence of DPA has been found in a green alga (Gribanovski Sassu, 1972). Similar intermediates with a hydroxyl group at C-4 could not be detected in *M. fulvus*. A stimulation of carotenoid glycoside synthesis in the presence of DPA as has been reported by Ogawa *et al.* (1970) for the blue-green alga *Anabaena variabilis* was also not observed in *M. fulvus*.

The menaquinone content of *M. fulvus* was only very slightly affected by DPA if at all which is in accordance with the findings of Salton and Schmitt (1967) for some Gram-positive bacteria.

DPA was taken up into the plasma membrane of *M. fulvus* as expected. This was shown with isolated membranes. Quantitative removal of DPA from DPA-treated bacteria by washing was not possible. Bacteria were grown for 16 h with 0.02 mM DPA, washed, and reincubated in fresh medium for another 8 h (more than one generation time). At the beginning of the reincubation period, 0.02 mg DPA were found in the extracted bacterial lipids, which corresponds to 6% of the originally applied amount. At the end of the reincubation period the same absolute amount was measured. During this time phytoene was almost the only carotenoid formed. At the end of reincubation, small amounts of myxobacton ester appeared, obviously due to the dilution of bound DPA by growth. This tight retaining within the membrane which is certainly due to the highly lipophilic nature of DPA makes this drug not so suitable for reversible inhibition kinetics in *M. fulvus*. For enrichment of the 7,8-dihydro derivative (III) and perhaps other minor components, however, DPA is very useful.

The Effect of San 6706

This herbicide inhibits the synthesis of colored carotenoids and causes accumulation of phytoene in wheat seedlings (Bartels and McCullough, 1972).

In *M. fulvus* the effect of San 6706 is very similar. After addition of 0.01 mM San 6706 the formation of all unsaturated carotenoids was im-

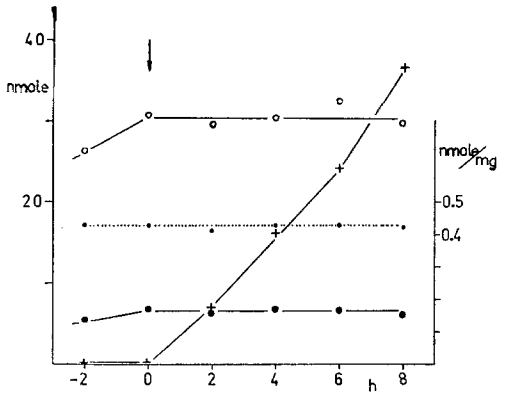


Fig. 5. Effect of San 6706 on carotenoid synthesis in *M. fulvus*. Carotenoid values are given in nmoles (from a 100 ml-culture). At 0 h (arrow) San 6706 (0.01 mM) was added to the cultures. –o–o– myxobacton ester (I); –•–•– ketotorulene; –+–+– phytoene; dotted line, specific carotenoid content (nmole per mg dry weight)

mediately prevented and phytoene, normally present only in trace amounts was the only carotenoid synthesized (Fig. 5). The absolute amounts of myxobacton ester and ketotorulene remained constant over the incubation period (Fig. 5) which is in accordance with the earlier finding that virtually no degradation or turnover of carotenoid pigments occurs in *M. fulvus* under the standard conditions used (Kleinig and Reichenbach, 1973b). At lower concentrations of the drug the synthesis of myxobacton ester was partly inhibited, intermediary pigments, however, were not detected. This indicated that San 6706 interferes very specifically with the dehydrogenation reaction on the phytoene level. At concentrations of about 0.1 mM San 6706, growth of bacteria was seriously inhibited.

San 6706 was easily removed from the bacteria by washing. In contrast to the DPA experiments, phytoene was not further accumulated after washing, and myxobacton ester was again formed. This easy removeability and the very distinct inhibitory effect makes this drug very promising for further studies on carotenogenesis and its regulation in *M. fulvus* which are in progress.

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