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Biosynthesis of Aryl Carotenoids: Studies on the Nature of the Precyclization Intermediate

Leslie Sise Leutwiler[1] and D. J. Chapman

Department of Biology, University of California, Los Angeles, California 90024, U.S.A.

The conversion of nicotine-induced pools of radioactive lycopene-^{14}C into chlorobactene was studied in *Chlorobium limicola*. Removal of the nicotine and resuspension of cells in radioactivity-free medium in the absence or presence of diphenylamine (to prevent further carotenoid synthesis) indicates that the lycopene is metabolized to chlorobactene. Results suggest that some older lycopene molecules, in the presence of nicotine, become inaccessible to the carotenogenic enzymes, and are not further metabolized upon removal of the inhibitor. Leucine is not effectively incorporated into carotenoids in *C. limicola*.

Key words: Biosynthesis (aryl carotenoid) — Carotenoid — Chlorobactene — *Chlorobium limicola* — Inhibitors (carotenogenesis).

The anaerobic photosynthetic bacteria synthesize carotenoids which differ in chemical structure from those found in aerobic organisms. The cyclic carotenoids characteristically contain aromatic end rings [e.g., chlorobactene (ϕ,ψ-carotene)] in contrast to the cyclohexenyl end rings of aerobic organisms [e.g., β-carotene (β,β-carotene)]. Cooper et al. (1963) have proposed that the aryl ring systems of carotenoids may be formed by the aromatization with accompanying methyl migration of acyclic precursor [e.g., γ-carotene (β,ψ-carotene) and β-carotene]. Moshier and Chapman (1973) showed that the aryl ring of chlorobactene isolated from *Prosthecochloris aestuarii* [formerly *Chloropseudomonas ethylica* (Pfennig and Biebl 1976)] is synthesized via the mevalonic acid pathway and involves the migration of one of the methyl groups originating from the C-3′ of mevalonic acid. The objective of this work was to determine the precyclization intermediate.

Previous work with *Chlorobium limicola* (Leutwiler and Chapman 1979) showed that nicotine inhibited the production of the aryl carotenoid, chlorobactene, and caused a corresponding accumulation of the acyclic lycopene (ψ,ψ-carotene). Removal of nicotine and resuspension in nicotine-free medium brought a small transient increase in the amount of γ-carotene, indicating the pathway for chlorobactene biosynthesis proceeds through lycopene and the alicyclic intermediate, γ-carotene. Almost all of the lycopene which accumulated during nicotine inhibition converted to chlorobactene on removal of the inhibitor. However, if de novo carotenogenesis was inhibited by adding DPA to cultures resuspended in fresh medium after removal of the nicotine, the lycopene pool that had accumulated in the presence of nicotine was not totally cyclized to chlorobactene. In order to more closely investigate this

Abbreviation: DPA, diphenylamine.

[1] Present address: Division of Biology, California Institute of Technology, Pasadena, California 91125, U.S.A.

possibility in *C. limicola*, radioactive labeling experiments were performed with acetate-2-^{14}C. These were designed to examine the conversion of nicotine-induced pools of lycopene-^{14}C on removal of the inhibitor. The results indicate that the pathway to chlorobactene proceeds via lycopene (ψ,ψ-carotene), and provide support for Kleinig's (1975) hypothesis of "assembly line" carotenoid biosynthesis.

Materials and Methods

Organism and culture conditions—Chlorobium limicola, f. thiosulfatophilum strain 6230 (DSM 249), generously provided by Drs. N. Pfennig and K. Schmidt, Institut für Mikrobiologie der Universität Göttingen, West Germany, was cultured on Pfennig's Thiorhodaceae medium (Leutwiler and Chapman 1979). Cultures (12 liters) were grown anaerobically in the light at 25°C for 1–4 days as previously described (Leutwiler and Chapman 1979). The appropriate amounts of a 1 M nicotine or 1 M DPA solution in ethanol were added aseptically at the time of inoculation.

Chemicals and solvents—Leucine-U-^{14}C in 0.01 N HCl (320 mCi/mmol) and sodium acetate-2-^{14}C (2 mCi/mmol) were obtained from New England Nuclear, Boston, MA, U.S.A. Organic solvents were either reagent grade or scintillation grade as appropriate, unless specified, and were obtained from Fisher Chemical, Los Angeles, CA, U.S.A. or J.T. Baker, Orangeburg, NJ, U.S.A.

Liquid-scintillation spectrometry—Color quenching by carotenoids was eliminated by bleaching, using a modification of the method of Walter and Purcell (1966). A 1 ml solution of the carotenoid in petroleum ether (b.p. 30–60°C) was added to 1 ml of a freshly prepared benzoyl peroxide solution (1 g/40 ml benzene). The vial was capped and placed in sunlight until the solution was colorless. Then 5 ml of the scintillation cocktail (3a70B from Research Products International Corp., Elk Grove Village, IL) was added and the sample was counted in a Beckman LS-150 liquid scintillation system.

Incubation with radioactive precursors—For labeling studies with inhibitors, the cells were incubated in 18 liter cultures for 96 hr with acetate-2-^{14}C (10 μCi/mmol in culture) in the presence of 2.5×10^{-5} M nicotine, harvested, washed free of the radioactive acetate and nicotine and resuspended in fresh medium (12 liters) containing either zero or 5×10^{-5} M diphenylamine for 24 or 48 hr, respectively.

Pigment extraction and purification—Cells were harvested according to the procedure of Leutwiler and Chapman (1978) modified from Nakamura (1965) and then extracted and saponified in methanol and acetone as described previously (Leutwiler and Chapman 1979). Carotenoids were purified to constant specific activity on magnesium oxide : MN-kieselgur G (1 : 1, w/w) and on aluminum oxide GA : MN kieselgur G (4 : 3, w/w) thin layer chromatographic plates and developed in acetone : benzene : petroleum ether (b.p. 30–60°C) (1 : 1 : 8, v/v/v) and 1–5% acetone in petroleum ether (v/v), respectively.

Pigments were initially identified from non-radioactive cultures by their electronic absorption spectra recorded on a Beckman Acta CIII recording spectrophotometer and their mass spectra recorded on an AEI MS-9 mass spectrometer at 70 eV ionizing voltage, 170°C inlet temperature (Leutwiler and Chapman 1979). Quantitative assay was performed using the $E^{1\%}_{1cm}$ values of Davies (1965).

Results

Uptake of ^{14}C-labeled substrate

It has been shown that leucine stimulates carotenogenesis in *Phycomyces blakesleeanus* (Goodwin and Lijinsky 1952) and was readily assimilated by growing cultures of *Chlorobium limicola* (Kelly 1976). The possibility that leucine could be an effective labeling precursor (see Goodwin 1971) for chlorobactene in these experiments was considered. Initial experiments, monitoring the residual activity in the medium compared the uptake of acetate-2-^{14}C and L-leucine-U-^{14}C into whole cells of *C. limicola* and incorporation of the label into chlorobactene. The uptake of acetate was linear, during which time approximately 70% was taken up from the medium. Thereafter, radioactivity reappeared in the medium. Leucine uptake followed a similar pattern of uptake. Acetate was not only effectively taken up by the cells but also incorporated into chlorobactene. No radioactivity could be detected in the chlorobactene isolated from cells grown in the presence of L-leucine-U-^{14}C.

The uptake of acetate-2-^{14}C is illustrated in Fig. 1. At 72 hr, when growth rate begins to decline, the culture was harvested by centrifugation and the cells resuspended in medium lacking the radioisotope. The residual radioactivity adhering to the cells on resuspension at 84 hr was plotted for convenience as zero percent uptake (or 100% in medium). This remaining activity was continuously taken up by the cells to about 192 hr after which the radioactivity in the supernatant began to increase as was apparent in the previous experiments. A similar phenomenon of reappearance of label in the medium has previously been observed with mevalonic acid (Moshier and Chapman 1973). These experiments confirmed that the obligate anaerobe *C. limicola* could withstand short-term aerobic exposure during harvesting and resuspension without apparent impairment of growth or functioning. All subsequent experiments were carried out with acetate-2-^{14}C for a duration not exceeding 144 hr.

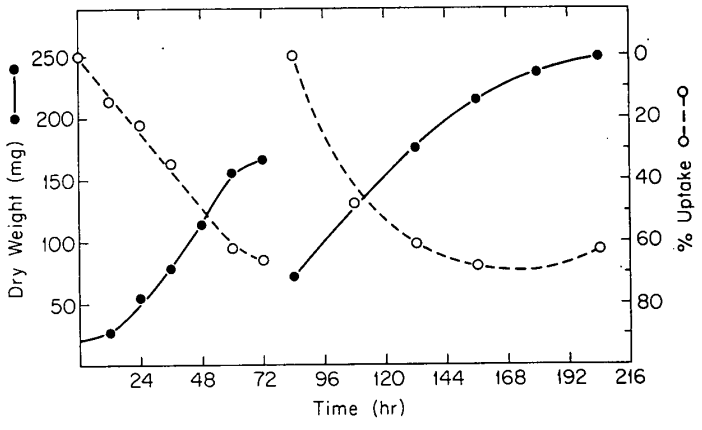

Fig. 1 Growth and acetate-2-^{14}C uptake curves for *Chlorobium limicola*. *Chlorobium limicola* was grown for 72 hr in the presence of acetate-2-^{14}C, during which time growth (as dry weight) and percent uptake of label (from measurements of percent label remaining in medium) were calculated. The cells were then harvested and transferred to fresh medium lacking radioactive acetate. Residual activity adhering to cells, and thus present in the fresh medium, was arbitrarily taken as 100% or 0% uptake at time 84 hr. Dry weight and percent uptake were then recorded as before for an additional 120 hr.

Table 1 Changes in carotenoid content of *Chlorobium limicola* growtn first for 96 hr in medium wih acetate-2-^{14}C and 2.5×10^{-4} M nicotine (a) then washed free of label and nicotine and reincubated in a medium containing either zero (b) for 24 hr or 5×10^{-5} M diphenylamine (c) for 48 hr

	Nicotine Conc. (M)		
	2.5×10^{-4}	0	$0+5 \times 10^{-5}$ M DPA
Dry weight (g)	2.59	1.81	1.69
Carotenoid content			
Neurosporene			
Content (μg/g dry wt)	58 (11)	188 (8)	9 (7)
Activity (dpm)	60,000 (12)	65,000 (12)	5,000 (6)
Lycopene			
Content (μg/g dry wt)	334 (66)	31 (1)	16 (13)
Activity (dpm)	329,000 (66)	15,000 (3)	11,000 (14)
γ-Carotene			
Content (μg/g dry wt)	10 (2)	392 (17)	12 (10)
Activity (dpm)	11,000 (2)	219,000 (39)	11,000 (14)
Chlorobactene			
Content (μg/g dry wt)	106 (21)	1,703 (74)	89 (71)
Activity (dpm)	98,000 (20)	257,000 (46)	51,000 (66)

Values in parentheses indicate percent of either total carotenoids or of total dpm/culture.

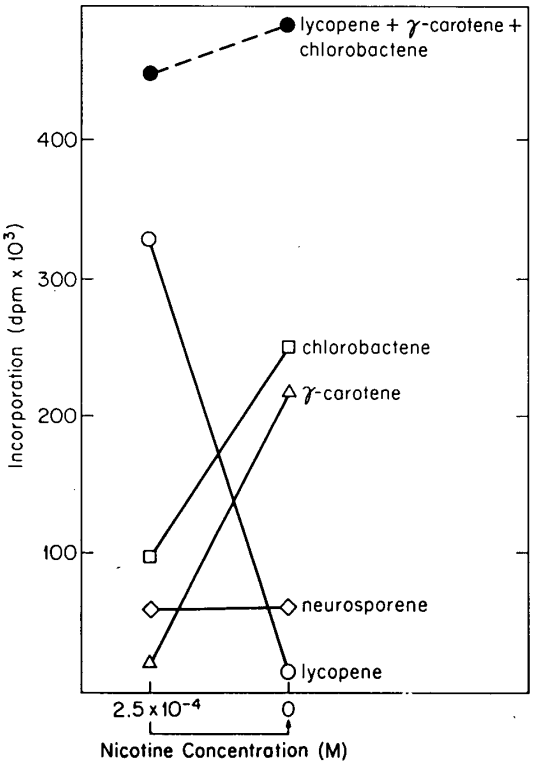

Fig. 2 Conversion of lycopene in *Chlorobium limicola*. Cultures were initially grown in the presence of acetate-2-^{14}C and 2.5×10^{-4} M nicotine, harvested and resuspended in medium lacking the radioactive acetate.

Radioisotope experiments demonstrating lycopene conversion

Prior experiments (Leutwiler and Chapman 1979) did not conclusively demonstrate that the decrease in the amount of lycopene and increase in the amount of chlorobactene, upon removal of the nicotine, was due to conversion in vivo rather than degradation of lycopene along with synthesis de novo of chlorobactene. Therefore, cultures were grown in the presence of acetate-2-^{14}C and 2.5×10^{-4} M nicotine for 4 days, harvested, washed free of the radioactive acetate and nicotine, and resuspended in fresh medium with or without DPA. The results of the experiment are given in Table 1.

The conversion of lycopene to chlorobactene can be seen in Fig. 2 where the radioactivity initially present in lycopene from cells grown at a high nicotine concentration (2.5×10^{-4} M) is found to be converted γ-carotene and chlorobactene when the inhibitor and radioactive acetate were removed. The 9% increase in total radioactivity (lycopene+γ-carotene+chlorobactene) on removal of the nicotine presumably is due to the residual activity remaining in early precursor pools. This small amount of radioactivity is insignificant when compared with the 80% increase in total amount of the three carotenoids when the inhibitor was removed (Table 1).

Discussion

Biosynthetic pathway

The present results are an extension of earlier experiments and confirm that the pathway for chlorobactene biosynthesis proceeds through lycopene, and by extension, presumably through γ-carotene. There was no evidence that neurosporene (7,8 dihydrolycopene) was the precyclization intermediate. When the inhibitor was removed and the cells resuspended in fresh medium, the amount of neurosporene actually increased, while the amount of lycopene decreased 11-fold (Table 1). If neurosporene were the precyclization intermediate, one would expect a decrease in the total amount and radioactivity on removal of the inhibitor. Furthermore, the activity initially present in lycopene during nicotine inhibition was found in both γ-carotene and chlorobactene on resuspension (Fig. 2) lending further credence for the idea of lycopene$\rightarrow\gamma$-carotene$\rightarrow$chlorobactene pathway. Conclusive proof, however, will depend upon experiments establishing that γ-carotene is converted to chlorobactene. Since γ-carotene is typically less than 5% of the total carotenoids (Leutwiler and Chapman 1979) in vivo experiments of this type might well prove inconclusive and in vitro experiments with labeled γ-carotene will need to be carried out.

Possible enzyme arrangement

Kleinig (1975) has proposed that in the nonphotosynthetic bacterium, *Myxococcus fulvus*, the enzymes are arranged in an "assembly line" which synthesizes carotenoids from early precursors. Intermediates (e.g., lycopene) which build up during abnormal conditions such as nicotine inhibition may not as readily gain access again to the enzyme sites under normal conditions as newly synthesized molecules. Only when earlier biosynthetic steps are blocked by the inhibitor Sandoz 6706 [4-chloro-5-(dimethylamino)-2-α,α,α-(trifluoro-*m*-tolyl)3-(2H)-pyrida-

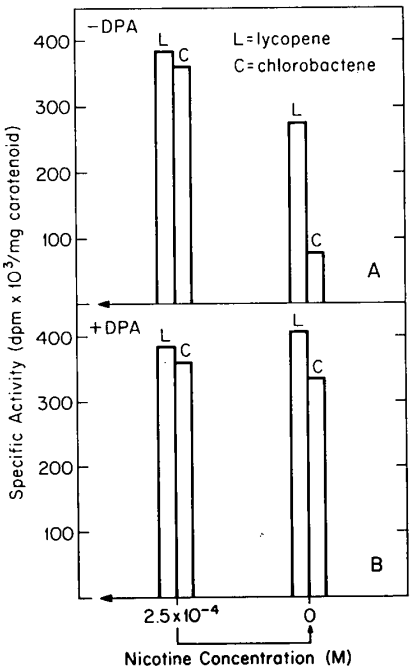

Fig. 3 Specific activities for lycopene and chlorobactene of *Chlorobium limicola*. Cultures were initially grown in the presence of acetate-2-^{14}C tnd 2.5×10^{-4} M nicotine, harvested and resuspended in medium lacking the radioactive acetate but in the absence (A) or presence (B) of 5×10^{-5} M DPA.

zinone] can the accumulated intermediates gain access again to the enzyme assembly line. A similar situation was found to exist in the photosynthetic bacterium, *Rhodomicrobium vannielii* (Britton et al. 1977).

The present data from *C. limicola* also appear to support Kleinig's theory on the arrangement of enzymes. When the cells were transferred to nicotine and radioactive acetate-free medium (Fig. 3A), the specific activity of chlorobactene decreased by 77% indicating a dilution of the activity by newly synthesized unlabeled chlorobactene. The specific activity for lycopene also decreased; however, the decrease was only 28% indicating the presence of a labeled pool of lycopene molecules which had built up in the presence of nicotine (2.5×10^{-4} M) and that was not readily cyclized when the inhibitor was washed out. Rather, newly synthesized unlabeled lycopene molecules were preferentially cyclized. In the presence of DPA (Fig. 3B), which inhibited this dilution effect, the specific activities for lycopene and chlorobactene show a 7% increase and 6% decrease, respectively, which may be considered insignificant. Under these conditions, the total activities for both lycopene and chlorobactene decrease (Table 1). However, lycopene shows a 15-fold greater decrease than chlorobactene suggesting that some of the accumulated lycopene is cyclized to chlorobactene when previous biosynthetic steps are blocked.

These results support the concept of "inaccessible precursors" accumulating during biosynthetic inhibition, at least in bacterial systems. This further compounds the difficulty of interpreting in vivo studies with inhibitors and labeled precursors, and emphasizes the need for in vitro carotenoid systems.

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