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ALTERNATIVE PATHWAYS OF CAROTENE CYCLISATION IN *PHYCOMYCES BLAKESLEEANUS*

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Key Word Index—*Phycomyces blakesleeanus*; Mucoraceae; β-carotene biosynthesis; carotenes-[^{14}C].

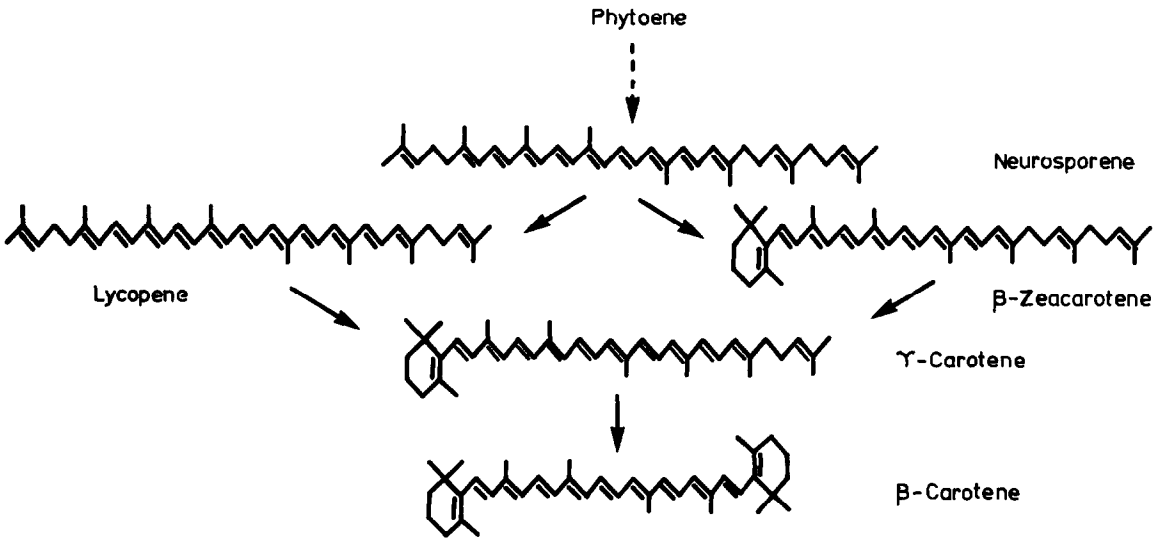
Abstract—Three carotene substrates, lycopene-[^{14}C], γ-carotene-[^{14}C] and neurosporene-[^{14}C] were each incorporated into β-carotene using a cell extract of the C115 *car-42 mad-107*(−) mutant of *P. blakesleeanus*. Neurosporene-[^{14}C] was also converted into β-zeacarotene and lycopene. Addition of either unlabelled lycopene or β-zeacarotene with neurosporene-[^{14}C] to the cell extract caused approximately equal reductions of incorporation into β-carotene, indicating that both cyclisation pathways are of equivalent quantitative importance.

INTRODUCTION

The two alternative pathways for the conversion of acyclic carotenes into β-carotene (β,β-carotene, Scheme 1) have been postulated mainly on evidence from the isolation of these compounds in nature and on *in vivo* studies involving inhibitors of cyclisation, such as diphenylamine [1], nicotine [2–5] and 2-(*p*-chlorophenylthio)triethylamine hydrochloride (CPTA) [6, 7]. Most of these investigations showed that lycopene (ψ,ψ-carotene) accumulated on inhibition of β-carotene formation, the latter being formed at the expense of lycopene on removal of the inhibitor.

Of the relatively few conversions of acyclic carotenes into cyclic products *in vitro* the majority have used higher plant systems. For example, lycopene-[^{3}H] has been converted into the cyclic carotenes of both spinach and tomato plastids [8, 9] and also into β-carotene of *Phaseolus vulgaris* plastids and tomato fruit [10]. There have been no reports to date, however, of the conversion of neurosporene (7,8-dihydro-ψ,ψ-carotene) or β-zeacarotene (7′,8′-dihydro-β,ψ-carotene) to β-carotene in any cell-free system.

In a previous publication [11] we showed that the addition of the unlabelled carotenes γ-carotene (β,ψ-caratene), neurosporene, lycopene and β-zeacarotene to a cell extract from the C115 *car-42 mad-107*(−) mutant of *P. blakesleeanus*, which synthesises β-carotene from mevalonic acid-[2-^{14}C], significantly reduced the incorporation of radioactivity into β-carotene, thus implicating both pathways in the formation of this end product.



Scheme 1. Possible cyclisation pathways for the formation of β-carotene. – – → Two or more reactions → Direct reactions.

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The availability of lycopene-[^{14}C] and γ-carotene-[^{14}C] from the C9 (lycopene-accumulating) mutant of *P. blakesleeanus*, and the isolation of neurosporene-[^{14}C] from cultures of *Rhodopseudomonas sphaeroides* incubated with 7 mM nicotine and succinic acid -[2,3-^{14}C], has enabled us to carry out a series of experiments using these substrates in a β-carotene-synthesising system, in order to assess the relative importance of the two alternative pathways for the formation of β-carotene.

RESULTS AND DISCUSSION

Three carotene substrates, lycopene-[^{14}C], γ-carotene-[^{14}C] and neurosporene-[^{14}C], solubilised in Tween 80 (2.5 mg), were incubated with the crude enzyme system from the C115 mutant of *P. blakesleeanus*. Although Tween 80 decreases the *in vitro* incorporation of MVA-[2-^{14}C] into unsaponifiable components of *P. blakesleeanus* [11], the direct incorporations of carotenes are less affected. In every case radioactivity was recovered on reisolation of the carrier β-carotene, which was purified to constant specific activity (Table 1). A boiled enzyme preparation failed to convert any of these substrates into β-carotene.

It appears from this series of experiments that the percentage incorporation of a given carotene substrate into β-carotene depends on the concentration of the substrate used; for both lycopene-[^{14}C] and neurosporene-[^{14}C] the percentage incorporations at a substrate concentration of approximately 25 μg/ml are of the same order, but increase as the amount of substrate decreases. This is probably due to the better percentage solubilisations obtained with low concentrations of carotene, so that high incorporations can only be achieved when substrates of very high specific activity are used. Under such conditions, a 6.9% incorporation of neurosporene-[^{14}C] into β-carotene was demonstrated, but this does not necessarily imply that neurosporene is a more efficient precursor of β-carotene than is lycopene, since at equivalent substrate concentrations they have comparable efficiencies. Using the same argument, however, it is clear that γ-carotene gives the best incorporation, as would be expected from a substrate which is the immediate biosynthetic precursor of the product.

In order to assess whether the neurosporene/β-zeacarotene route or the lycopene/γ-carotene pathway is operating in this organism, it is necessary either to compare β-zeacarotene-[^{14}C] and lycopene-[^{14}C] as substrates or to dilute out the incorporation of neurosporene-[^{14}C] by the separate additions of unlabelled β-zeacarotene and lycopene. Since labelled β-zeacarotene is not readily available, the latter method has been used.

In a series of experiments neurosporene-[^{14}C] was incubated alone, with unlabelled β-zeacarotene or with unlabelled lycopene. On radioassay of the diluents and the reisolated carrier β-carotene it was found that β-zeacarotene and lycopene have approximately equal effects in diluting out the incorporation of neurosporene-[^{14}C] into β-carotene (Table 2).

These data indicate that both cyclisation pathways are of equal quantitative importance in this enzyme system. The lower incorporations into β-carotene in the presence of added carotenes may be due to the substrate having a lower solubility at the higher total carotene concentration, but the trapping of equal amounts of radioactivity by the β-zeacarotene and the lycopene again leads to the conclusion that the two pathways are of equal significance. The labelling of the two added carotenes also demonstrates the *in vitro* conversion of neurosporene into both lycopene and β-zeacarotene.

Thus both this present study using carotenes-[^{14}C] and our previous investigation [11], which employed unlabelled carotenes as diluents for the incorporation of MVA-[2-^{14}C] into β-carotene, point to the actual operation of the two alternative pathways of cyclisation in C115 under the experimental conditions employed. A recent study on heterokaryons of *Phycomyces* indicates that β-carotene is produced via β-zeacarotene at late stages of growth [12] while the existence of a caroteno-

Table 1. Incorporations of lycopene-[^{14}C], γ-carotene-[^{14}C] and neurosporene-[^{14}C] into β-carotene by a crude enzyme system from C115 *car-42 mad-107*(−)

[^{14}C] substrate	Substrate concentration		Specific activity of substrate (dpm/mg)	Enzyme concn. (mg/ml)	Specific activity of β-carotene (dpm/mg)	Percentage incorporation into β-carotene
	μg/ml	nmol/ml				
Lycopene	23.5	43.8	3.32×10^5	13.8	7778a 7789b	2.6
Lycopene	2.8	5.2	3.25×10^6	9.0	50000a 43000b 44000c	3.6
γ-Carotene	4.6	8.6	2.70×10^6	8.3	37717a 36775b	5.4
Neurosporene	28.8	53.5	8.21×10^5	18.8	19064a 17021b	3.3
Neurosporene	23.2	43.3	9.54×10^5	9.3	29278a 27281b	2.4
Neurosporene	0.79	1.5	1.16×10^7	10.7	55167a 37059b 39853c	6.9

Incubation mixture (1 ml) also contained Tween 80 (2.5 mg) and cofactors described in the text. a. Chromatography on columns of alumina. b. Column chromatography and TLC on MgO/Kieselgel G (1:1) with petrol–benzene (2:1). c. As 'b' plus TLC on MgO with petrol–benzene (3:1).

Table 2. Effect of added carotenes on the incorporations of neurosporene-[^{14}C] into β-carotene by an enzyme system from C115 *car-42 mad-107*(−)

Substrate and addition	Radioactivity (dpm) recovered as:			
	β-Carotene	Lycopene	β-Zeacarotene	Neurosporene
Neurosporene-[^{14}C] only	529	—	—*	13913
Neurosporene-[^{14}C] and β-Zeacarotene	94	—	243	—
Neurosporene-[^{14}C] and Lycopene	112	297	—	—

The incubation mixtures each contained neurosporene-[^{14}C], 22300 dpm and 44 nmol; Tween 80, 2.5 mg; enzyme protein 12.95 mg/ml and the cofactors described in the text. β-Zeacarotene and lycopene were added to give final concentrations of 121 and 111 nmol/ml respectively. * Radioactivity not determined.

genic enzyme aggregate with two cyclases is proposed in another study on quantitative complementation in *P. blakesleeanus* [13]. The presence of an enzyme aggregate would explain the relatively low incorporations in our experiments of carotenes-[^{14}C] into β-carotene, since lycopene, neurosporene and β-zeacarotene are all intermediates of the pathway which would have phytoene (7,8,11,12,7′,8′,11′,12′-octahydrolycopene) as its initial substrate.

A final answer on whether two cyclisation pathways are operative in carotenogenic organisms or whether this is an example of low-substrate specificity must await the isolation and study of the individual enzymes concerned.

EXPERIMENTAL

Radiochemicals. DL-Mevalonic acid—[2-^{14}C] lactone, acetic acid—[U-^{14}C] and succinic acid-[2,3-^{14}C] were purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

Organisms and cultural conditions. The C5 *car-R21*(−) and C115 *car-42 mad-107*(−) strains of *P. blakesleeanus* were kindly provided by Prof. Max Delbrück, Division of Biology, California Institute of Technology, Pasadena, California, U.S.A. and named according to the recommendations of the Caltech Group on Phycomyces Genetics Nomenclature [14]. Growth and maintenance conditions were the same as those for the C5 *car-10*(−) strain [15]. *Rhodepseudomonas sphaeroides* (NCIB 8253) was cultured anaerobically in completely-filled Al-capped Roux bottles (1.2 l.) in the light (tungsten, 4300 lx) at 29° on a standard medium [16].

Preparation of carotenes-[^{14}*C*]. (a) *Neurosporene-*[^{14}*C*]. In a typical procedure ethanolic nicotine was added at inoculation of *Rps. sphaeroides* to give a final nicotine concentration of 7 mM (EtOH <1‰, v/v). While the culture was in the logarithmic phase of growth (43 hr, cell density 1.2 g/l.) the cells were harvested by centrifugation (8000 *g*, 20 min), washed 2× with 0·1M K_2HPO_4–KH_2PO_4 buffer (pH 6.8) and resuspended in the same buffer in a 50 ml Pyrex bottle. Nicotine (as above, final concentration 7 mM) and succinic acid-[2,3-^{14}C] (2 ml soln in phosphate buffer; 10 μCi/ml; sp. act. 20 mCi/mmol) were added, the bottle was filled with buffer and stoppered, and the suspension was incubated under the above conditions for 6 hr. The suspension was harvested by centrifugation (8000 *g*, 20 min) and the cells washed 2× with 100 ml vols. of the same buffer and then extracted and the extract saponified by procedures described previously for *R. rubrum* [17]. The unsaponifiable fraction (2175 600 dpm; 4.9% incorporation) was chromatographed on a column (15 × 1 cm) of Al_2O_3 (Woelm neutral, Brockmann activity grade III) when neurosporene was eluted with 2% Et_2O in petrol (bp 40–60°). This crude neurosporene-[^{14}C] contained 7.2% of the radioactivity of the unsaponifiable fraction and 0.35% of that of the added succinate-[2,3-^{14}C]. The crude neurosporene-[^{14}C] (70.5 μg; 156600 dpm; sp. act. 2.22 × 10^6 dpm/mg) was first purified by LC on a smaller column (10 × 0.8 cm, Al_2O_3 grade III, 2% Et_2O in petrol). The recovered sample (64.7 μg; 142600 dpm; sp. act. 2.20 × 10^6 dpm/mg) was subjected to TLC purification (Merck Si gel G; C_6H_6) to yield 57.3 μg (122500 dpm) neurosporene-[^{14}C] of sp. act. 2.14 × 10^6 dpm. (b) *Lycopene-*[^{14}*C*] was biosynthesised from either MVA-[2-^{14}C] using an enzyme system from the C5 *car R21*(−) mutant or by adding acetate-[U-^{14}C] to a growing culture of this organism. The latter technique was also used for the synthesis of γ-carotene-[^{14}C] [18].

Isolation of diluent carotenes. β-Zeacarotene was isolated from the C115 strain grown in the presence of 50 μM diphenylamine and purified by the chromatographic techniques described elsewhere [19]. Lycopene was obtained from the C9 *car R21*(−) mutant as described previously [19].

Preparation of cell extract, incubation conditions, solubilisation of carotenes and isolation and purification of radioactive carotenes. As described in detail in a previous publication [11].

Radioassay. All samples were assayed by liquid scintillation counting using the corrections for colour quenching described elsewhere [20].

Specific activities were calculated after spectrophotometric determination of the carotene in petrol [bp 40–60°] and radioassay of small aliquots.

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