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β-CAROTENE BIOSYNTHESIS BY EXTRACTS OF THE C115 MUTANT OF *PHYCOMYCES BLAKESLEEANUS*

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Key Word Index—*Phycomyces blakesleeanus*; Mucoraceae; mutant; β-carotene biosynthesis; MVA-[2-^{14}C].

Abstract—A cell extract of the yellow C115 *car-42 mad-107*(−) mutant of *Phycomyces blakesleeanus*, capable of converting MVA-[2-^{14}C] into isoprenoids, was used to investigate the formation of β-carotene. The incorporation of radioactivity into β-carotene was reduced by the addition of unlabelled carotenes, solubilised using detergent, to the incubation mixtures. On reisolation of these carotenes after anaerobic incubations, they were found to carry radioactivity. The relative efficiencies of these carotenes as trapping agents are discussed in relation to the pathways of carotene cyclisation and to the apparent operation of a system for the negative feedback control of carotene biosynthesis.

INTRODUCTION

Although a general mechanism for the conversion of an acyclic (ψ) carotene end group into a β-ring has been established in *Phycomyces blakesleeanus* [1], the situation regarding the actual nature of the cyclisation substrate is no clearer in this organism than in others (Scheme 1).

Considerable evidence can be cited in support of the cyclisation of lycopene (**2**; ψ,ψ-carotene) to β-carotene (**5**; β,β-carotene) with γ-carotene (**4**; β,ψ-carotene) as an intermediate. For instance, direct evidence has come from the conversion of labelled lycopene into β-carotene by higher plant systems [2–4], while studies on fungi have yielded further, but less direct evidence. The addition of such cyclisation inhibitors as 2-(*p*-chlorophenylthio)triethylamine hydrochloride (CPTA) [5] or nicotine [6, 7] to cultures of fungi which normally form β-carotene results in the accumulation of lycopene instead. Experiments with *Rhizophlyctis rosea*, however, indicated that lycopene could not be a precursor of γ-carotene [8].

Evidence for the alternative pathway, i.e. the cyclisation of neurosporene (**1**; 7,8-dihydro-ψ,ψ-carotene) to β-zeacarotene (**3**; 7′,8′-dihydro-β,ψ-carotene) and the dehydrogenation of this to yield γ-carotene, is rather circumstantial. The existence of β-zeacarotene in fungi, both under conditions of diphenylamine inhibition in *Rhodotorula* [9] and *P. blakesleeanus* [10] and in normal cultures of *Neurospora crassa* [11], means that these organisms have enzymes capable of cyclising neurosporene. Significantly, the removal of diphenylamine from inhibited cultures of *P. blakesleeanus* resulted in the formation of β-carotene, apparently at the expense of β-zeacarotene [12]. The conversion of β-zeacarotene into γ- and β-carotenes has not been demonstrated directly.

These apparently conflicting results presumably arise, at least in part, from the use of different experimental approaches on different organisms. It may also be the case, however, that both alternative pathways operate.

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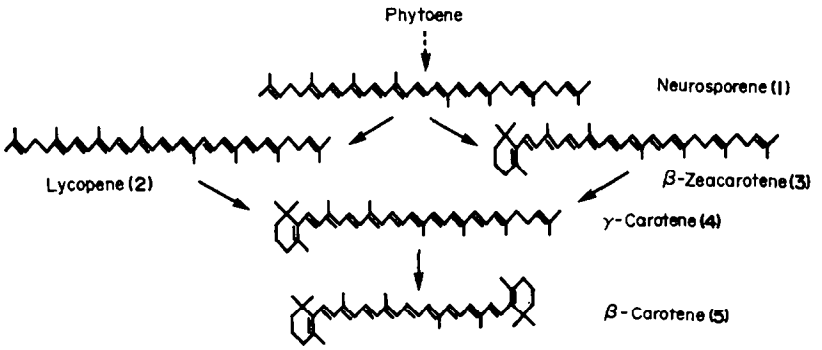


Scheme 1. Possible cyclisation pathways for the formation of β-carotene: ——→ two or more reactions; ——→ direct reactions.

With the development of a crude enzyme system from the C115 (high β-carotene) mutant of *P. blakesleeanus* [13], capable of incorporating the radioactivity of MVA-[2-^{14}C] into β-carotene, it has become possible to investigate the cyclisation problem in a more direct way. This paper reports the effects of adding various postulated unlabelled carotene intermediates on the *in vitro* incorporation of MVA-[2-^{14}C] into β-carotene. It is the first full report of this work; some of the results have already been quoted in a review [7].

RESULTS

In order to ascertain the quantity of diluent carotene that would reduce the conversion of MVA-[2-^{14}C] into β-carotene, yet not unduly affect the overall efficiency of incorporation, four incubations were carried out containing different amounts of γ-carotene (from 0 to 0.10 mM) as this carotene would be expected to dilute out the incorporation irrespective of the cyclisation pathway (Scheme 1). The incorporations of MVA-[2-^{14}C] into both β-carotene and γ-carotene were assessed (Table 1). A boiled enzyme control failed to incorporate radioactivity into any of the fractions.

Since the highest concentration of γ-carotene used (0.1 mM) completely inhibited the incorporation of MVA-[2-^{14}C] into β-carotene, and the incorporation was hardly affected by 0.01 mM γ-carotene, the intermediate concentration (0.05 mM) was chosen for subsequent studies. In these, other unlabelled carotenes and related compounds were added, each at a concentration of 0.05 mM, to a series of anaerobic incubations of MVA-[2-^{14}C] with the *P. blakesleeanus* system. The results of radioassays on β-carotene reisolated after the addition of carrier and on the recovered diluent carotenoids are shown in Table 2.

Table 1. Radioactivities of β-carotene and γ-carotene formed from MVA-[2-^{14}C] by a cell extract from C115 *car-42 mad-107*(−)

Additions per incubation	dpm incorporated β-carotene	γ-carotene
Tween 80 alone	1186	515
+ 0.01 μmol γ-carotene	1158	333
+ 0.05 μmol γ-carotene	114	1522
+ 0.10 μmol γ-carotene	0	1529

Incubation mixtures (1 ml) contained 11.9 mg protein and 2.5 mg Tween 80; other conditions are described in the text.

Table 3. Ratios of radioactivities recovered in diluent carotenes and in β-carotene after incubation of cell extracts from C115 *car-42 mad-107*(−) with MVA-[2-^{14}C]

Carotenoid added to incubation mixture	dpm in diluent dpm in β-carotene
γ-Carotene	34.70 1
Neurosporene	5.12·1
Lycopene	2.12·1
β-Zeacarotene	0.74.1
Spirilloxanthin	0 01 1

Conditions as in Table 2.

The only carotenoid that did not dilute out the incorporation of radioactivity from MVA-[2-^{14}C] into β-carotene was spirilloxanthin (1,1'-dimethoxy-3,4,3',4'-tetradehydro-1,2,1',2'-tetrahydro-ψ,ψ-carotene), which, as it is not a fungal carotenoid [14], was included as a control. The addition of squalene as a diluent also caused a reduction in the amount of radioactivity found in β-carotene. Since the values in Table 2 are uncorrected with respect to the total incorporation into the unsaponifiable lipid, which varies from incubation to incubation, they are not directly comparable. A better comparison can be made from the ratios of radioactivity recovered in each case on reisolating and purifying the diluting carotenoid and β-carotene (Table 3).

These figures show that γ-carotene and neurosporene trap the highest proportions of radioactivity, while β-carotene inhibits its own formation to some extent, causing the accumulation of some ^{14}C-labelled neurosporene and lycopene. Purification and radioassay of phytoene and squalene from the incubations shown in Table 2 indicated that the addition of certain carotenes to a system forming β-carotene and other isoprenoids affects the relative amounts of squalene and phytoene that are produced (Table 4). The ratio of phytoene to squalene which is normally 0.8:1 in control experiments is unchanged by the addition of squalene, phytoene or spirilloxanthin, but the addition of neurosporene, β-zeacarotene, lycopene or γ-carotene increases the ratio by a factor of at least 2. The effect of β-carotene is less marked than those of other coloured carotenes. Although the addition of squalene or phytoene does not cause a change in this ratio, there are parallel reductions in the incorporation

Table 2. Effect of adding unlabelled terpenoids on the incorporation of MVA-[2-^{14}C] into β-carotene by a cell extract from C115 *car-42 mad-107*(−)

Addition	dpm in recovered carotenoids* β-carotene	γ-carotene	Neu	Lyc.	β-zea.	Spx.
Tween 80 alone	506	226	662	185	—†	—
+ γ-carotene	26	902	—	—	—	—
+ neurosporene	464	—	2378	—	—	—
+ lycopene	67	—	—	142	—	—
+ β-zeacarotene	325	—	—	—	240	—
+ β-carotene	175	—	477	493	—	—
+ total phytoene	28	—	—	—	—	—
+ 15-*cis* phytoene	381	—	—	—	—	—
+ spirilloxanthin	546	—	—	—	—	6
+ squalene	73	—	—	—	—	—

Incubations (1 ml) contained 13.4 mg protein. Each terpenoid (0.05 μmole) added with 2.5 mg Tween 80; other conditions are described in the text.

* Values of dpm are not corrected with respect to total incorporation into unsaponifiable fractions. † Radioactivity not determined. Abbreviations: Neu. = neurosporene; lyc. = lycopene; β-zea = β-zeacarotene; spx. = spirilloxanthin.

Table 4. Effect of terpenoid additions on the radioactivities incorporated into phytoene and squalene after incubation of MVA-[2-^{14}C] with cell extracts of C115 *car-42 mad-107*(−)

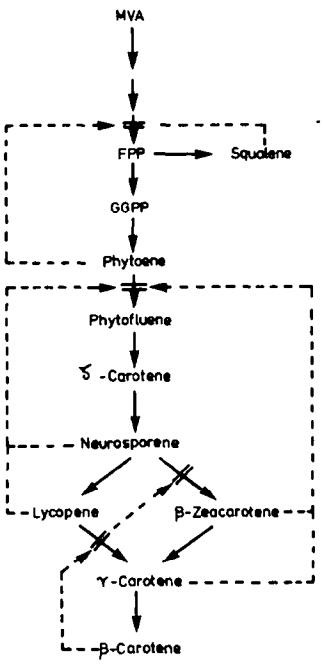
Addition	dpm in phytoene	dpm in squalene	dpm phytoene: dpm squalene
Tween 80 alone	19360	24200	0.80:1
+ squalene	9570	11990	0.80:1
+ total phytoene	8910	10450	0.85:1
+ 15-*cis* phytoene	15950	20240	0.79:1
+ neurosporene	28930	16170	1.79:1
+ lycopene	30690	19880	1.55:1
+ β-zeacarotene	26070	14080	1.85:1
+ γ-carotene	47410	13310	3.56:1
+ β-carotene	10450	11440	0.91:1
spirilloxanthin	21690	26741	0.81:1

Conditions as in Table 2.

of MVA-[2-^{14}C] into both squalene and phytoene by 51.5 and 55.6%, respectively (Table 4).

DISCUSSION

In order to include diluent carotenoids in an incubation mixture, some solubilisihg agent must be added. In these experiments the non-ionic detergent Tween 80 was chosen. Although the presence of Tween 80 caused a substantial reduction in the incorporation of MVA-[2-^{14}C] into carotenoids (Table 1), it is preferable to other solubilising agents such as bovine serum albumin since they cause varying incorporation patterns of MVA-[2-^{14}C] into unsaponifiable lipids [15]. The radioactivity incorporated into β-carotene from MVA-[2-^{14}C] in the presence of various carotenes shows that γ-carotene and then neurosporene trap the highest proportions of radioactivity (Tables 2 and 3). This situation is consistent both with γ-carotene and neurosporene being on the pathway to β-carotene, irrespective of whether β-zeacarotene or lycopene is intermediate between them, and with γ-carotene being the immediate precursor of β-carotene. The ratios obtained with lycopene and with β-zeacarotene are both lower than for neurosporene; this can only mean that neither β-zeacarotene nor lycopene, individually, is an obligatory intermediate in β-carotene formation and that alternative routes, through either lycopene or β-zeacarotene, actually operate in *P. blakesleeanus* (Scheme 1). From this experiment at least, it may also be concluded that the route through lycopene is quantitatively the more important.



Scheme 2. Postulated feedback control mechanisms for carotene biosynthesis in *P. blakesleeanus*: ——→ biosynthetic pathway; ——→ feedback control.

Apart from providing evidence for the cyclisation pathways leading to β-carotene formation, the addition of individual carotenes and squalene to cell extracts of the C115 mutant have revealed the presence of what may be negative feedback inhibition in this biosynthetic sequence. The increase in the ratio of dpm in phytoene:dpm in squalene (Table 4) can be interpreted in terms of negative feedback inhibition of phytoene desaturation by neurosporene, lycopene, β-zeacarotene and γ-carotene. Additional evidence for the inhibition of phytoene metabolism by lycopene was obtained in our previous investigations with the C9 mutant [13]. The rather lower ratio (0.91) obtained on the addition of β-carotene indicates that no such inhibition is actuated by the end product of the pathway and parallels the failure of this carotene to cause phytoene accumulation in the later stages of fungal growth [13]. β-Carotene does, however, inhibit its own cyclisation leading to the accumulation of radioactivity in lycopene and neurosporene (Table 2). The presence of either squalene or phytoene (a mixture of both isomers) has no such effect on the ratio of these two terpenoids, but the decrease in their combined formation indicates feedback inhibition at an earlier stage in the pathway, i.e. before the formation of farnesyl pyrophosphate (FPP). Thus the accumulation of either phytoene or squalene can slow down the overall rate of terpenoid biosynthesis by inhibiting one or more of the enzymes between MVA and FPP. The postulated feedback mechanisms are shown in Scheme 2.

In conclusion, these experiments give the first indication that the biosynthetic sequences of carotene formation are controlled by at least negative feedback inhibition, perhaps similar to that exhibited by another terpenoid pathway, cholesterol biosynthesis [16, 17]. The addition of unlabelled carotenes as diluents has shown that the cyclisation of β-carotene can occur by both alternative pathways in *P. blakesleeanus*. Evidence for the direct conversion of these precursors to β-carotene will be presented in a future publication.

EXPERIMENTAL

Biochemical. DL-Mevalonic acid-[2-^{14}C] lactone (10.3 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. It was converted into Na salt prior to use by the addition of the requisite quantity of aq. NaOH solution.

Organism and culture conditions. The C115 *car-42 mad-107*(−) mutant strain of *P. blakesleeanus* was kindly provided by Prof. Max Delbrück, Division of Biology, California Institute of Technology, Pasadena, California, USA; it is named according to the recommendations of the Caltech Group on Phycomyces Genetics Nomenclature [18]. Growth and maintenance were as described for the C5-*car-10*(−) strain [19].

Preparation of cell extracts and incubation conditions. These were carried out as described previously [13]. Incubation mixtures (1 ml) contained Tween 80 (2.5 mg), the appropriate unlabelled carotenoid or squalene as specified for individual experiments, in addition to the normal components [13]. Anaerobic conditions were obtained by successive evacuation (oil vacuum pump) and purging of the Thunberg tubes with argon [13].

Solubilisation of terpenoids in aqueous incubation mixtures. Each terpenoid, dissolved in acetone (1 ml), was added to the incubation mixture containing all the components except for the cell extract. To this mixture was added a soln of Tween 80 (polyoxyethylene sorbitan monooleate) in Me_2CO (1 ml, 2.5 mg/ml). The mixture was stirred vigorously and the Me_2CO removed under a stream of N_2. Once the solvent had been removed completely, the emulsion was stirred well again before the addition of the cell extract. Any terpenoid which adhered to the sides of the Thunberg tube was removed by the addition of a small volume of Me_2CO, which itself was removed before the addition of the enzymic preparation.

Isolation and purification of unlabelled carotenoids. Carotenes were isolated from mutants of *P. blakesleeanus*; lycopene, γ-carotene, and phytoene from the C9 mutant and β-carotene from the C115 mutant. β-Zeacarotene was isolated from the C115 strain grown in the presence of 50 μM diphenylamine and purified by the chromatographic techniques previously described [10]. Spirilloxanthin was isolated from *Rhodospirillum rubrum* using documented procedures [20], while neurosporene was obtained from *Rhodopseudomaus sphaeroides* grown in the presence of nicotine [21]. *Cis*-phytoene was separated from total phytoene as described previously [19]. The identity and purity of these pigments was confirmed by their chromatographic behaviour and by a comparison of their absorption spectra with those previously documented [22].

Extraction and purification of radioactive polyenes. Extraction of ^{14}C-labelled polyenes from incubation mixtures was achieved by the techniques described earlier [13]. Purification of β-carotene, lycopene, γ-carotene, neurosporene, phytoene and squalene was made by a combination of column chromatography and TLC [13]. Spirilloxanthin was purified by the techniques of Davies [20] while β-zeacarotene was chromatographed initially on columns of Si gel G [10] and final purification on thin layers of MgO–Si gel G (1:1 w/w) using light petrol–C_6H_6 (2:1) as the developing solvent. The radiochemical purity of β-carotene was ascertained by recrystallisation [13].

Radioassay. All samples were assayed by liquid scintillation counting using the corrections for colour quenching described elsewhere [23].

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