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Carotenoid Biosynthesis in *Rhodotorula glutinis*[1]

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It was determined that lycopene could be cyclized directly by *Rhodotorula glutinis*. It was also shown the the temperature effect (i.e., increased β-carotene synthesis in response to lower incubation temperatures) in *R. glutinis* was controlled by changes in enzyme concentration.

The various carotenes are derived by a sequential pathway involving three dehydrogenation reactions which result in the production of neurosporene from phytoene. The pathway for the conversion of neurosporene is, however, in question. Evidence has indicated that in some carotenogenic systems neurosporene was cyclized to either α-zeacarotene or β-zeacarotene (2, 3, 6, 9, 15, 18, 20, 22, 24), while in others it was further dehydrogenated to lycopene prior to cyclization to γ-carotene (7, 8, 10-13; L. W. Wells, W. J. Schelble, and J. W. Porter, Fed. Proc. **23**:426, 1964). Still other investigators (5, 17, 19, 21, 23) indicate that both cyclization and dehydrogenation of neurosporene may occur in the same organism.

The yeast *Rhodotorula glutinis* (Fres.) Harrison 48-23T has been reported to accumulate neurosporene and β-zeacarotene but not lycopene. The presence of β-zeacarotene constitutes presumptive evidence for the cyclization of neurosporene. This, together with the absence of lycopene, indicated that *R. glutinis* was capable of the direct cyclization of the major portion of neurosporene. However, treatment of this organism with 2-(4-chlorophenylthio)-triethylamine (CPTA) has been shown to cause the accumulation of lycopene. The ability of *R. glutinis* to metabolize this lycopene was also demonstrated. Thus, CPTA-treated cultures of *R. glutinis* offer a system for the study of the primary cyclization reaction in carotenogenesis.

In the present study an inhibitor of dehydrogenation reactions in carotenogenesis, diphenylamine, was utilized to determine the sequence of reactions involved in carotenogenesis cyclization.

When *R. glutinis* was cultured at 4 C instead of at 25 C, the relative concentrations of β-carotene, torulene, and torularhodin were markedly changed (14, 16). Each of these three carotenoids represents approximately 25% of the total carotenoids present at 25 C. Through these observations, the carotenogenic sequence in *R. glutinis* was determined to branch at γ-carotene (20). γ-carotene represents a branch point leading to the synthesis of either torulene or β-carotene and the relative concentrations of these compounds are altered by temperature changes. The carotenoids are enzymatically biosynthesized and any control mechanism should exert its influence through these enzymes.

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MATERIALS AND METHODS

Methods of culturing. *R. glutinis* (Fres.) Harrison 48-23T cultures (5 to 9 liters) were grown in a batch-type fermentor (14-liter capacity) on a medium of 5% glucose and 0.5% yeast extract (20) at 28 C. For growth at 4 C the fermentor was placed in a cold room at 4 C, 24 h after inoculation.

Harvesting and reincubation. Cells were centrifuged for 10 min at 11,000 × *g* and washed consecutively with distilled water, 0.1 M sodium citrate buffer (pH 5.0), and 0.9% NaCl in sterile distilled water, until all traces of CPTA were removed as determined by the absence of the characteristic ultraviolet spectrum of CPTA (λ_{max} at 254 nm). Washed cells were suspended in sterile 0.9% NaCl in distilled water. In some cases this suspension medium also contained 0.5 mM diphenylamine.

Disruption. Cells were lyophilized prior to disruption in an Omni Mixer (Ivan Sorvall, Inc., Newton, Conn.). The cells were disrupted in a solvent system consisting of acetone:light petroleum (75:25, vol/vol) which contained glass beads (120 μm average diameter). The mixer was operated at full speed for 20 min.

Extraction and determination of carotenoids. Cells were extracted several times with acetone and

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light petroleum. Methods used for the analysis of these carotenoids have been described (4, 20).

RESULTS

Cyclization reaction. Tables 1 and 2 present data for the effects of diphenylamine upon the conversion of lycopene and other carotenes by washed, CPTA-treated cultures of *R. glutinis* that were further incubated at 4 and 25 C.

Comparison of treatments II and III with treatment I (Table 1) shows that diphenylamine had no discernible effect upon the conversion of lycopene from washed CPTA-treated cells. Diphenylamine did inhibit the formation of torulene while increasing the concentration of γ-carotene obtained from the washed cultures. The proportion of torulene in the total carotenoids decreased from 40.1% in treatment II to 6.0% in treatment III.

Due to the lower temperatures of incubation, not all the lycopene disappeared in treatments II and III (Table 2). Values of 15.1 and 18.2% for lycopene with and without diphenylamine in the incubation mixtures, respectively, indicate that diphenylamine did not affect lycopene conversion.

Temperature effect. Table 3 shows the effects of the temperature of incubation of CPTA-treated cultures of *R. glutinis*. In treatment I cells were incubated at 4 C after the removal of CPTA from cultures maintained at 25 C. Only 3.8% β-carotene was obtained while the normally high values (for 25 C) of torulene and torularhodin were obtained. Treatment II (cells incubated at 25 C prior to and after the removal of inhibitor) served as a control, and normal levels for β-carotene, torulene, and torularhodin (13.5 to 40.1%) in CPTA-incubated cells were obtained. Treatment III (incubation at 25 C after culturing in the presence of CPTA at 4 C) resulted in elevated levels of β-carotene (53%) and somewhat decreased levels (as compared to treatments I and II) of torulene plus torularhodin (21.8 and 4.6%). Treatment IV, a control for the 4 C experiment, contained only 34.2% β-carotene but very little torulene and torularhodin.

DISCUSSION

Cyclization reaction. Lycopene formed in response to the application of CPTA to cultures of *R. glutinis* is converted to cyclic carotenoids. The simplest explanation for this conversion is the direct cyclization of lycopene to λ-carotene. In untreated *R. glutinis*, however, lycopene is not known to occur. Another possibility for the conversion of lycopene to the cyclic carotenoids in *R. glutinis* involves first hydrogenation of lycopene to yield neurosporene. The neurosporene thus formed could be transformed by the known systems of *R. glutinis* to β-zeacarotene, λ-carotene, etc. This second pathway was previously proposed (Wells, Schelble, and Porter, Fed. Proc. **23**:426, 1964). Also, labeled β-carotene has been traced back to lycopene (7) in a cell-free system, indicating the general possibility for the reversal of the carotenogenic pathway.

The second pathway contains a dehydrogenation step (the conversions of β-zeacarotene to λ-carotene). The conversion of lycopene by the first pathway contains no dehydrogenation re-

TABLE 1. *Effect of diphenylamine upon disappearance of lycopene in CPTA-treated Rhodotorula glutinis cells at room temperature*

Substance	I[a]		II[a]		III[a]	
	A[b]	B[c]	A[b]	B[c]	A[b]	B[c]
Phytoene	18.9/2.7	8.1/3.8	5.0/9.6	5.2/9.4	17.6/8.7	3.9/12.3
Phytofluene	1.5/—	1.3/—	0.1/0.8	0.3/1.1	1.8/—	1.2/—
ζ-Carotene	1.0/0.3	1.1/0.3	—/—	—/—	—/—	—/—
Neurosporene	0.4/—	2.5/—	—/—	—/—	0.7/—	1.4/—
Lycopene	49.7/—	9.1/—	—/—	—/—	—/—	—/—
β-Zeacarotene	0.3/1.0	0.2/1.3	0.5/2.0	1.4/1.3	0.8/3.6	1.3/0.5
γ-Carotene	12.1/15.7	3.6/3.4	25.8/14.8	5.2/6.1	39.2/19.0	0.9/1.6
β-Carotene	1.0/27.0	2.1/3.3	17.6/31.0	7.6/0.2	24.6/28.9	8.8/4.2
Torulene	—/22.8	—/10.4	40.1/12.6	10.6/5.9	6.0/17.2	7.6/4.7
Torularhodin	13.1/30.1	4.3/11.9	13.5/29.2	7.1/7.0	14.7/22.3	5.9/6.1

[a] Treatment I: 200 μg of CPTA per ml added to media 24 h after inoculation and incubated for 4 days. Treatment II: same as I, harvested, washed, and suspended in 0.9% NaCl and incubated for 4 days. Treatment III: same as I, harvested, washed, and suspended in 0.5 mM diphenylamine in 0.9% NaCl and incubated for 4 days.

[b] A = (% of total carotenoids of CPTA-treated sample)/(% of total carotenoids for CPTA-free sample).

[c] B = (standard deviation [S_x] of CPTA-treated sample)/(standard deviation [S_x] of CPTA-free sample).

TABLE 2. *Effect of diphenylamine upon the disappearance of lycopene in CPTA-treated Rhodotorula glutinis cells at 4 C*

Substance	I[a]		II[a]		III[a]	
	A[b]	B[c]	A[b]	B[c]	A[b]	B[c]
Phytoene	22.8/2.0	1.3/7.5	7.2/2.2	6.1/3.2	26.6/5.6	11.7/0.2
Phytofluene	0.6/0.2	0.8/0.3	1.0/—	0.7/—	3.4/—	1.7/—
ζ-Carotene	0.8/1.6	1.2/1.6	—/—	—/—	—/—	—/—
Neurosporene	5.5/0.8	0.4/0.5	1.8/—	2.6/—	1.9/1.0	2.7/1.3
Lycopene	57.7/—	6.1/—	18.2/—	2.8/—	15.1/—	1.1/—
β-Zeacarotene	—/4.8	—/4.6	0.7/4.8	1.0/6.8	—/5.4	—/3.3
γ-Carotene	8.0/8.3	3.7/5.3	31.2/2.4	1.6/3.5	31.8/3.9	5.6/2.7
β-Carotene	—/78.6	—/9.5	34.2/84.4	12.0/18.8	19.0/77.7	14.3/1.6
Torulene	—/0.9	—/0.9	—/0.7	—/1.0	—/1.0	—/0.0
Torularhodin	4.5/6.9	1.5/5.4	5.4/5.4	1.0/4.5	2.1/5.4	1.5/2.1

[a] Treatment I: 400 μg of CPTA per ml added to media 24 h after inoculation and incubated for 14 days. Treatment II: same as I, harvested, washed, and suspended in 0.9% NaCl and incubated for 4 days. Treatment III: same as I, harvested, washed, and suspended in 0.5 mM diphenylamine in 0.9% NaCl and incubated for 4 days.

[b] A = (% of total carotenoids of CPTA-treated sample)/(% of total carotenoids for CPTA-free sample).

[c] B = (standard deviation [S_x] of CPTA-treated sample)/(standard deviation [S_x] of CPTA-free sample).

TABLE 3. *Effect of temperature changes upon carotenogenesis in CPTA-treated Rhodotorula glutinis*

Substance	I[a]		II[a]		III[a]		IV[a]	
	A[b]	B[c]	A[b]	B[c]	A[b]	B[c]	A[b]	B[c]
Phytoene	13.4	1.8	5.0	5.2	14.4	20.3	7.2	6.1
Phytofluene	1.2	1.6	0.1	0.3			1.0	0.7
ζ-Carotene	0.7	1.0						
Neurosporene							1.8	2.6
Lycopene	6.8	9.6					18.2	2.8
β-Zeacarotene	0.4	0.6	0.5	1.4			0.7	1.0
γ-Carotene	42.5	5.3	25.8	5.2	6.4	3.5	31.2	1.6
β-Carotene	3.8	1.8	17.6	7.6	53.0	22.8	34.2	12.0
Torulene	13.8	1.6	40.1	10.6	21.8	2.3		
Torularhodin	22.8	4.6	13.5	7.1	4.6	1.3	5.4	1.0

[a] Treatment I: 200 μg of CPTA per ml added to media 24 h after inoculation and incubated at 25 C for 4 days. Cells were harvested, washed, and suspended in 0.9% NaCl at 4 C for 4 days. Treatment II: 200 μg of CPTA per ml added to media 24 h after inoculation and incubated at 25 C for 4 days. Cells were harvested, washed, and suspended in 0.9% NaCl at 25 C for 4 days. Treatment III: 400 μg of CPTA per ml added to media 24 h after inoculation and incubated at 4 C for 14 days. Cells were harvested, washed, and suspended in 0.9% NaCl at 25 C for 4 days. Treatment IV: 400 μg of CPTA per ml added to media 24 h after inoculation and incubated at 4 C for 14 days. Cells were harvested, washed, and suspended in 0.9% NaCl at 4 C for 4 days.

[b] A = % of total carotenoids.

[c] B = standard deviation (S_x) of A above.

action, requiring only that lycopene be cyclized directly to λ-carotene. Diphenylamine is a known inhibitor of dehydrogenation reactions in carotenogenesis (5). Since diphenylamine did not prevent the conversion of lycopene, then neurosporene is not an obligatory intermediate in the transformation and direct cyclization of lycopene must occur.

Similar results have been obtained (1) with nictone inhibition of *Mycobacterium marium* and ferricyanide addition after removal of the cyclase inhibitor. The inability to remove CPTA in the *M. marium* experiments might be attributed to use of a basic pH buffer rather than to a species difference between *R. glutinis* and *M. marium*. The basic buffer would make CPTA (an amine) water-insoluble and its removal more difficult.

In treatment III of Table 1, inhibition of torulene formation (6.0%) and accumulation of λ-carotene (39.2%) indicate that the diphenylamine was effective in preventing dehydrogenations in this experiment since torulene is formed by the dehydrogenation of λ-carotene in the

3',4' position. The effectiveness of diphenylamine in the latter stages of the carotenoid biosynthetic sequence had not previously been demonstrated.

R. glutinis cultured at 4 C showed a change in the relative concentrations of the cyclic carotenes. Studies performed at this lower temperature provided a check of the previous experiment, since different relative concentrations of the cyclic carotenes are expected. Table 2, treatment III suggests that diphenylamine does not appreciably inhibit the cyclization of lycopene. Thus lycopene must also be cyclized directly at 4 C.

In *R. glutinis*, either neurosporene or lycopene, depending on experimental conditions, can act as the substrate for cyclization. In other carotenogenic systems, either lycopene or neurosporene might act as the substrate for cyclization depending on the pool size of the two carotene substrates. These relative pool sizes in various carotenogenic systems may be controlled by the relative activities and concentrations of the neurosporene cyclizing and dehydrogenating enzymes. In *R. glutinis*, under normal growth conditions, the activity of the neurosporene-cyclizing enzyme appears to be greater than that of the neurosporene-dehydrogenating enzyme.

Temperature effect. CPTA was used in these experiments to chemically inhibit the formation of the substrate (λ-carotene) for the two enzymes (a β-carotene synthetase and a torulene synthetase) whose control mechanisms were under study. This inhibitory effect can be easily removed by washing the cells, thus permitting the production of substrate (λ-carotene). If the effect of temperature is dependent upon enzyme activity alone, then the temperature of incubation prior to CPTA removal should not affect the relative concentrations of λ-carotene, torulene, and torularhodin. Likewise, if the effect of temperature is a result of enzyme concentration, then the temperature of incubation after removal of CPTA should not make an essential difference.

In treatment I (Table 3), low levels of λ-carotene and much higher levels of torulene and torularhodin were obtained. This is consistent with the assumption that 25 C affects the concentrations of some enzymes. The higher levels of β-carotene and torulene in treatment II were due to the higher temperature of incubation after inhibitor removal, allowing for faster and more complete conversion of the accumulated lycopene.

In treatment III (Table 3), higher levels of β-carotene were obtained. Similarily high values for β-carotene in treatment IV were not obtained because suspension 4 C slowed the rate of reaction. That the λ-carotene left would have been converted to β-carotene, not to torulene and torularhodin, was indicated by the absence of torulene and the low level of torularhodin.

These results indicate that the temperature effect of *R. glutinis* is controlled by enzyme concentration since it does not involve enzyme activity. The change in enzyme concentration may be due to effects at the levels of transcription or translation, or to a decrease in the labile nature of one of the enzymes involved.

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