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EFFECTS OF CPTA UPON CAROTENOGENESIS AND LIPOIDAL CONSTITUENTS IN *RHODOTORULA* SPECIES*

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Abstract—The effects of 2-(4-chlorophenyl)-triethylamine (CPTA) upon carotenogenesis in *Rhodotorula glutinis*, and upon various lipoidal constituents of *R. rubra* were studied. CPTA was found to cause the accumulation of lycopene and γ-carotene and to inhibit the formation of fatty acids and ergosterol. Upon removal of the inhibitor lycopene was metabolized to the cyclic carotenes and the levels of ergosterol increased. It was proposed that CPTA inhibits the cyclase enzyme of carotenogenesis, inhibits the formation of ergosterol, and causes the build-up of intermediates of these compounds.

INTRODUCTION

RECENT investigations[1–7] into the effects of 2-(4-chlorophenyl)-triethylamine (CPTA) upon a wide range of the protista and plants indicates an inhibition of the cyclase enzyme in the carotenogenic sequence. Lycopene has been shown to accumulate in organisms where lycopene is normally either not detected or is formed at relatively low levels. Two reports[1, 5] have shown that lycopene was metabolized once the CPTA was removed but another[7] states that the inhibition of the cyclization process is not reversible. Investigations dealing with the effects of CPTA upon other aspects of cellular metabolism have been limited to one report[6] in which it was observed that CPTA had no effect upon the concentration of terpenoid oils in the peel of citrus fruit.

It was the purpose of this study to determine the effects of CPTA upon carotenogenesis in *Rhodotorula glutinis* and to study the conditions under which the effect of CPTA can be reversed. A study was also made to discern the extent of the inhibition of CPTA on other lipoidal constituents.

RESULTS

Table 1 shows the effects of CPTA upon carotenogenesis in *R. glutinis*. Without the addition of CPTA the cyclic carotenes, γ-carotene, β-carotene, torulene, and torularhodin

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predominate. Traces of the acyclic carotenes phytoene and ζ-carotene were isolated, but lycopene was not normally detected. Upon inclusion of CPTA in the media, lycopene was observed as the predominate pigment (49·7%). This is accompanied by the accumulation of significant levels of phytoene (18·9%), a decrease in the cyclic carotenes (β-carotene, torulene, and torularhodin), and a relatively unchanged concentrations of γ-carotene.

TABLE 1. EFFECT OF CPTA ON CAROTENOGENESIS IN *Rhodotorula glutinis*

	% Total carotenoids*			
Treatment	None†	CPTA†	None + reincubation‡ in 0·9% NaCl	CPTA + reincubation‡ in 0·9% NaCl
Phytoene	2·7 ± 3·8	18·9 ± 8·1	9·6 ± 9·4	5·0 ± 5·2
Phytofluene	—	1·5 ± 1·3	0·8 ± 1·1	0·1 ± 0·3
ζ-Carotene	0·3 ± 0·3	1·0 ± 1·1	—	—
Neurosporene	—	0·4 ± 2·5	—	—
Lycopene	—	49·7 ± 9·1	—	—
β-Zeacarotene	1·0 ± 1·3	0·3 ± 0·2	2·0 ± 1·3	0·5 ± 1·4
γ-Carotene	15·7 ± 3·4	12·1 ± 3·6	14·8 ± 6·1	25·8 ± 5·2
β-Carotene	27·0 ± 3·3	1·0 ± 2·1	31·0 ± 0·2	17·6 ± 7·6
Torulene	22·8 ± 10·4	—	12·6 ± 5·9	40·1 ± 10·6
Torularhodin	30·1 ± 11·9	13·1 ± 4·3	29·2 ± 7·0	13·5 ± 7·1

* Values are ± one s.d. (s_x).
† 4 days growth.
‡ 4 days growth: washed and 4 more days growth.

Table 1 also shows the effect of resuspension in 0·9% NaCl of *R. glutinis* cells in the maximum stationary phase of growth followed by 4 days further metabolism. In the cells which were free of treatment with CPTA, no significant changes are noted. In the CPTA-treated cells, however, large alterations in the relative concentrations of the carotenoids were obtained. Lycopene which had accounted for nearly half of all the carotenoids disappeared. It would appear that the lycopene which had accumulated in the presence of CPTA was converted to the cyclic carotenes (torularhodin, torulene, β-carotene, and γ-carotene) upon its removal.

With the increased number and types of compounds analyzed in the experiments summarized in Table 2, *R. rubra* was utilized because of its relatively less complex carotenoid distribution. The concentration of fatty acids decreased with treatment with CPTA, as did the ergosterol levels. Upon reincubation, however, the fatty acid concentrations did not increase as did the ergosterol levels (both with and without treatment with CPTA). Because of the variability of the results as depicted by the large standard deviations, no conclusions as to significant changes in concentration of phospholipids, squalene, and lanosterol can be made.

Similar patterns of carotenogenesis were obtained with *R. rubra* as were obtained with the previously described experiments with *R. glutinis*.

DISCUSSION

CPTA and various nitrogenous heterocyclic compounds were shown to alter the biosynthetic formation of carotenes in some *Phycomyces blakesleeanus* mutants.[8] If a

[8] ELAHI, M., CHICHESTER, C. O. and SIMPSON, K. L. (1973) *Phytochemistry* **12**, 1627.

compound inhibited β-carotene formation it invariably caused an increase in the level of phytoene, γ-carotene and lycopene. β-Zeacarotene was usually not detected in cultures where β-carotene synthesis was inhibited.

TABLE 2. EFFECTS OF CPTA ON LIPID CONCENTRATIONS IN *Rhodotorula rubra*

Treatment	Lipid μg/g dry wt*			
	None—8 days	None—4 days	CPTA—8 days	CPTA—4 days
Reincubation in 0·9% NaCl	None	4 days	None	4 days
Fatty acids	(0·37 ± 0·02) × 10^6	(0·38 ± 0·02) × 10^6	(0·28 ± 0·01) × 10^6	(0·25 ± 0·01) × 10^6
Phospholipids	36·3 ± 10·6	14·8 ± 3·1	24·1 ± 7·7	17·2 ± 10·8
Squalene	9·0 ± 3·7	12·3 ± 2·6	7·2 ± 0·4	10·3 ± 2·8
Lanosterol	1·6 ± 1·9	—	1·0 ± 0·1	—
Ergosterol	1685 ± 361	2241 ± 118	932 ± 11	2383 ± 246
Carotenoids:				
Phytoene	4·2 ± 0·0	—	4·4 ± 6·6	—
Phytofluene	0·8 ± 0·1	—	0·4 ± 0·5	—
ζ-Carotene	—	—	1·1 ± 0·0	0·5 ± 1·0
Lycopene	—	—	81·2 ± 14·9	—
γ-Carotene	8·6 ± 1·5	3·3 ± 0·1	7·6 ± 1·2	7·8 ± 0·7
β-Carotene	7·9 ± 0·0	7·6 ± 0·5	0·4 ± 0·1	17·1 ± 3·1
Torulene	89·9 ± 1·6	93·2 ± 1·2	—	44·4 ± 0·8
Torularhodin	93·8 ± 12·6	87·3 ± 7·2	75·3 ± 11·7	44·4 ± 0·8
Total Carotenoids	205·3 ± 9·4	191·3 ± 5·4	170·4 ± 21·2	167·0 ± 0·2

* Values ± one s.d. (s_x).

In yeast the levels of γ-carotene and β-zeacarotene are relatively high and γ-carotene once formed can be transformed to other carotenoids without further cyclization reactions. Thus lycopene accumulates in CPTA-treated cells together with the inhibition of β-carotene, but the level of γ-carotene remained the same or slightly lower. The simplest explanation is that γ-carotene in yeast can be converted to torulene which can in turn be converted to torularhodin. The results shown in Table 1 are consistent with this interpretation.

It remains to be seen why all of the torulene should be converted to torularhodin in CPTA-treated cells whereas non-treated cells contain significant amounts of torulene.

It is possible that a control mechanism is in operation between torulene and torularhodin that is related to the level of β-carotene. The control was not operative when β-carotene synthesis was inhibited. When the cells were washed free of CPTA and resuspended, the lycopene level dropped and the levels of γ-carotene, β-carotene and torulene increased. The slow return of torularhodin over the period of incubation would seem best explained by a control mechanism activated by the active β-carotene synthesis.

When *R. glutinis*[9] was cultured in the presence of β-ionone vapors, the concentration of β-carotene decreased from 63·0 to 24·3%. Under these conditions the torulene to torularhodin ratio changed from 1·04 to 0·26. These results would also be consistent with the hypothesis that the transformation of torulene was related to β-carotene synthesis.

The metabolization of the bioaccumulated lycopene along with the concomitant increase in concentration of the cyclic carotenes upon washing out of the CPTA, indicated that

[9] SIMPSON, K. L. (1963) Ph.D. Thesis Univ. of Calif., Davis, California.

CPTA was acting at the level of enzyme activity of the cyclase enzyme. It was reported,[7] however, that the removal of the effects of CPTA by washing was not possible in *Mycobacterium marium*. Others[1,5] have shown that the effects of CPTA were reversed by washing. This difference was explained as a tighter binding of CPTA by the enzyme of *M. marium* than occurs in the other organisms studied. These authors report no difficulty, however, in the washing out of nicotine (an inhibitor of the same enzyme) and one might expect the same tight binding. These differences in results may be explained by the experimental procedures used for the washing out of the CPTA with pH 8·0 phosphate buffer. CPTA, an amine, is insoluble in water at alkaline pHs. On occasion some difficulty was observed in the reversal of the effects of CPTA in *R. glutinis* until a citrate buffer, pH 5·0, washing was included in the procedure. The effects of CPTA were reversed by the washing of the inhibitor from the yeast cells and reports otherwise might be due to incomplete washing procedures. The metabolization of lycopene was contrary to the postulated inertness of the end product of the carotenogenic enzyme aggregate toward further carotenogenic transformation.[10]

Upon treatment with CPTA a partial inhibition of both fatty acid and ergosterol synthesis was observed. This, together with the alteration of carotenogenic distribution and a previous report[5] describing the induction of enzymes of the lycopene pathways, indicates a more complex site of action for CPTA than for some other chemical effectors of carotenogenesis. Upon reincubation of the cells the concentration of fatty acids decreased in the CPTA-treated sample but not in the untreated sample. Reincubation also caused an increase in concentration of ergosterol in both samples. It is interesting to note that nearly the same level of ergosterol was obtained upon reincubation of both samples even though widely differing values were obtained before reincubation. This level of ergosterol (*ca* 2·3 mg/g) may represent a maximal level of ergosterol accumulation in *R. rubra* as controlled by either feedback inhibition or feedback repression. The more lively burst of synthesis of ergosterol upon reincubation of the CPTA-treated sample as compared to the untreated sample may indicate an accumulation of the isoprenoid precursors such as farnesyl pyrophosphate, geranyl pyrophosphate, etc. in response to some inhibitory effect of CPTA. CPTA may be effecting an enzyme such as HGM-Co A reductase, causing an increase in isoprenoid or terpenoid synthesis at the expense of acetyl-Co A derived compounds. Further investigation in this area is underway.

EXPERIMENTAL

Method of culturing. Rhodotorula glutinis and *R. rubra* were grown in a batch-type fermentor on a medium of 5% glucose and 0·5% yeast extract. CPTA was added as the hydrochloride 24 hr after innoculation. Cells were harvested after specified growth periods.

Harvesting and reincubation. Cells were centrifuged for 10 min at 11000 *g*. If the cells were to be reincubated and had not been inhibited by CPTA, they were washed 3 × in H_2O. If the cells had been inhibited with CPTA, they were washed consecutively with: H_2O, 0·1 M sodium citrate buffer, pH 5·0, and 0·9% NaCl in H_2O until all traces of CPTA were removed as determined by the absence of the characteristic UV spectrum of CPTA with a λ_{max} at 254 nm. The washed cells were then examined after a further 4·0 days growth.

Disruption. Cells were lypholized prior to disruption in an Omni Mixer (Ivan Sorvall, Inc., Newton, Conn.). Disruption was accomplished at full speed operation of the mixer for 20 min. Disruption was aided by the addition of glass beads (120 μ av. dia.) to the disrupting solvent of 3 : 1, acetone–light petroleum.

Extraction. The cellular debris was washed with acetone and petrol, until all traces of color were absent in the washings. In those cases in which lipoidal material other than carotenoids were to be identified, the residue of cellular debris was placed in a Soxhlet extractor thimble and subjected to an additional overnight extraction with 2 : 1, $CHCl_3$–MeOH.

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Separation and determination of lipoidal constituents. The methods used for analysis of these carotenoids have been described.[9,11] In those experiments in which it was desired to analyze other compounds in addition to the carotenoids, an alteration of methods was used. Both extracts (petrol.–acetone and $CHCl_3$–MeOH) were saponified separately prior to removal of the torularhodin by column chromatography as was done otherwise. The pH of the saponifiable fraction was fraction was adjusted to *ca* 2 with 3 N HCl, and the resulting sol. extracted 3 × with 100 ml portions of petrol. Torularhodin was determined spectrophotometrically. Fatty acid levels in the petrol. layer were determined gravimetrically after evaporation of the solvent. The amount of phosphate in the acidified aqueous portion of the saponifiable material was determined by complexing with a molybdate reagent and reduction with $SnCl_2$.[12] This was taken as a measure of the phospholipid concentration of the original sample. The nonsaponifiable fraction was analyzed for carotenoids as described above. The combined nonsaponifiable fraction (including all isolated carotenes except phytoene, the 2 : 1 $CHCl_3$–MeOH extract of the remaining portions of the MgO–Supercell column used for the separation of carotenoids, and the nonsaponifiable portion of the $CHCl_3$–MeOH extract from the Soxhlet extractor) was made to a vol. of 50 ml with petrol. An aliquot (2 ml in most cases) was utilized for the determination of ergosterol by digitonin precipitation[13] and subsequent spectrophotometric analysis. The remaining portion of the nonsaponifiable material was chromatographed on 1mm thick layers of silica gel G (Stahl) with 10% MeOH in petrol. as a developing solvent. The band which chromatographed with a similar R_f as did an authentic sample of lanosterol (as determined by relative distance from the ergosterol band which flouresced under UV light) was scraped, eluted, and analyzed gas chromatographically for lanosterol as described below. Squalene was contained in the crude phytoene fraction was also analyzed as described below.

Gas chromatography conditions. Column 15 cm × 3 mm o.d. stainless steel packed with 3% OV 1 on gaschrom Q, 60–80 mesh, N_2 = 50 ml/min, H_2 = 25 ml/min, injector port and detector = 315°. In the case of squalene, a temp. program of 8°/min, with initial and final temps. of 200 and 295°, respectively, was used. For the determination of lanosterol the chromatograph was operated isothermally at 295°. Detection of both compounds was through use of a FID.

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