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CAROTENOID BIOSYNTHESIS IN *BLAKESLEA TRISPORA**

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Abstract—2-(4-Chlorophenylthio)triethylamine hydrochloride (CPTA) has a profound effect on the carotenogenesis in *Blakeslea trispora*. When cultures were treated with CPTA, lycopene accumulated as the principle pigment with the concomitant increase in γ-carotene. CPTA is suggested to have multiple functions in carotenogenesis. It might act as an inhibitor of the cyclase(s) and additionally act as a derepressor of a gene regulating the synthesis of a specific enzyme(s) in the lycopene pathway.

INTRODUCTION

2-(4-CHLOROPHENYLTHIO)TRIETHYLAMINE HYDROCHLORIDE (CPTA) was found to have a profound effect on the induction of lycopene in a wide array of carotenogenic tissues: fruits, roots or the mycelium of certain plants.[1] A complete alteration of the carotenoid pattern was observed in the trigeneric citrus hybrid, *Sinton citrangequat*. Lycopene accumulated and formation of the normally present methylketone carotenoids was inhibited.[2] Also a stimulation of the lycopene pathway by CPTA was found in Marsh seedless grapefruit and navel orange.[3] In the present investigation certain aspects of the effect of CPTA in stimulating lycopene synthesis in mated *Blakeslea trispora* are reported; also the mechanism of action of CPTA is considered.

RESULTS AND DISCUSSION

Nature and Amount of Pigment Produced in the Mated Strains treated with CPTA

The pigments of the mycelia of mated *Blakeslea trispora* are essentially hydrocarbon in nature. It acquires a yellow color because of the β-carotene accumulation.[4] As shown in Table 1, the main pigments are phytoene and β-carotene which constitute 36·39 and 26·28% of the total pigment respectively. γ-Carotene constitutes 13·43%, lycopene 7·6% and phytofluene, β-zeacarotene and neurosporene constitute the remainder. In CPTA (35 ppm) treated culture, the mycelia carotenoid composition was altered. Lycopene accumulated up to 53·36% of the total pigment and is responsible for the red color. The total polyene

* Part IV in the series "Chemical Regulation of Carotenoid Biosynthesis". For Part III see *Phytochem.* **11**, 1721 (1972).

[1] C. W. COGGINS, JR., G. L. HENNING and H. YOKOYAMA, *Science* **168**, 1589 (1970).
[2] H. YOKOYAMA, C. W. COGGINS, JR. and G. L. HENNING, *Phytochem.* **10**, 1831 (1971).
[3] H. YOKOYAMA, C. W. COGGINS, JR., G. L. HENNING and C. DEBENEDICT, *Phytochem.* **11**, 1721 (1972).
[4] D. M. THOMAS and T. W. GOODWIN, *Phytochem.* **6**, 355 (1967).

production was increased by 250%. However, the lycopene level was increased by 16-fold. In addition, an increase in the γ-carotene level was observed. Its absolute amount was raised by about 4·5-fold and constituted 25·37% of the total. The concentrations of β-carotene and phytoene were reduced.

TABLE 1. EFFECT OF CPTA (35 ppm) ON THE CAROTENOGENESIS IN MATED *B. trispora*

Pigment	Control		CPTA treated	
	μg/g fr. wt	% of total	μg/g fr. wt	% of total
Phytoene	118·86	36·39	72·03	9·32
Phytofluene	22·94	7·02	9·02	1·17
β-Carotene	86·10	26·28	52·06	6·74
β-Zeacarotene	20·49	6·27	9·76	1·26
ζ-Carotene	trace	—	trace	—
γ-Carotene	43·83	13·43	196·06	25·37
Neurosporene	6·00	1·83	11·38	1·47
Lycopene	24·87	7·60	412·41	53·36

CPTA was added at the time of inoculation cultures were incubated for two weeks before harvesting.

Both lycopene and neurosporene have been proposed for the immediate precursor of cyclic carotenoids.[5,6] Decker and Uehleke[7] reported the conversion of lycopene-^{14}C into β-carotene by spinach chlorophlasts and the reverse conversion of β-carotene to lycopene by tomato slices. Kushwaha *et al.* demonstrated the conversion of lycopene-^{3}H to cyclic carotenes by soluble extracts of spinach chloroplasts and Hi-β and Hi-δ tomato fruit plastids[8] and red tomato.[9] Recently Howes and Batra showed the accumulation of lycopene in nicotine treated mycobacterium cultures and the conversion of accumulated lycopene to β-carotene upon removal of the nicotine.[10] All the above reports provide direct evidence for the participation of lycopene in β-carotene synthesis. The accumulation of lycopene and γ-carotene in the CPTA treated culture suggest that these two pigments might be the substrates for β-carotene synthesis, γ-carotene serves as an intermediate between lycopene and β-carotene and CPTA acts as an inhibitor of the cyclase(s). The speculation of inhibitory effect of CPTA was supported by finding a complete alteration of carotenoid pattern in CPTA treated citrus fruits. When CPTA was applied to the immature or mature green fruits of citrus hybrid-*Sinton citrangequat* and navel orange which were then allowed to ripen to full maturity and color, lycopene accumulated and the normally present methylketone carotenoids (in *Sinton citrangequat*) and secondary carotenoids including epoxides (in navel orange) were absent,[2,3] suggesting that CPTA inhibited the synthesis of cyclic carotenoids.

In order to further investigate the hinibitory effect of CPTA on the β-carotene synthesis, *B. trispora* was cultured under different concentrations of CPTA. In this series, higher concentrations of CPTA were required to produce comparable accumulation of lycopene than in previous experiments. The results (Fig. 1a) show that at lower concentration

[5] T. W. GOODWIN, in T. W. GOODWIN, *Chemistry and Biochemistry of Plant Pigments*, p. 143, Academic Press, London (1965).
[6] J. W. PORTER and D. G. ANDERSON, *Ann. Rev. Plant Physiol.* **18,** 197 (1967).
[7] K. DECKER and H. UEHLEKE, *Hoppe-Seyler's Z. Physiol. Chem.* **323,** 6 (1961).
[8] S. C. KUSHWAHA, C. SUBBARAYAN, D. A. BEELER and J. W. PORTER, *J. Biol. Chem.* **244,** 3635 (1969).
[9] S. C. KUSHWAHA, G. SUZUE, C. SUBBARAYAN and J. W. PORTER, *J. Biol. Chem.* **245,** 4708 (1970).
[10] C. D. HOWES and P. P. BATRA, *Biochim. Biophys. Acta* **222,** 174 (1970).

(50 ppm) of CPTA γ-carotene accumulates as the major pigment (1000 μg/g dry wt). It constituted 38% of the total; lycopene constituted only 12% while β-carotene dropped from 56 to 12%. At higher concentrations of CPTA (100–300 ppm) lycopene accumulates as the main pigment, accompanied by increase in γ-carotene concentration. Above 300 ppm the total polyene synthesis was reduced somewhat; however, the relative concentrations of lycopene, γ-carotene and β-carotene remained almost constant (Fig. 1b). These data indicate that, if CPTA acts as an inhibitor of cyclase(s), it will not only inhibit the first cyclization reaction between the lycopene and γ-carotene but also the second one between the γ-carotene and β-carotene. However, the data do not entirely rule out the route via β-zeacarotene. (Fig. 2).

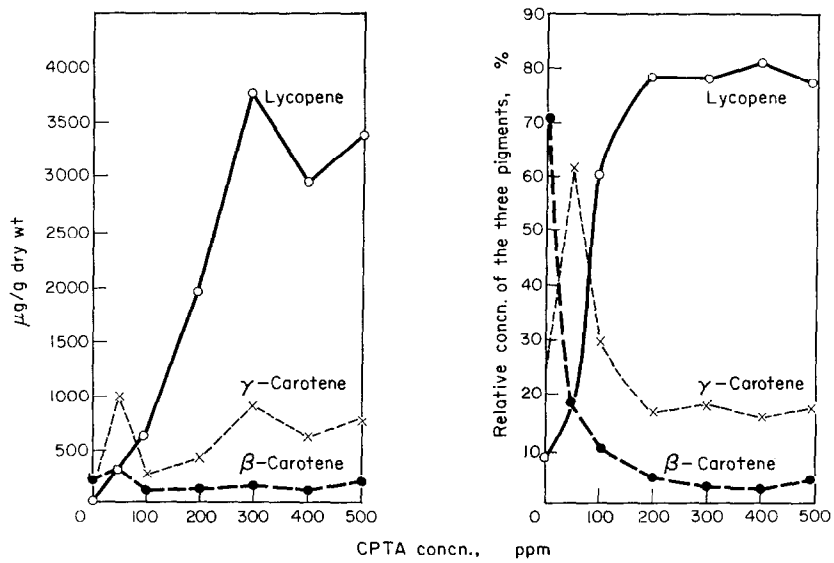

FIG. 1. EFFECT OF CPTA CONCENTRATION ON THE SYNTHESIS OF VARIOUS CAROTENOIDS IN *B. trispora*. CPTA was added at the time of inoculation and the cultures were incubated for 10 days.

Complete inhibition of β-carotene synthesis was not observed even at higher concentrations of CPTA (100–500 ppm). Also the β-carotene concentration remains almost constant at CPTA concentrations from 100 to 500 ppm while the lycopene levels were increased six fold (Fig. 1a). These results suggest that CPTA might not just act as an inhibitor but also as an activator or inducer in the lycopene synthesis pathway.

Diphenylamine Studies

Diphenylamine is known to inhibit the synthesis of the highly unsaturated polyenes β-carotene, γ-carotene, and lycopene and also cause the accumulation of the precursor phytoene in *Phycomyces blakesleeanus*,[11,12] *Blakeslea trispora*[13] and *Rhodospirillum rubrum*.[14] An experiment was carried out to examine the effect of diphenylamine on the accumulation of lycopene in CPTA treated culture.

[11] J. A. OLSON and H. KNIZLEY, JR., *Arch. Biochem. Biophys.* **97**, 138 (1962).
[12] T. W. GOODWIN, in *Biosynthetic Pathways in Higher Plants* (edited by J. B. PRIDHAM and T. SWAIN), Academic Press, New York (1965).
[13] D. M. THOMAS and T. W. GOODWIN, *Phytochem.* **6**, 355 (1967).
[14] S. L. JENSEN, G. COHEN-BAZIRE, T. O. M. NAKAYAMA and R. Y. STANIER, *Biochim. Biophys. Acta* **29**, 477 (1958).

The results (Table 2) show that diphenylamine at a concentration of 0·1 mM inhibits the formation of β-, γ-carotene and lycopene and stimulates phytoene synthesis. When cultures were incubated with both diphenylamine (0·1 mM) and CPTA (35 ppm) not only the phytoene accumulated but also γ-carotene and lycopene; total polyene production was also

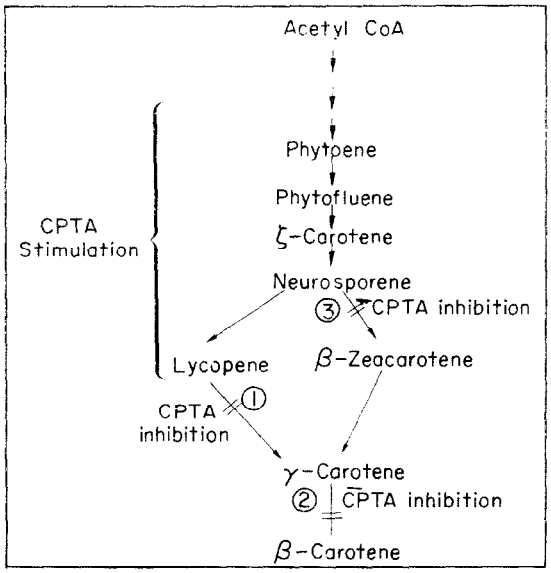

Fig. 2. The possible sites of CPTA action on carotenoid biosynthesis in mated *B. trispora*.

stimulated about fourteen fold. Diphenylamine is known to have two functions on carotenogenesis: inhibition of the dehydrogenase[15,16] and stimulation of phytoene synthesis.[17] If we assume that CPTA and diphenylamine act on the same pathway, the accumulations of the lycopene and γ-carotene found in cultures treated with both agents suggests that CPTA

Table 2. Effect of diphenylamine (DPA) (0·1 mM) and CPTA (35 ppm) on the carotenogenesis in mated *B. trispora*

Pigment	Control		DPA treated		DPA + CPTA treated	
	μg/g fr. wt	% of total	μg/g fr. wt	% of total	μg/g fr. wt	% of total
Phytoene	118·86	36·39	177·69	58·36	1227·15	28·07
Phytofluene	22·94	7·02	14·75	4·84	198·42	4·54
β-Carotene	86·10	26·28	50·90	16·72	165·74	3·80
β-Zeacarotene	20·49	6·27	15·01	4·93	77·40	1·76
ζ-Carotene	trace	—	trace	—	trace	—
γ-Carotene	43·83	13·43	22·45	7·37	540·38	12·36
Neurosporene	6·00	1·83	8·76	2·88	219·55	5·03
Lycopene	24·87	7·60	14·96	4·90	1886·46	43·14

Diphenylamine and CPTA were added at the time of inoculation. Cultures were incubated for 2 weeks before harvesting.

[15] T. W. Goodwin, M. Jamikorn and J. S. Willmer, *Biochem. J.* **53**, 531 (1953).
[16] H. C. Rilling, *Arch. Biochem. Biophys.* **110**, 39 (1965).
[17] T. W. Goodwin, *Advances in Enzymol.* **21**, 295 (1959).

might compete with diphenylamine for the dehydrogenases, allowing the normal dehydrogenations to proceed in the cultures and then CPTA further acts at the cyclization steps to cause accumulation of lycopene and γ-carotene. If the cultures have the normal dehydrogenase activity in the presence of a mixture of diphenylamine and CPTA, then there would not be an accumulation of phytoene, since the phytoene formed would be readily dehydrogenated to lycopene. However, a large accumulation of phytoene was observed in cultures treated with both reagents, accumulation being probably due to the stimulatory effect of diphenylamine.[17]

Cycloheximide Studies

Evidence from the previous experiments indicates that CPTA may either act as an inhibitor of the cyclases or as an activator or inducer for the enzyme(s) leading to lycopene synthesis. The net synthesis of carotenoid pigments in grapefruit ceases during the maturation process.[18] The synthesis of lycopene and its precursors found in the CPTA treated Marsh seedless grapefruit of any stage or fully matured fruits of *Sinton citrangequat* and navel orange[2,3] strongly indicates that a stimulation of lycopene synthesis occurs after treatment with CPTA. The question should then be raised whether CPTA activates the already existing enzyme(s) or induces *de novo* synthesis of the enzyme(s) for the lycopene pathway. Experiments have, therefore, been carried out to determine the effect of protein synthesis inhibitor cycloheximide on the stimulatory effect of CPTA on the lycopene synthesis in mated *Blakeslea trispora.*

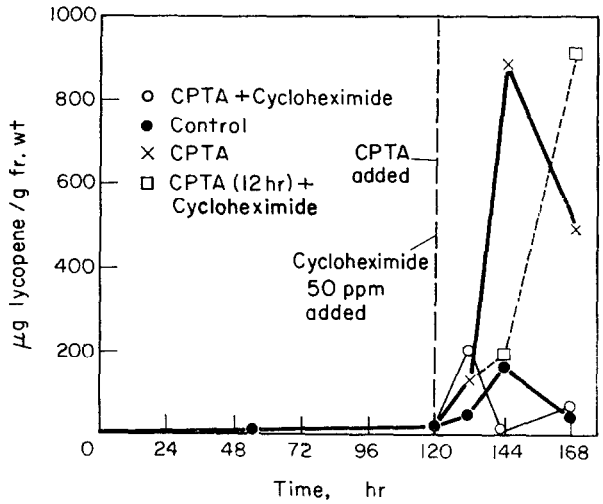

FIG. 3. THE EFFECT OF CYCLOHEXIMIDE (50 ppm) ON CPTA (175 ppm) STIMULATED LYCOPENE PRODUCTION IN *B. trispora.*
120-hr cultures were treated in the following manner and reincubated for a further 48 hr.

Cycloheximide, which is known to be an extremely effective inhibitor of protein synthesis in *B. trispora*[19] was studied together with CPTA. A final concentration of 50 μg/ml medium (50 ppm) was found to nullify the stimulatory effect of CPTA on the lycopene accumulation (Fig. 3). Cultures (120-hr) were divided into four groups; each group was treated in a

[18] H. YOKOYAMA and M. J. WHITE, *J. Agric. Food. Chem.* **65**, 693 (1967).
[19] D. M. THOMAS, R. C. HARRIS, J. T. O. KIRK and T. W. GOODWIN, *Phytochem.* **6**, 361 (1967).

different manner and reincubated for an additional 48 hr. Group *A* was the control, group *B* was treated with CPTA alone, group *C* was treated with CPTA and cycloheximide together, while group *D* was treated with CPTA first for 12 hr and then cycloheximide was added. In the control, maximal production of lycopene was observed after 144 hr of incubation, and then lycopene content declined. The same phenomenon occurs in CPTA treated culture: a slight increase in lycopene content during the first 12 hr followed by sharp increase in the next 12 hr and then decrease. When cultures were treated with a mixture of CPTA and cycloheximide, the production of lycopene reached its maximum at 12 hr after the treatment and then decreased. Maximal concentrations of lycopene were the same as in the control, indicating the nullifying effect of cycloheximide on the stimulatory effect of CPTA. CPTA is also acting as an inducer (or derepressor) rather than as an activator for the pre-existing enzyme(s). The synthesis of lycopene in the treated cultures is probably due to the inhibitory effect of CPTA on the pre-existing cyclase(s) in the system. Results of the group *D* experiment show that if the cultures are treated with CPTA first, lycopene induction thus stimulated is not affected by the following addition of cycloheximide. Although there is a lag phase of 12 hr before the lycopene is rapidly accumulated, the maximal concentration of lycopene in groups *B* and *D* cultures are close. Once the enzyme(s) participating in lycopene synthesis have been formed under the influence of CPTA, cycloheximide does not affect their activity. A similar phenomenon was observed for the stimulatory effect of trisporic acid on the carotenogenesis in *B. trispora*.[19]

Cycloheximide is known to act at the ribosomal level; it inhibits the formation of messenger RNA and then in turn inhibits protein synthesis.[20] The nullifying effect of cycloheximide on the effect of CPTA on lycopene synthesis suggests that CPTA may act as a derepressor of a gene regulating the synthesis of a specific enzyme or enzymes in the lycopene pathway. Previous studies have shown that in the ripening grapefruit, a large accumulation of phytoene is observed owing to the inhibition of the dehydrogenation steps in the sequential carotenogenic pathway.[18] On treatment with CPTA the fruits accumulated a large amount of lycopene.[3] This evidence strongly supports the view that CPTA may act as a derepressor.

EXPERIMENTAL

Organism. The (+) strain (NRRL 2456) and (−) strain (NRRL 2457) of *Blakeslea trispora* were obtained from Dr. Alex Ciegler of the Northern Regional Research Laboratory, USDA. Both strains were maintained on agars slopes consisting of per l., glucose 20 g; yeast extract 4 g; agar 15 g; and thiamine–HCl 0·0002%.

Culture. The medium was inoculated with the liquid obtained from the surface of the agar slopes by using the wire loop technique. The organism was cultured in Erlenmeyer flasks in a gyrotary shaker at 30°. The culture medium contained per l. glucose 20 g; potato extract 20 g and thiamine–HCl 0·0002%.

Extraction of lipid and preparation of unsaponifiable matter. The washed cultures were homogenized and the disruptured mycelia were extracted with acetone and methanol. The lipid was saponified and the unsaponifiable material extracted by standard procedures.[21]

Separation and identification of pigments. The unsaponifiable matter, dissolved in light petroleum (30–60°) was chromatographed on MgO: Hyflo-Supercel (1:1, w/w), and the various fractions were eluted with light petroleum (30–60°) with increasing amount of acetone. The pigments were identified by their UV and visible spectra and adsorption behavior relative to known compounds.

Quantitative determination. The method used has been described by Davies.[21]

Acknowledgement—CPTA was supplied by Amchem. Products, Inc., Ambler, Pa.

[20] D. BOULTER, *Ann. Rev. Plant Physiol.* **21**, 107 (1970).

[21] B. H. DAVIES, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), Academic Press, New York (1965).