Phytochemistry, 1973, Vol. 12, pp. 1633 to 1639. Pergamon Press. Printed in England.

EFFECT OF CPTA AND CYCOCEL ON THE BIOSYNTHESIS OF CAROTENOIDS BY *PHYCOMYCES BLAKESLEEANUS* MUTANTS*

MANZOOR ELAHI†, TERRENCE H. LEE‡, KENNETH L. SIMPSON and CLINTON O. CHICHESTER
Department of Food and Resource Chemistry, University of Rhode Island, Kingston, RI 02881, U.S.A.

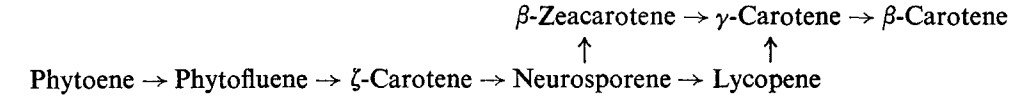
(*Received* 14 *December* 1972. *Accepted* 24 *January* 1973)

Key Word Index—*Phycomyces blakesleeanus*; Fungi; carotenoid biosynthesis; effect of trialkylammonium chlorides; biochemical mutants.

Abstract—CPTA and cycocel cause accumulation of lycopene and γ-carotene, simultaneously inhibiting the formation of β-carotene and β-zeacarotene in *Phycomyces blakesleeanus* mutant strain C115. Phytoene synthesis is enhanced. CPTA is more effective than cycocel. Kinetic studies show that with increasing concentrations of CPTA, lycopene and γ-carotene increase with the concomitant decrease in β-carotene, the total of these three carotenes being almost equal to β-carotene present in the control. When CPTA-treated mycelium is washed free of the chemical and resuspended in phosphate buffer solution containing 2·5% glucose (pH 5·6), β-carotene is formed at the expense of both γ-carotene and lycopene. β-Zeacarotene, which is not present in the mycelium, reappears upon resuspension. These results indicate that CPTA is inhibiting the enzymes causing cyclization both at neurosporene and lycopene levels. Studies on the effect of CPTA on the high lycopene mutant strain C9 reveal that with increasing concentrations of the compound, lycopene increases slightly and both β-carotene and γ-carotene decrease. Phytoene synthesis is stimulated up to a certain level of CPTA and then becomes steady. In the albino mutant strain C5, there is a slight increase in phytoene formation on the addition of CPTA to the medium. No other carotenoid is formed, suggesting that CPTA cannot remove the block caused by genetic mutation and exerts its influence in an already existing biosynthetic pathway.

INTRODUCTION§

ACCORDING to the revised scheme of Porter and Anderson,[1] there are two pathways for the formation of cyclic carotene:



The initial cyclization may occur at either neurosporene or lycopene levels. There are circumstantial evidences in support of both of these possibilities.[2] While lycopene[3,4] has

* Contribution No. 1456 of the Rhode Island Agricultural Experiment Station.
† Post-doctoral fellow, PCSIR Laboratories, Lahore-16, Pakistan.
‡ Department of Food Technology, University of New South Wales, Kensington, N.S.W., Australia.
§ Abbreviations used: CPTA, 2-(4-chlorophenylthio)-triethylamine hydrochloride; cycocel, (2-chloroethyl)-trimethylammonium chloride.

[1] PORTER, J. W. and ANDERSON, D. G. (1962) *Arch. Biochem. Biophys.* **97**, 520.
[2] GOODWIN, T. W. (1971) in *Carotenoids* (ISLER, O., ed.), p. 594, Birkhauser, Basel.
[3] KUSHWAHA, S. C., SUBBARAYAN, C., BEELER, D. A. and PORTER, J. W. (1969) *J. Biol. Chem.* **244**, 3635.
[4] DECKER, K. and UEHLEKE, H. (1961) *Z. Physiol. Chem.* **323**, 61.

been cyclized, there is no such direct experimental evidence for the cyclization of neurosporene. Although the basic mechanism of cyclization has been proposed,[2] the stage at which acyclic carotenes cyclize is a point that has yet to be established.

Recently it has been reported that in some carotenogenic systems, CPTA,[5,6] cycocel[7] and other chemicals[8,9] stimulate the bionsynthesis of lycopene and inhibit β-carotene formation, irrespective of whether the system produces little or no lycopene.

In this paper, the effect of CPTA and cycocel on the biosynthesis of carotenoids by *Phycomyces blakesleeanus* mutants is reported. This work was undertaken with the view of obtaining an insight into first, the stage at which acyclic carotenes cyclize and second, the possible mode of action of CPTA in *P. blakesleeanus* mutants.

RESULTS AND DISCUSSION

Table 1 shows the effect of CPTA and cycocel on the biosynthesis of carotenoids by *Phycomyces* strain C115 at two different concentrations. It will be observed that although CPTA, of the two, is more effective, both bring about changes, such as: (a) stimulation of phytoene formation; (b) little or no change in the intermediates like phytofluene, ζ-carotene and neurosporene despite the availability of the precursor phytoene in larger quantities; (c) accumulation of lycopene and γ-carotene with the simultaneous decrease in β-carotene; and (d) inhibition of β-zeacarotene formation.

TABLE 1. EFFECT OF VARIOUS CONCENTRATIONS OF CPTA AND CYCOCEL ON THE BIOSYNTHESIS OF CAROTENOIDS BY *Phycomyces blakesleeanus* STRAIN C115

Carotenoids μg/g dry basis	Control	CPTA (ppm) 25	CPTA (ppm) 100	Cycocel (ppm) 25	Cycocel (ppm) 100
Phytoene	142	200	315	162	270
Phytofluene	32	33	49	28	29
ζ-Carotene	10	10	13	8	8
Neurosporene	19	20	18	17	16
Lycopene	4·5	40	135	8	15
γ-Carotene	—	31	87	11	21
β-Carotene	1243	1128	1075	1195	1151
β-Zeacarotene	14	7	—	10	8
Total	1464·5	1469	1692	1439	1518

It remains to be seen whether the stimulation of phytoene biosynthesis occurs at the expense of other metabolites such as steroids and lipids, or by the stimulation of the overall biosynthetic pathway. It has been observed in the present studies, however, that in the presence of CPTA, steroid formation is somewhat inhibited. Results of the effects of CPTA on the synthesis of lipids and steroids will be reported separately. Both CPTA and cycocel have almost no effect on the formation of total carotenoids in this strain at a concentration of 25 ppm but an increase is observed at concentrations greater than 100 ppm.

[5] COGGINS, C. W., HENNING, G. L. and YOKOYAMA, H. (1970) *Science* **168,** 1589.
[6] HSU, W. J., YOKOYAMA, H. and COGGINS, C. W., JR. (1972) *Phytochemistry* **11,** 2985.
[7] KNYPL, J. S. (1969) *Naturwissenschaften* **56,** 572.
[8] HOWES, C. D. and BATRA, P. P. (1970) *Biochem. Biophys. Acta* **222,** 174.
[9] NINET, L., RENAUT, J. and TISISER, R. (1969) *Biotech. Bioengng* **11,** 1195.

Results of kinetic experiments (Table 2) in strain C115 show that phytoene synthesis increases with the increasing concentrations of CPTA up to 400 ppm. The intermediates phytofluene, ζ-carotene and neurosporene are also increased. β-Carotene and β-zeacarotene decrease and both lycopene and γ-carotene increase. At each concentration of CPTA, the

TABLE 2. EFFECT OF VARIOUS CONCENTRATIONS OF CPTA ON THE BIOSYNTHESIS OF CAROTENOIDS BY *Phycomyces blakesleeanus* STRAIN C115

Carotenoids μg/g dry basis	CPTA concentrations (ppm) 0	25	100	200	400	600	800	1000	1000*
Phytoene	138	180	225	408	460	427	432	420	1800
Phytofluene	38	46	50	67	76	95	96	104	305
ζ-Carotene	—	24	25	25	38	53	40	54	108
Neurosporene	12	14	15	20	23	28	31	28	86
Lycopene	6	57	157	254	416	440	687	792	1326
γ-Carotene	—	40	90	138	200	264	224	202	200
β-Carotene	1483	1302	1167	1017	838	702	485	362	75
β-Zeacarotene	12	Trace	—	—	—	—	—	—	—
Total	1689	1663	1729	1929	2051	2009	2003	1762	3900

* CPTA added immediately after inoculation.

total of lycopene, γ-carotene and β-carotene is nearly equal to that of β-carotene present in the control, suggesting that the former are increased at the expense of the latter. The ratio of levels of lycopene and γ-carotene to β-carotene is directly proportional to the CPTA concentration.

TABLE 3. EFFECT OF VARIOUS CONCENTRATIONS OF CPTA ON THE BIOSYNTHESIS OF CAROTENOIDS BY *Phycomyces blakesleeanus* STRAIN C9

Carotenoids μg/g dry basis	CPTA concentrations (ppm) 0	25	50	100	200	400	600	800
Phytoene	225	464	510	513	602	552	598	541
Phytofluene	95	100	94	97	101	113	114	107
ζ-Carotene	38	39	40	44	51	56	54	42
Neurosporene	21	19	23	22·5	27	26	22	20
Lycopene	656	700	642	681	735	725	718	722
γ-Carotene	38	41	34	33	31	21	13	6·5
β-Carotene	12	10	8	5·5	5	2·5	Trace	Trace
Total	1085	1373	1351	1396	1552	1495·5	1519	1438·5

The effect of various concentrations of CPTA was also studied on strain C9 which produces lycopene as its major pigment. CPTA (Table 3) caused an increased synthesis of phytoene; however, only negligible changes in the concentrations of phytofluene, ζ-carotene and neurosporene could be observed. The total carotenoids were increased. Both β-carotene

and γ-carotene decreased. The increase in lycopene was very small in this mutant which produces large amounts of lycopene in contrast to the large increase observed in a mutant that produces little or no lycopene.

TABLE 4. EFFECT OF VARIOUS CONCENTRATIONS OF CPTA ON THE BIOSYNTHESIS OF CAROTENOIDS BY *Phycomyces blakesleeanus* STRAIN C5

CPTA concns. (ppm)	Phytoene (μg/g dry basis)	CPTA concns. (ppm)	Phytoene (μg/g dry basis)
0	550	200	590
25	635	400	570
50	670	600	578
100	653	800	570

The albino mutant C5 which produces only phytoene was treated with increasing concentrations of CPTA. Although there was a slight increase in the level of phytoene, no other pigments were isolated (Table 4). Thus, CPTA is only effective in an already existing biosynthetic pathway and cannot remove a block caused by a genetic mutation.

TABLE 5. DISTRIBUTION OF CAROTENOIDS IN *Phycomyces blakesleeanus* STRAIN C115 GROWN NORMALLY AND IN THE PRESENCE OF CPTA (1000 ppm) FOR 4 DAYS

	Mycelium							
	Normal				CPTA-treated			
Carotenoids	Time (days)				Time (days)			
μg/g dry basis	1	2	3	4	1	2	3	4
Phytoene	220	265	130	175	220	264	400	285
Phytofluene	—	15	26	45	—	67	65	53
ζ-Carotene	—	4	15	30	—	28	51	41
Neurosporene	—	12	6·5	13	—	15	36	25
Lycopene	—	1	1	3	—	284	360	495
γ-Carotene	—	—	—	—	—	125	100	155
β-Carotene	440	915	1128	1135	440	337	746	462
β-Zeacarotene	—	5	5	3	—	—	—	—
Total	660	1217	1311·5	1404	660	1120	1758	1516

In order to develop information on the cyclization stage of acyclic carotenes and the mode of action of CPTA, the biosynthetic pathway was followed in strain C115 both with and without CPTA (Table 5). The mold was harvested at daily intervals up to 4 days. In the untreated mycelium, only phytoene and β-carotene could be detected after 1 day. After that period intermediates appear and the β-carotene content was increased. β-Zeacarotene which is present in the untreated mycelium after 2 days disappears in the presence of CPTA. Both lycopene and γ-carotene increase and β-carotene decrease in CPTA-treated mycelium; the total of the three was almost equal to β-carotene in the untreated mycelium at all stages of growth.

[10] LEE, T. C. and CHICHESTER, C. O. (1969) *Phytochemistry* **8**, 603.
[11] SIMPSON, K. L., NAKAYAMA, T. O. M. and CHICHESTER, C. O. (1964) *J. Bacteriology* **88**, 1688.

Four-day-old mycelium of strain C115 (both CPTA-treated and untreated) were washed and resuspended in fresh medium and the changes in carotenoids were determined at intervals of 24 hr. All the carotenoids increased after the second day onward.

In the case of CPTA-treated and resuspended mycelium, about 75% of each of lycopene and γ-carotene present in the inoculum was lost after 1 day with the simultaneous increase in β-carotene. β-Zeacarotene which is absent in the inoculum, reappears in significant amounts indicating its possible role in the formation of β-carotene. Similar results have been reported[12] when diphenylamine-treated mycelium of *P. blakesleeanus* was washed and resuspended in a glucose–phosphate solution.

The increase in β-carotene with the simultaneous decrease in lycopene and γ-carotene in washed and resuspended cells shows that lycopene can be converted to cyclic carotenes once the CPTA block is removed. However, the results indicate possible alternate pathways to γ-carotene via either lycopene or β-zeacarotene.

Washed mycelia of both untreated and CPTA-treated were resuspended in phosphate buffer or standard medium devoid of yeast autolysate and thiamine hydrochloride at pH 5·6.

While the changes in the levels of phytoene, phytofluene, ζ-carotene, and neurosporene would appear to be minor there is a conversion of lycopene and γ-carotene into β-carotene. The role of β-zeacarotene in β-carotene formation would appear to be negligible where there was no significant net synthesis of carotenes. However, when the cells are transferred to a complete medium (Table 6) the level of lycopene drops significantly.

TABLE 6. DISTRIBUTION OF CAROTENOIDS WHEN WASHED MYCELIUM (BOTH NORMAL AND CPTA-TREATED) OF *Phycomyces Blakesleeanus* STRAIN C115 ARE SUSPENDED IN FRESH MEDIUM

Carotenoids μg/250 ml of medium	Normal mycelium					CPTA-treated mycelium				
	Amount present in inoculum	Time (days) 1	2	3	4	Amount present in inoculum	Time (days) 1	2	3	4
Phytoene	89	163	375	545	800	400	142	460	675	810
Phytofluene	40	26	63	107	114	64	35	71	111	174
ζ-Carotene	41	2	17	27	35	33	—	23	25	39
Neurosporene	16	trace	16	25	51	24	11	23	31·5	31·5
Lycopene	4–5	—	—	12	16	300	81	81	78	72
γ-Carotene	—	—	—	5	19	65	15	11	12	21
β-Carotene	517	570	1310	2382	3072	261	572	1441	2048	2679
β-Zeacarotene	24	14	56	72	93	—	27·5	64	78	46·3
Total	704	775	1837	3175	4200	1147	883·5	2174	3058·5	3875

The initial report by Coggins *et al.*[5] on the effect of CPTA showed that the chemical caused the accumulation of lycopene in a wide spectrum of carotenogenic systems such as fruits, vegetables and microorganisms. Recently, these authors have reported in more detail the effect of CPTA on citrus[13,14] and *Blakeslea trispora*.[6] It would appear from their studies that CPTA not only inhibits the cyclases but also causes a stimulation of the synthesis of the other polyenes. In *B. trispora*[6] a large accumulation of lycopene and γ-carotene with a drop in β-carotene and β-zeacarotene was reported. By the use of cycloheximide, an inhibitor of protein synthesis, these authors found that when used in combination with CPTA the

[12] DAVIES, B. H., VILLOUTREIX, J., WILLIAMS, R. J. H. and GOODWIN, T. W. (1963) *Biochem. J.* **89**, 96P.
[13] YOKOYAMA, H., COGGINS, C. W. JR. and HENNING, G. L. (1971) *Phytochemistry* **10**, 1831.
[14] YOKOYAMA, H., COGGINS, C. W. JR., HENNING, G. L. and DEBENEDICT, C. (1972) *Phytochemistry* **11**, 1721.

stimulatory effect of CPTA on lycopene accumulation was nullified. It was suggested that CPTA acts 'as a depressor of a gene regulating the synthesis of a specific enzyme or enzymes in the lycopene pathway'.

A comparison of the data on *B. trispora* and the mutants of *Phycomycetes* allows a number of observations to be made. In the present study, CPTA was normally added 24 hr after inoculation because of lower growth when the chemical is added at inoculation. Table 2 shows that even up to 1000 ppm CPTA the sum total of carotenoids is nearly that of the control. However, when the 1000 ppm was added immediately after inoculation the amount of cell mass at harvest was less (0·6 g/l. vs. 7·5 g/l. dry wt), but the amount of carotenoids was more than doubled. The effect on the polyenes phytofluene, ζ-carotene, and neurosporene was minimal where the chemical was added at 24 hr. However, when added at the time of inoculation the level of these polymers increased 2–3 times that of the level observed with the later addition of CPTA. Thus, there is a stimulation of carotene synthesis and an increase in lycopene proportional to the total increase in carotene formation. This increase may occur because of a shift away from sterol formation to carotene formation at the farnesol phyrophosphate, squalene or steroid levels. It has been a consistent observation that the amounts of steroids is reduced in both the CPTA-treated *Rhodotorula* and *Phycomycetes* cultures. It would appear that once these enzymes are formed the stimulatory effect of CPTA is less.

The addition of CPTA on strain C5 at 24 hr had no effect on phytoene formation. In the high lycopene strain C9 the increased total level of polyenes was due to an increase in phytoene, not to a large increase in lycopene.

TABLE 7. DISTRIBUTION OF CAROTENOIDS WHEN WASHED MYCELIUM (BOTH NORMAL AND CPTA-TREATED) OF *Phycomyces blakesleeanus* STRAIN C115 ARE RESUSPENDED IN PHOSPHATE BUFFER AND MINERAL SOLUTION

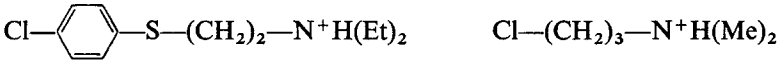
Carotenoids μg/g dry basis	Normal mycelium time (days)			CPTA-treated mycelium time (days)				
				Phosphate buffer			Mineral solution	
	0	1	2	0	1	2	1	2
Phytoene	273	320	346	481	500	560	615	522
Phytofluene	40	51	33	93	80	68	71	80
ζ-Carotene	10	8	Trace	42	45	21	Trace	12
Neurosporene	16	15	16	25	24	26	20	23
Lycopene	6	3·5	3	500	354	325	340	290
γ-Carotene	—	—	—	165	116	93	84	69
β-Carotene	1144	1167	1151	427	720	750	721	729
β-Zeacarotene	22	30	26	—	Trace	—	15	Trace
Total	1511	1594·4	1575	1733	1839	1853	1866	1725

The fate of the lycopene once formed in CPTA-treated cultures has not been determined. Tables 6 and 7 show that once the chemical is removed lycopene can be converted to γ- and β-carotenes. These results show the probably conversion of lycopene to γ-carotene and β-zeacarotene's active role in β-carotene synthesis. It remains to be seen whether one route or both routes to β-carotene are operative in *Phycomycetes*.

Recently de la Guardia *et al.*[15] reported on the quantitative analysis of carotenoids in

[15] DELAGUARDIA, M. D., ARAGÓN, C. M. G., MURILLO, F. and CERDÁ-OLMEDO, E. (1971) *Proc. Nat. Acad. Sci.* **68**, 2012.

heterokaryotic C2*C9 myclelia. The results of genetic analyses of nuclear proportions were interpreted to fit the hypothesis that carotenogenesis is performed by linearly-organized enzyme aggregates. These authors proposed that intermediates could not be transferred from one aggregate to another nor could an intermediate be further transformed once released from an aggregate. While an enzyme aggregate rather than free enzymes are probably involved in carotene synthesis, our results show that when lycopene and γ-carotene are released due to the action of cyclase inhibitors they can be further acted upon. Thus, both water soluble compounds such as CPTA and cycocel and insoluble compounds such as lycopene would appear to have access to the enzyme aggregate. Although CPTA (I) and cycocel (II) are structurally different, they exert the same effect (Table 1).



(I) CPTA (II) Cycocel

These compounds are derivatives of trialkyl ammonium chlorides and have one thing in common, i.e. quaternary ammonium ion. Thus, by analogy with pyridine,[16,17] the inhibitory action may be attributed to this quaternary ammonium ion. The difference in the activity of these compounds may be due to the difference in permeability of the cell wall to these compounds or to some structural feature.

EXPERIMENTAL

Materials and methods. Phycomyces blakesleeanus strains C5, carrying the mutation *carB*10 (previously Albino 10), C115 (previously Ph 107) and C9, carrying mutation *carR*21 (previously R1) were kindly supplied by Dr. M. Delbrück of the California Institute of Technology, Pasadena, Calif., U.S.A. All reagents used were of analytical grade. Other materials were obtained as follows: CPTA from Amchem Products, Inc., Ambler, Pa., U.S.A.; cycocel from Eastern Organic Chemicals, Rochester, N.Y., U.S.A. Solvents were distilled before use.

Mold growth conditions. The sterile standard medium[10] (250 ml) contained in one liter flasks was inoculated with equal vol. of spore suspension of the respective molds and allowed to grow in a controlled environmental shaker for 24 hr prior to the addition of the CPTA or cycocel under the following conditions: shaker speed, 200 rpm; temp., 20°; illumination, fluorescent lamps giving a light intensity of 3000 lx at the liquid surface. The mold was grown for 3 additional days before harvesting. In experiments to study the rate of biosynthesis of carotenoids by strain C115 grown both normally and in the presence of CPTA, the mold was harvested at 24-hr intervals starting after 1 day. For resuspension experiments, strain C115 was allowed to grow both with and without CPTA for 4 days as described above, harvested by transferring onto a Buchner funnel and washed free of CPTA with sterile H_2O. The washed mycelium both normal and CPTA-treated, was resuspended (in equal amounts on dry basis) in fresh sterile medium (250 ml) and allowed to grow. The washed mycelia as obtained above were also resuspended in 250 ml of each of phosphate buffer pH 5·6 containing 2·5% glucose and standard medium[10] devoid of yeast autolysate and thiamine hydrochloride adjusted to pH 5·6 with KOH. The rate of biosynthesis of carotenoids was followed by harvesting the mold after intervals of 24 hr.

Extraction and chromatographic separation of pigments. A portion of each harvested mold was used for moisture determination. The method of extraction, saponification, chromatographic separation and estimation of carotenoids had been reported earlier.[10,11]

Acknowledgements—This work was supported by Grants Nos. 7R01AM 15483-01 (C.O.C.) and R01/NB08516-01 (K.L.S.) from the National Institutes of Health. The *Phycomycetes* mutants were kindly supplied by Dr. M. Delbrück of California Institute of Technology.

[16] ATKINSON, M. R., ECKERMAN, G. and LILLEY, R. M. (1967) *Biochem. J.* **104,** 872.
[17] ELAHI, M., CHICHESTER, C. O. and SIMPSON, K. L. (1973) *Phytochemistry* **12,** 1627.