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# BIOSYNTHESIS OF CAROTENOIDS BY *PHYCOMYCES BLAKESLEEANUS* MUTANTS IN THE PRESENCE OF NITROGENOUS HETEROCYCLIC COMPOUNDS*

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**Key Word Index**—*Phycomyces blakesleeanus*; fungi; carotenoid biosynthesis; effect of nitrogen heterocytes; pyridine derivatives; enzymes of cyclization.

**Abstract**—Pyridine, imidazole and some of their derivatives stimulate lycopene and γ-carotene synthesis-simultaneously inhibiting β-carotene formation in *Phycomyces blakesleeanus* Strain C115. Isonicotinoylhydrazine has a toxic effect on Strains C9 and C115 and 1-methylimidazole on Strain C115 in the concentrations of 1 g/l. Compounds which cause an accumulation of lycopene and γ-carotene usually cause an increase in phytoene synthesis and the disappearance of β-zeacarotene. The effect of succinimide, 4-hydroxypyridine, and isonicotinoylhydrazine on Strain C9 has also been studied. When β-picoline and 2-methylimidazole treated C115 mycelia were washed and resuspended in phosphate buffer at pH 5·6, β-zeacarotene reappeared and β-carotene increased with the simultaneous decrease in lycopene and γ-carotene. The sum of β-carotene, γ-carotene up to 3 days of resuspension was almost equal to the total of these at zero time. These results show that the inhibitory action of these compounds is on the enzymes responsible for cyclization of acyclic carotenes. This inhibition varies with the nature of the substituent on the heterocyclic ring and pyridine derivatives having $pK_a$ values of $6 \pm 1$ show the greatest degree of inhibition.

## INTRODUCTION

IN A previous communication the effects of CPTA on the biosynthesis of carotenoids by *Phycomyces blakesleeanus* have been reported.[1] CPTA is known to cause accumulation of lycopene and γ-carotene in many carotenogenic systems.[2–5] Cycocel [(2-chloroethyl)-trimethyl ammonium chloride] stimulates lycopene biosynthesis in pumpkin cotyledons[6] and *P. blakesleeanus*.[1] Nicotine causes accumulation of lycopene in *Myocbacterium marinum*.[7]

Ninet *et al.*[8] studied the biosynthesis of carotenoids by *Blakeslea trispora* in the presence of various organic compounds. Pyridine, imidazole and some of their derivatives were found to stimulate the synthesis of lycopene while isonicotinoylhydrazine, succinimide and 4-hydroxypyridine enhanced β-carotene synthesis. These authors restricted their studies to the end products (lycopene and β-carotene) and did not report on the effect of these chemicals on the formation of the intermediates.

In the present paper the biosynthesis of carotenoids by *P. blakesleeanus* mutants have been studied in the presence of nitrogenous heterocyclic compounds and the results are discussed in relation to present theories on carotene biosynthesis.

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## RESULTS AND DISCUSSION

Table 1 shows the effect of some nitrogenous heterocyclic compounds on the biosynthesis of carotenoids by *P. blakesleeanus* Strain C115. All compounds tested except 2-acetylpyridine, 4-hydroxypyridine, 1-methylimidazole, 2-methylpyrazine, nicotinic acid, nicotinamide and isonicotinoylhydrazine cause accumulation of lycopene and γ-carotene with the simultaneous decrease in the β-carotene content. The sum of β-carotene, γ-carotene and lycopene in the treated mycelium was considerably less than that of β-carotene in the control.

TABLE 1. EFFECT OF NITROGENOUS HETEROCYCLIC COMPOUNDS ON THE BIOSYNTHESIS OF CAROTENOIDS BY *Phycomyces blakesleeanus* STRAIN C115

| Carotenoids μg/g dry basis | Nitrogenous compound* 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Phytoene | 191 | 263 | 396 | 325 | 253 | 203 | 315 | 202 | 187 |
| Phytofluene | 33 | 48·5 | 68 | 59 | 57 | 36 | 54·5 | 37 | 33 |
| ζ-Carotene | 10·5 | 22 | 33 | 30·5 | 20 | 6 | 30 | 7·5 | 9 |
| Neurosporene | 12 | 15·5 | 20 | 17·5 | 10 | 10 | 14 | 11 | 10 |
| Lycopene | 5 | 30 | 108 | 124 | 15 | 6 | 53 | 7 | 5 |
| β-Zeacarotene | 20 | — | — | — | T | 14 | — | — | 18 |
| γ-Carotene | T | 23·5 | 63·5 | 65 | 18 | T | 21 | T | T |
| β-Carotene | 1200 | 844 | 753 | 790 | 990 | 1029 | 1062 | 1170 | 1090 |
| Totals | 1471·5 | 1246·5 | 1441·5 | 1411·0 | 1363 | 1304 | 1549·5 | 1449·5 | 1352 |
| Wt of mycelium, dry basis, g/250 ml medium | 2·15 | 2·01 | 2·04 | 2·02 | 2·25 | 2·2 | 1·95 | 2·22 | 2·2 |

| Nitrogenous compound 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|
| 210 | 231 | 193 | 231 | 113 | 435 | 112 | 298 | 319 |
| 30 | 42 | 35 | 43·5 | T | 82·5 | — | 58 | 42 |
| 11 | 6 | 9 | 21 | — | 42·5 | — | 20 | 24 |
| 11 | 10·5 | 15 | 13·5 | — | 20 | — | 11 | 14 |
| 4 | 8 | 4·5 | 130 | T | 617 | — | 96 | 45 |
| 20 | 24 | — | — | — | — | — | — | — |
| T | T | T | 68 | — | 52·5 | — | 32 | 42 |
| 1120 | 1076 | 1250 | 700 | 366 | 64·5 | 1225 | 590 | 804 |
| 1406 | 1397·5 | 1506·5 | 1207·0 | 489 | 1314·0 | 1337 | 1105 | 1290 |
| 2·1 | 2·04 | 2·13 | 2·15 | 0·6 | 2·01 | 0·7 | 2·2 | 2·04 |

* *Key.* *T*—trace; 1—Control; 2—Pyridine (12·7)†; 3—β-Picoline (10·7); 4—2,4-Dimethyl-pyridine (9·3); 5—2,4,6-trimethyl-pyridine (8·3); 6—2-Acetyl-pyridine (8·3); 7—3-Hydroxypyridine (10·5); 8—4-Hydroxypyridine (10·5); 9—Nicotinic acid (8·1); 10—Nicotinamide (8·2); 11—2-Methylpyrazine (10·6); 12—Succinimide (10·1); 13—Imidazole (14·8); 14—1-Methylimidazole (12·2); 15—2-Methylimidazole (12·2); 16—Isonicotinoylhydrazine (7·3); 17—Piperidine (11·8); 18—Nicotine (6·2).

† mM/l. added.

Concentration of each compound used—1 g/l. of medium.

The weight of dry mycelium/250 ml of the medium was almost the same irrespective of whether these compounds were added, except in the case of isonicotinoylhydrazine and 1-methylimidazole. All compounds which cause an accumulation of lycopene also bring about a significant increase in phytoene. β-Zeacarotene was significantly decreased in the presence of all the compounds which cause the accumulation of lycopene.

1-Methylimidazole has been reported to stimulate lycopene synthesis and was more effective than 2-methylimidazole in *B. trispora*.[8] In *P. blakesleeanus* Strain C115 it shows a rather toxic effect as the growth was retarded significantly and the color of the mycelium turns a greyish-green after 4 days of growth. β-Carotene and phytoene are present in much smaller amounts and traces of phytofluene and γ-carotene were detected.

In the study reported in Table 1, 1 g/l. concentration was used rather than equal molar concentrations in order that a comparison could be made with previous work. The molar amounts added are listed in Table 1. Preliminary experiments showed that small differences in concentration did not give significantly different results. The small differences in molar concentrations within sets thus was not found to be significant. The 3- and 4-hydroxypyridines and 1- and 2-methylimidazole isomers were added at the same concentration on either basis and their inhibition is seen to be based on structural differences.

TABLE 2. EFFECT OF SOME NITROGENOUS HETEROCYCLIC COMPOUNDS ON THE BIOSYNTHESIS OF CAROTENOIDS IN *Phycomyces blakesleeanus* STRAIN C9

| Carotenoids μg/g dry basis | Control | Succinimide | 4-Hydroxy-pyridine | Isonicotinoyl-hydrazine |
|---|---|---|---|---|
| Phytoene | 340 | 386 | 261 | 200 |
| Phytofluene | 60 | 64 | 50 | — |
| ζ-Carotene | 27 | 30 | 21 | — |
| Neurosporene | 17·5 | 17 | 15·5 | — |
| Lycopene | 621 | 700 | 556 | 745 |
| γ-Carotene | 18 | 23 | 13 | — |
| β-Carotene | 4 | 3·5 | 3·5 | — |
| Total | 1087·5 | 1223·5 | 920·0 | 945·0 |
| Wt of mycelium dry basis g/250 ml medium | 2·10 | 2·03 | 2·14 | 0·49 |

It has been reported[8] that isonicotinoylhydrazine, 4-hydroxypyridine and succinimide, when used in the concentrations of 1, 3 and 4 g/l., increase the synthesis of β-carotene in *B. trispora*. In the present study the last two compounds at 1 g/l. were observed to have almost no effect on carotene formation. Isonicotinoylhydrazine was found to retard the growth of the mold and only phytoene and β-carotene were isolated from the mold where this compound was used. At 0·1% the other compounds were not observed to inhibit growth as judged by the weight of mycelia produced.

Table 2 shows the effect of the above-mentioned compounds on the biosynthesis of carotenoids by *P. blakesleeanus* Strain C9. This study was made to obtain information on whether the increase in β-carotene content, as reported earlier,[8] is at the expense of lycopene or the

activation of the overall biosynthesis. It will be noted that succinimide has slightly enhanced lycopene formation and has almost no effect on β-carotene synthesis. 4-Hydroxypyridine was observed to be slightly inhibitory to the formation of the carotenoids. Isonicotinoylhydrazine caused an increase in lycopene on the basis of 1 g of dry mycelium, but has a somewhat toxic effect on the growth of this mutant because only 0·49 g of mycelium is obtained as compared to 2·0–2·14 g in the presence of other compounds. No other carotenoid except phytoene and lycopene was isolated.

In the previous study,[8] 1, 2 and 4 g/l. of each chemical was used. Table 2 lists only the use of 1 g/l. as 2 and 4 g/l. gave essentially the same result. Only about half as much mycelium was obtained (0·28 g/flask) with isonicotinoylhydrazine as that obtained at 1 g/l.

Cultures of Strain C115 were allowed to grow for 4 days in the presence of β-picoline and 2-methylimidazole and the mycelium was washed free of the chemicals and resuspended in phosphate buffer. Table 3 shows that there was a loss of total carotenoids on resuspending the control in buffer. The percentages loss of lycopene, γ-carotene, β-carotene and β-zeacarotene was slightly greater than that of the total loss due mainly to the slight increase in the level of phytoene. While there was an increase in the total carotene content on resuspending the mycelia from the treated samples, the increase in lycopene and the cyclic compounds was relatively less. Again, the increase was mainly due to increased synthesis of phytoene. Some new synthesis of polyenes occurred, as can be seen from the synthesis of β-zeacarotene, but these increases cannot explain the large decrease in lycopene and γ-carotene and increase in β-carotene. These results clearly show that the lycopene formed, due to the inhibition of the inhibitors, can be converted to β-carotene on resuspension of the mycelia. β-Zeacarotene increases up to 2 days and then decreases in the control and it reappears in trace amounts in the case of β-picoline-treated mycelium, which shows the inhibition of cyclization of neurosporene. No β-zeacarotene has been detected in the case of 2-methylimidazole-treated mycelium.

TABLE 3. DISTRIBUTION OF CAROTENOIDS WHEN WASHED MYCELIUM (WITH NORMAL AND β-PICOLINE, 2-METHYLIMIDAZOLE TREATED) OF *Phycomyces blakesleeanus* STRAIN C115 ARE RESUSPENDED IN PHOSPHATE BUFFER CONTAINING 2·5% GLUCOSE AT pH 5·6

| Carotenoids kg/g dry basis | Control time (days) | | | | β-Picoline-treated time (days) | | | | 2-Methylimidazole-treated time (days) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| Phytoene | 192 | 190 | 240 | 244 | 340 | 380 | 435 | 420 | 410 | 464 | 450 | 470 |
| Phytofluene | 28 | 28 | 25 | 20 | 86 | 54 | 50 | 50 | 70 | 62 | 57 | 54 |
| ζ-Carotene | 10 | — | — | — | 28 | 30 | 33 | 18 | 29 | 14 | 24 | 31 |
| Neuorsporene | 11 | 17 | 17 | 8 | 17 | 18 | 13 | 20 | 14 | 20 | 20 | 12 |
| *Lycopene | 5 | — | — | — | 100 | 25 | 21 | 17 | 457 | 345 | 239 | 216 |
| *γ-Carotene | trace | — | — | — | 61 | 27 | 18 | 20 | 94 | 70 | 70 | 55 |
| *β-Carotene | 1130 | 1045 | 1015 | 1028 | 840 | 988 | 1025 | 1020 | 111 | 283 | 366 | 410 |
| *β-Zeacarotene | 22 | 31 | 33 | 18 | — | — | 5 | 7 | — | — | — | — |
| Totals | 1398 | 1311 | 1330 | 1318 | 1472 | 1522 | 1600 | 1572 | 1185 | 1258 | 1226 | 1248 |
| % Increase of (*) carotenes | | −7·0 | −9·4 | −9·5 | | 4·0 | 6·2 | 5·6 | | 5·4 | 1·9 | 2·8 |
| % Increase of all polyenes | | −6·2 | −4·8 | −5·7 | | 3·3 | 8·6 | 6·9 | | 6·2 | 3·4 | 5·3 |

The disappearance of β-zeacarotene on treating the mold with β-picoline and 2-imidazole and its reappearance on resuspension of the β-picoline-treated mycelium shows the inhibition

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

of cyclization at neurosporene level. No β-zeacarotene has been detected on resuspending the 2-methylimidazole mycelium.

Pyridine, imidazole and other heterocyclic nitrogenous compounds which can direct the biosynthetic pathway towards lycopene do so by inhibiting the enzymes responsible for cyclization. Both pyridine[11,12] and imidazole[13] have been reported to inhibit the yeast alcohol dehydrogenases. The inhibition by pyridine has been attributed to the pyridinium ion and not the unionized pyridine. Similar results have been obtained in the present studies as the inhibitory action of pyridine varies with the nature of the substituents. However, the degree of inhibition of cyclization reaction was opposite to that of the alcohol dehydrogenase reaction, as the greater the electronegativity of the side chain, the weaker the inhibition. This can clearly be seen in Fig. 1 where the level of inhibition of some pyridine derivatives are plotted against their $pK_a$ values. In this series the highest level of inhibition occurs in the $pK_a$ value range of 5–7. 2,4-Dimethyl- and 2,4,6-trimethylpyridine (Fig. 1) would appear to show that at $pK_a$ values greater than 7 the level of inhibition decreases. This may be due to steric factors since nicotine and piperidine with $pK_a$ values greater than 8 are effective inhibitors.

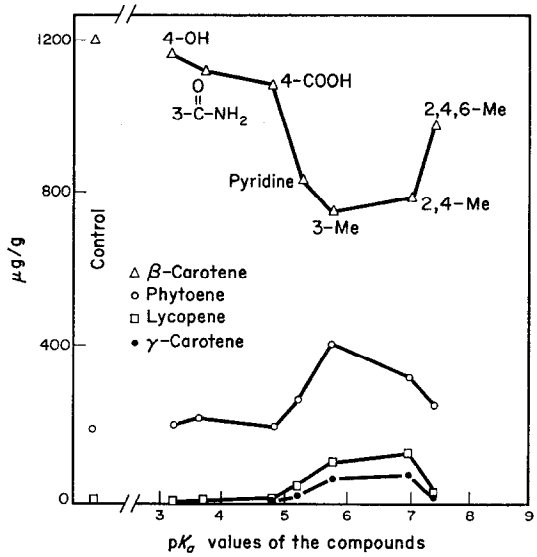


FIG. 1. THE INHIBITION OF CAROTENE SYNTHESIS BY PYRIDINE DERIVATIVE AS RELATED TO THEIR $pK_a$ VALUES. PIGMENT LEVELS EXPRESSED ON μg/g DRY WT BASIS.

While 3-hydroxypyridine causes accumulation of lycopene, 4-hydroxypyridine does not. Ninet *et al.*[8] has reported similar results with *B. trispora*. 4-Hydroxypyridine forms the ketonic tautomer, γ-pyridines, and has a low $pK_a$ value, whereas 3-hydroxypyridine does not form such tautomers and has a higher $pK_a$ value. Both imidazole and 1-methylimidazole have $pK_a$ values of 6·95. Imidazole treatment results in the stimulation of lycopene and γ-carotene with the inhibition of β-carotene. 2-Methylimidazole treatment results in a much

[11] EYS, J. V. and KAPLAN, N. O. (1957) *Biochem. Biophys. Acta* **23**, 574.
[12] ATKINSON, M. R., ECKERMANN, G. and LILLEY, R. M. (1967) *Biochem. J.* **104**, 872.
[13] MCKINLEY-MCKEE, J. S. (1964) *Progr. Biophys. Molec. Biol.* **14**, 225.

greater effect, whereas the 1-methylimidazole treatment results in a large reduction of carotenoid formation and growth. Both 1-methylimidazole and isonicotinoylhydrazine treatments result in the rather curious situation of only phytoene and the end product, β-carotene, being isolated.

The mechanism of the cyclization reaction has been outlined by Goodwin,[14] based on hydrogen loss and retention on carbons 4 and 6 of the β and ε rings. One might expect the cyclase to have negative and positive areas in the active center corresponding to an intermediate compound electron rich at C-2 and deficient at C-5. One could speculate on the basis of the correlation between $pK_a$ values and cyclase inhibition that the point of inhibition is at the positive area of the active center. Until the cyclase has been isolated and purified, one could not rule out the simple explanation of the compounds having varying degrees of permeability to the cell wall or access to the enzymes.

Recently, DeLaGuardia *et al.*[15] reported results on the analyses of the carotenoids in heterokaryotic *Phycomyces* mycelia with different nuclear proportions as fitting a linearly organized enzyme aggregate. In their model these authors proposed that each enzyme receives its substrate from the previous enzyme and passes its product to the next enzyme. Once a compound was discharged from the aggregate, according to the model it could not be returned. In the previous[1] as well as the present paper, data are presented which show that lycopene can be acted upon once the inhibitor has been removed. The availability of lycopene as a substrate reported here would tend to argue against the explanation given by these authors for the non-involvement of β-zeacarotene in *Rhizophlyctis rosea*. The action of isonicotinoylhydrazine and 1-methylimidazole on carotene synthesis in *Phycomyces* would tend to support the enzyme aggregate theory. In some manner these compounds prevent the discharge of intermediates and only the final product is observed. These results would be hard to explain on the basis of a system consisting of random action of free enzymes.

## EXPERIMENTAL

*Materials and methods. Phycomyces blakesleeanus* Strains C9, carrying mutation *carR*21 (previously R1) and C115 (previously Ph 107) were obtained from Dr. M. Delbrück, California Institute of Technology, Pasadena, Calif., U.S.A. Chemicals were purchased commercially.

*Mold growth conditions.* Equal vol. of spore suspension of the respective strains of *P. blakesleeanus*, in sterile $H_2O$, were added to equal vols. (250 ml) of standard medium[9] contained in a 1-l. flask. The mold was allowed to grow for 24 hr in a controlled environmental incubator at 20° as described previously.[1] The standard solution of chemicals (sterilized by filtration) were added in the concentrations of 1 g/l. of medium. The mold was allowed to grow for 3 days further before harvesting. In resuspension experiments, Strain C115 was allowed to grow for 4 days in the presence of β-picoline and 2-methylimidazole (1 g/l.) as described above. The mold was then aseptically washed free of the chemicals. The washed mycellium was then resuspended in 0·067 M phosphate buffer (pH 5·6) containing 2·5% glucose. Samples were taken for analysis at 24-hr intervals.

*Extraction and chromatographic separation of carotenoids.* Extraction, saponification and chromatographic separation were made according to the methods described earlier.[1,8,10]

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[14] GOODWIN, T. W. (1971) in *Carotenoids* (ISLER, O., ed.), p. 596, Birkhauser, Basel.

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