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STIMULATION OF CAROTENOGENESIS BY PENICILLIN IN *BLAKESLEA TRISPORA*

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Abstract—Penicillin (1 mg/ml) specifically caused an increase in the synthesis of lipids, (carotenes and sterols) in *Blakeslea trispora*. The increase in cartenogenesis was associated with the increase in mevalonate kinase activity. The stimulation of carotenogenesis by penicillin was sensitive to iodoacetamide (40 μM). For penicillin mediated stimulation of carotenogenesis *de novo* protein synthesis is not essential. The accumulation of carotenes in the cultures of *B. trispora* grown in the presence of penicillin seems to be the result of increased synthesis of carotenes due to stimulation of early steps of isoprene pathway and decreased degradation of already synthesized carotenes to trisporic acids and other products.

INTRODUCTION

The different physical and chemical factors affecting carotenogenesis in various systems have been reviewed by various authors [1–5]. A variety of chemicals have been studied for their effect on carotenogenesis. Some of the important groups are terpenes, ionones, amines, alkaloids and antibiotics. The effect of antibiotics on carotenogenesis has been studied only in seedlings [6, 7], *Euglena gracilis* [8] and *Phycomyces blakesleeanus* [9] and that also with respect to a few antibiotics, e.g. streptomycin, chloramphenicol, terramycin and penicillin. During the present studies on carotenogenesis in moulds, the effect of some antibiotics on carotenogenesis was observed in *Neurospora crassa* and *Blakeslea trispora*. Among the antibiotics tested only penicillin and ampicillin stimulated carotenogenesis in *B. trispora* [10, and our unpublished observation]. It was of interest to study the effect of penicillin on the physiology of the mould *B. trispora* and to understand the mechanism of stimulation of carotenogenesis by penicillin.

RESULTS AND DISCUSSION

The gross chemical composition of the mycelia of the cultures grown in the absence and presence of penicillin was determined (Table 1). There were no significant changes in total protein and carbohydrate contents of both types of mycelia. However, lipid constituents were greater in the case of cultures grown on media with penicillin. There was a 50% increase in total lipids and the carotenoids and sterols were about 50% and 30% more resectively. The results indicate that there is an overall increase in lipid synthesis and further suggest that carotenes are not synthesized at the expense of sterols, as both share a common pathway (up to C_{15} farnesyl pyrophosphate) for their biogenesis. It further suggests that penicillin exerts its stimulatory effect during the early stages of the isoprene biosynthetic pathway.

The activity of the enzyme mevalonate kinase (one of the early enzymes of the isoprene pathway) nearly doubled reaching 5.8 units/mg protein in the cultures grown in the presence of penicillin, compared to 3.25 units/mg protein in the corresponding control cultures. This observation also explained the increased carotenogenesis in the presence of penicillin and suggested that penicillin may be stimulating the early steps of C_5 formation and thereby stimulating the overall pathway of carotenogenesis.

The results (Table 1) indicate the possibility of the activation of early steps of the isoprene pathway in the cultures grown in the presence of penicillin. It was of interest to study the effect of iodoacetamide on carotenogenesis in the cultures grown in the absence and presence of penicillin (Table 2). Carotenogenesis was completely inhibited at iodoacetamide concentrations above 0.15 mM 150 μM). There was *ca* 60% inhibition of carotenogenesis at 40 μm iodoacetamide. Inhibition was restric-

Table 1. Carotene, lipid, sterol, protein and carbohydrate contents of mated *Blakeslea trispora*

No.	Incubation condition	Growth dry mycelia/100 ml medium mg	Carotene μg/g	Lipid mg/g	Sterol mg/g	Total protein mg/g	Total carbohydrates mg/g
1.	Without penicillin	347 ± 11	1.650 ± 102	101 ± 13	3.92 ± 0.22	352 ± 40	496 ± 50
2.	With penicillin	352 ± 12	2.560 ± 155	156 ± 19	5.15 ± 0.3	335 ± 35	494 ± 55

Penicillin (1 mg/ml) was added at 24 hr, cultures were harvested at 96 hr.

Table 2. Effect of iodoacetamide on carotenogenesis in *Blakeslea trispora* grown in the absence (A) and presence (B) of penicillin*

Iodoacetamide addition† (μM)	Carotene (μg/g mycelia)‡	
	Group A	Group B
Nil	200	295
20	200	225
40	85	200
100	30	35
150	15	20
200	15	15

* Penicillin was added at 24 hr. † Iodoacetamide was added at 48 hr. ‡Cultures were harvested at 96 hr and analysed.

ted to *ca* 30% with the same concentration of iodoacetamide (40 μM) in the penicillin added cultures. Iodoacetamide is known to inhibit carotenogenesis [11, 12]. From the data it is also evident that at 40 μM iodoacetamide concentration, the increase in carotenogenesis which would take place in the presence of penicillin was inhibited. This suggests that the stimulation of carotenogenesis is iodoacetomide sensitive.

The cell free extracts prepared from the cultures grown in the presence and in the absence of penicillin were compared with respect to the net synthesis of carotenes *in vitro*. The results (Table 3) show that the net synthesis of carotenes was more in the case of extracts prepared from cultures grown in the presence of penicillin than in the extracts of cultures grown without penicillin. This indicated the presence of stimulatory factors of carotenogenesis when cultures are grown in the presence of penicillin. We further observed that penicillin caused stimulation of carotenogenesis irrespective of the presence of asparagine which was the sole source of nitrogen in the medium to which the mould was transferred. In the absence of a nitrogen source protein synthesis does not occur [13], even though the stimulation of carotene synthesis is possible. This was confirmed using cycloheximide which is known to inhibit protein synthesis in *B. trispora* [13, 14]. When cycloheximide was employed to inhibit protein synthesis, the penicillin mediated stimulation remained unaffected (Table 4). This suggests that the synthesis of new proteins may not be an essential requirement for penicillin mediated stimulation of carotenogenesis in *B. trispora*.

The net increase in the carotene content may be due to one or both of the following reasons viz. (i) increased synthesis and/or, (ii) decreased degradation. The first possibility is discussed above and the data (Tables 1–3)

Table 3. Carotenogenesis by cell-free extracts of mated *B. trispora* grown in the absence (A) and presence (B) of penicillin

Cell-free extracts from	Net synthesis of carotene (nmol/flask*)	
	Control	Control + MVA†
A	2.80 ± 0.21	5.64 ± 0.71
B	4.00 ± 0.35	9.25 ± 1.15

* The incubation systems and conditions are described in Experimental. The values are means of 5 independent determinations. † The sodium mevalonate concentration was 25 μmol.

Table 4. Effect of penicillin on carotenogenesis by *B. trispora* (−) grown in the presence of cycloheximide

Addition*	Growth† dry mycelial wt (mg/flask)	Carotene† (μg/g mycelia)	Stimulation by penicillin (%)
Nil	340	148	—
Penicillin (100 mg)	350	202	33
Cycloheximide (2.5 mg)	330	110	—
Cycloheximide (2.5 mg) + penicillin (100 mg)	335	145	30

* Additions were made aseptically to 72 hr old cultures. † Growth and carotene were determined at 96 hr.

also support it. To check the validity of the second possibility the effect of penicillin was investigated on carotene degradation or its stability under different incubation conditions. A known amount of authentic β-carotene was incubated under various conditions described in Table 5. It was observed that there was 40% degradation of β-carotene during incubations with washed cells suspended in buffer. The presence of penicillin resulted in only a 15% degradation of β-carotene under the same conditions. This suggests that penicillin may be protecting β-carotene from biological degradation. There are reports on the destruction of carotenoids in the extracts of mango [15] and alfalfa leaves [16]. This observation was further checked in the growing cultures of mated *B. trispora* by estimating trisporic acid from the medium of the mated cultures grown in the absence and presence of penicillin. Trisporic acid is one of the degradation products of β-carotene and series of oxidative degradation steps are postulated for trisporic acid formation form β-carotene [17–19].

There was a decrease in trisporic acid formation in the cultures grown in the presence of penicillin (Table 6) which suggests that penicillin may be protecting β-carotene from oxidative degradation. Further, preincubations of individual cultures with penicillin before mating resulted in low yields of trisporic acid. The results (Tables 5 and 6) support the second hypothesis viz. penicillin may be protecting β-carotene against degradation and thereby increasing the carotene content of the cultures. It seems therefore that penicillin stimulates carotenogenesis on one hand and protects carotene degradation on the other hand, thus causing an increase in the carotene content of the mould.

EXPERIMENTAL

The plus (+) (NRRL, 2895) and minus (−) (NRRL, 2896) strains of *B. trispora* (obtained U.S. Dept. of Agric., Peoria, Illinois), were individually maintained as described in ref. [20].

Culture media. The mould was grown on the synthetic mucor medium (SMM) (pH 6.2) described in ref. [21] containing per l. glucose 40 g; asparagine 2 g; KH_2PO_4 0.5 g; $MgSO_4.7H_2O$ 0.25 g; and thiamine hydrochloride 10 mg.

Inoculum preparation and cultivation. Mycelial growth from 5 to 6-day-old agar slants was transferred to 100 ml of culture medium (SMM) and incubated for 48 hr at 28° ± 2° on a rotary shaker (150 rpm). The mycelium after washing was macerated

Table 5. Effect of penicillin on β-carotene degradation by the cultures* of *B. trispora*

Sr. no.	System	β-carotene (μg)		Carotene destruction (%)
		Before incubation	After incubation‡	
1.	Culture* + buffer	Nil	Nil	Nil
2.	Culture + buffer	54	31	37
3.	Culture + buffer + penicillin (1 mg/ml)	54	43	14
4.	Buffer	54	50	—
5.	Buffer + pencillin (1 mg/ml)	54	51	—
6.	Spent medium†	54	44	12
7.	Spent medium + penicillin (1 mg/ml)	54	42	16

* 72 hr-old cultures of *B. trispora* (−) were washed with sterile water and reincubated in 100 ml of sterile 0.1 M K–Pi buffer pH 7.2. † Spent medium is the after fermentation medium collected aseptically from 72-hr-old cultures of *B. trispora* (−). ‡ All the flasks were incubated at 28 ± 2° in dark on a rotary shaker 150 rpm for 12 hr. The duplicate set was analysed before the initiation of incubation.

Table 6. Carotene and trisporic acid yields in mated cultures of *B. trispora**

Set no.	Penicillin addition (1 mg/ml)			Growth	Yields	
	Before mating†		After mating‡		Carotenes	Trisporic acid
	Plus culture	Minus culture		(g/flask)	(μg/flask)	(μg/flask)
1	−	−	−	1.30	959	4610
2	−	−	+	1.24	1460	2100
3	−	+	−	1.28	935	970
4	+	−	−	1.21	918	1120
5	+	+	−	1.28	955	1080

* All the sets of cultures were incubated in dark, at 28 ± 2°. Growth, carotene and trisporic acids are expressed per flask (200 ml medium). † The penicillin additions were made to 24-hr-old + and − cultures growing individually, on 100 ml medium per 250 ml flasks. ‡ The 72-hr-old individual cultures were combined aseptically (to set No. 2, penicillin was added) in 500 ml flasks and were further incubated for 48 hr before being analysed for growth carotene and trisporic acids.

vigorously in a sterilized flask for 5 sec. Vol. was made up with H_2O to prepare about 10%(w/v) homogenates. For single cultures, 1 ml of homogenate of respective strain was used to inoculate 250 ml conical flasks containing 100 ml of culture medium. For mated cultures 0.5 ml of each strain was used to inoculate 100 ml of cultivation medium in 250 ml conical flasks. The single and mated cultures were further incubated as described above, unless stated otherwise.

Cultivation in the presence of penicillin. Benzyl penicillin at a final concn of 1 mg/ml of cultivation medium when added to 24-hr-old cultures of *B. trispora* stimulated carotenogenesis to the maximum extent [10]. During the present studies penicillin additions were made as above, unless specified, and cultures were incubated further along with corresponding control cultures (to which penicillin was not added) before being analysed.

Growth Measurement. Mycelial growth was harvested by filtration and washed with H_2O until free from medium. Mycelia from duplicate set were dried to constant wt at 50° and growth is expressed as dry wt of mycelia per flask. Alternatively, the lipid-free mycelia (after carotene extraction) were dried and weighed. To this wt, the wt of lipid was added; the sum total of both was considered as mycelial yield per culture flask and expressed as growth per flask or per vol. of culture medium therein.

Trisporic acid (TA) preparation and estimation. TA was extracted from the fermentation medium of mated cultures by the method described in ref. [22]. Final $CHCl_3$ extract (acid fraction) was evaporated to dryness at below 0° under red. pres. The TA residue was dissolved in 0.1 M Tris–H_2SO_4 buffer (pH 7.5) and estimated using $E^{1\%}_{1cm}$ value of 700 at 325 nm.

Extraction and estimation of carotenes. Carotenoids were extracted in Me_2CO–Et_2O (1:1) by homogenizing the washed mycelia (either fresh or frozen). Mycelia from the extracts were separated by filtration through Whatman No. 1. Me_2CO was removed from combined extract (above filtrate) by washing with H_2O. Traces of moisture were removed by treating the ethereal extract with Na_2SO_4. The extract was evaporated to dryness under red. pres. at low temp. The residue was dissolved in petrol (bp 60–80°) and total carotenes were estimated as β-carotene by using $E^{1\%}_{1cm}$ value of 2500 at 450 nm as described in ref. [23].

Lipid estimation. Total lipids were extracted in $CHCl_3$–MeOH (1:1) and estimated gravimetrically by the method of ref. [24].

Determination of sterols. Total sterols were extracted in Me_2CO–EtOH (1:1) and pptd using digitonin (0.2% soln) as described in ref. [25]. The ppts were dried and dissolved in $CHCl_3$. Total sterols were estimated as ergosterol, by the colorimetric method described in ref. [26] and spectrophotometrically using $E^{1\%}_{1cm}$ value of 340 at 280 nm for ergosterol [27].

Determination of total proteins. These were determined by assying total N_2 by the micro-Kjeldahl method and applying the factor 6.38 [28].

Determination of carbohydrates. Free and total carbohydrate before and after hydrolysis by M HCl at 60° for 30 min were estimated as glucose, using anthrone by the method of ref. [29].

Preparation of cell-free extract. Mycelia were harvested by filtration and washed with H_2O. A 30% (w/v) extract was prepared in 0.1 M Tris–HCl buffer (pH 7.4) by grinding the mycelia with pyrex glass powder (2:1 w/w) using an unglazed mortar and pestle between 0–4°, until complete cell breakage occurred (checked microscopically). The homogenate was centrifuged at 15000 *g* for 15 min and the supernatant filtered through double layers of cheese cloth to remove floating scum usually present in the extract. The filtrate after dialysis, unless specified, against 5 mM Tris–HCl buffer (pH 7.4) for 2–3 hr between 0–4° with constant stirring and changing the buffer frequently, used as the cell-free extract for enzyme assays. Protein content of the cell free extract was determined by the method of ref. [30], using BSA as standard.

Mevalonate kinase (ATP: mevalonate 5-phosphotransferase (EC 2.7.1.36) was assayed by the procedure of ref. [31]. The assay system contained: Tris–HCl buffer (pH 8), 100 μmol; Na mevalonate (prepared from mevalonic acid lactone by treatment with $NaHCO_3$ and incubation for 30 min at 37°), 5 μmol; cysteine HCl (pH 7), 2.5 μmol; $MgCl_2$, 5 μmol; ATP, 10 μmol; NADH, 0.1 μmol; phosphoenolyruvate, 0.15 μmol; lactate dehydrogenase (Sigma-type IL 2375, 40–100 units/mg protein), 25 μg; pyruvate kinase, (Sigma-type IP 1381, 40–80 units/mg protein), 15 μg and appropriate amount of enzyme in a final vol. of 1.5 ml. The change in *A* at 340 nm was monitored. All the components of the assay system were kept at 0° except the assay buffers which were kept at 25°. The assay system was equilibrated at 25° before the assays. During enzyme assays optimal concs of the enzyme protein, co-enzymes and cofactors were determined for the substrate concn described in assay system. There was no activity in the absence of co-enzyme ATP and NADH. There was negligible or no activity in absence of substrate. Enzyme reaction was started by adding substrate, a control system, i.e. a substrate blank was also run side by side. The reactions were linear up to 15–20 min. A unit of enzyme is defined as the amount of enzyme which caused ΔA 0.001 per min at 340 nm at 25° under the conditions of assay.

Cell free carotenogenesis. Studies on cell free carotenogenesis were carried out according to the method developed in ref. [32]. For these *in vitro* studies, 50% w/v cell free extract prepared in 0.1 M Pi buffer (pH 7) containing 0.5 M sucrose, was used. The 5 ml of the extract (*ca* 10 mg protein) was incubated at 25° in 250 ml conical flasks containing $MgCl_2$, 5 μmol; ATP, 10 μmol; NaF, 250 μmol; in 25 ml. of 0.1 M Pi buffer (pH 5.5) in dark for 18 hr. All the buffers and glassware used were sterilized and cooled before use. Additions were made aseptically. After the incubation, carotenoids were extracted in freshly dist. Et_2O. Moisture was removed by mixing the ethereal layer with Na_2SO_4. Et_2O was evapd to dryness under red. pres. at low temp. The carotenes were dissolved in 5 ml of petrol (bp 60–80°) and were determined as β-carotene by using a $E^{1\%}_{1cm}$ value of 2500 at 450 nm [23]. The net synthesis of carotenes was calculated by the difference between the amounts of carotenes before and after incubation.

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REFERENCES

1. Goodwin, T. W. (1959) *Advan. Enzymol.* **21**, 295.
2. Porter, J. W. and Anderson, D. G. (1967) *Ann. Rev. Plant Physiol.* **18**, 197.
3. Goodwin, T. W. (1971) *Carotenoids* (Isler, O. ed.) p. 577. Birkhauser, Basel.
4. Britton, G. (1971) *Aspects of Terpenoid Chemistry and Biochemistry* (Goodwin, T. W. ed.) p. 255. Academic Press, London.
5. Goodwin, T. W. and Goad, L. J. (1970) *Biochemistry of Fruits and their Products* (Hulme, A. H. ed.) Vol. I. Chap. 12. Academic Press, London.
6. Schopfer, W. H., Grob, E. C. and Besson, G. (1952) *Arch. Sci. (Geneva)* **5**. 5.
7. Schopfer, W. H., Grob. E. C. and Besson, G. (1952) *Arch. Sci. (Geneva)* **5**, 1.
8. Goodwin, T. W., Griffiths, L. A. and Modi, V. V. (1956) *Biochem. J.* **62**, 259.
9. Goodwin, T. W. and Jamikorn, M. (1954) *J. Protozool.* **1**, 216.
10. Desai, H. G. [illegible] and Modi V. V. (1975) *Proc. 16th Ann. Conf. [illegible] of India* p. 26. Rishikesh, India.
11. Lynen, F., Eggerer, H., Henning, U. and Kessel, I. (1958) *Agnew. Chem.* **70**, 783.
12. Yokoyama, H., Nakayama, T. O. M. and Chichester, C. O. (1962) *J. Biol. Chem.* **237**, 681.
13. Modi, V. V. and Patwa, D. K. (1960) *Experientia* **16**, 352.
14. Ben-Aziz, A., Grossman, S., Budowski, P. and Ascarelli, I. (1971) *Phytochemistry* **10**, 1823.
15. Austin, D. J., Bu'Lock, J. D. and Drake, D. (1970) *Experientia* **26**, 348.
16. Bu'Lock, J. D., Jones, B. E., Taylor, D. A., Winskill, N. and Quarrie, S. A. (1974) *J. Gen. Microbiol.* **80**, 801.
17. Bu'Lock, J. D. (1976) *Proc. 2nd Symp. on the Genetics and Ind. Microg.* Sheffield, England.
18. Thomas, D. M., Harris, R. C., Kirk, J. T. O. and Goodwin, T. W. (1967) *Phytochemistry* **6**, 361.
19. Hsu, W. J., Yokoyama, H. and Coggins, Jr. C. W. (1972) *Phytochemistry* **11**, 2985.
20. Anderson, R. F., Arnold· M., Nelson, G. E. N. and Ciegler, A. (1958) *J. Agric. Food Chem.* **6**, 543.
21. Hesseltine, C. W. and Anderson, R. F. (1957) *Mycologia* **49**, 449.
22. Sutter, R. P. (1970) *Science* **168**, 1590.
23. Davies, B. H. (1965) in *Chemistry and Biochemiestry of Plant Pigments* (T. W. Goodwin ed.) p. 489. Academic Press, London.
24. Folch. J., Lees, M. and Stanley, G. H. S. (1957) *J. Biol. Chem.* **226**, 497.
25. Berndt, J., Boll, M., Lowel, M. and Gaumert, R. (1973) *Biochem. Biophys. Res. Commun.* 51, 843.
26. Stadtman, T. C. (1957) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds) Vol. 3 p. 392. Academic Press, New York.
27. Bu'Lock, J. D. and Osagie, A. U. (1973) *J. Gen. Microbiol.* **76**, 77.
28. Bock and Benedict (1915) *J. Biol. Chem.* **20**, 47.
29. Trevelyan, W. E. and Harrison, J. S. (1952) *Biochem. J.* **73**, 127.
30. Lowry, O. H., Rosebrough, N. U., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
31. Levy, H. R. and Popjak, G. (1960) *Biochem. J.* **75**, 417.
32. Modi, V. V., Reddy, V. V. R. and Shah, D. V. (1965) *Indian J. Exp. Biol.* **3**, 145.