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STUDIES ON CAROTENOGENESIS IN *BLAKESLEA TRISPORA*—II.
THE MODE OF ACTION OF TRISPORIC ACID

D. M. THOMAS, R. C. HARRIS, J. T. O. KIRK and T. W. GOODWIN

Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth

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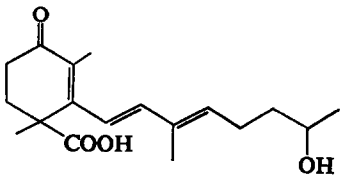
Abstract—The stimulatory effect of trisporic acid on carotenogenesis in the (−) strain of *Blakeslea trispora* is inhibited by actidione (50 μg/ml), which also inhibits the incorporation of DL-[1-^{14}C]leucine into protein. Actidione has no effect on respiration of the organism, nor on carotenogenesis if added some hours after trisporic acid. It is concluded that trisporic acid functions as a derepressor of an enzyme concerned with the carotenogenic sequence and which is normally rate-limiting. Chloramphenicol at high concentration (1 mg/ml) has only a slight effect on carotenogenesis and no obvious effect on protein synthesis in *B. trispora*. The lack of effect on DL-[1-^{14}C]leucine incorporation is probably caused by the ribosomes of the organism being insensitive to this antibiotic.

INTRODUCTION

UNMATED (+) and (−) strains of the heterothallic fungus *Blakeslea trispora* synthesize only small amounts of β-carotene, but when they are mated, synthesis of this and other carotenes is greatly stimulated (see Ciegler[1] for review). The mated strains produce a series of active factors, the trisporic acids, which stimulate carotenogenesis in the (−) strain but not in the (+) strain.[2,3] Although the main component, trisporic acid C, is a terpenoid (I) it is not itself incorporated into the additional carotene synthesized in its presence,[3] so the possibility existed that it either activated an enzyme system or stimulated the synthesis of an enzyme concerned in the carotenoid biosynthetic sequence. Experiments have, therefore, been carried out to determine the effect of protein synthesis inhibitors on the stimulatory effect of trisporic acid on carotenogenesis in *Blakeslea trispora* (−) strain.



(I)

RESULTS

Effect of Actidione on Protein Synthesis and Trisporic acid-stimulated Carotenogenesis in B. trispora

Actidione, which is known to inhibit protein synthesis in yeast,[5,6] is an extremely effective inhibitor of protein synthesis in *B. trispora* as measured by reduction of incorporation of

[1] A. CIEGLER, *Adv. Appl. Microbiol.* 7, 1 (1965).
[2] L. CAGLIOTTI, G. CAINELLI, B. CAMERINO, R. MONDELLI, A. PRIETO, A. QUILICO, T. SALVALORI and A. SELVA, *Chim. Ind. (Milan)* 46, 1 (1964).
[3] O. SEBEK and H. JAGER, *Abstr. 148th Meet. Am. Chem. Soc.*, p. 90 (1964).
[5] D. KERRIDGE, *J. Gen. Microbiol.* 19, 497 (1958).
[6] M. R. SIEGLER and H. D. SISLER, *Biochim. Biophys. Acta* 87, 83 (1964).

DL-[1-^{14}C]leucine (Table 1). A final concentration of 50 μg/ml medium decreases the incorporation some hundred times. It also completely nullifies the effect of trisporic acid on carotene synthesis (Fig. 1). The antibiotic on the other hand has no immediate effect on the general

TABLE 1. THE EFFECT OF ACTIDIONE ON INCORPORATION OF [^{14}C]LEUCINE INTO PROTEIN BY *B. trispora* (−) STRAIN

Culture No.*	Wt. of protein isolated (mg)	Total activity (counts/min)	Specific activity (counts/min/mg)
A1	2·09	41,400	19,800
A2	3·00	35,200	11,230
A3	3·12	36,300	11,630
B1	2·00	515	258
B2	4·08	695	170
B3	3·49	684	196

* A series are controls; B series with actidione, 50 μg/ml.

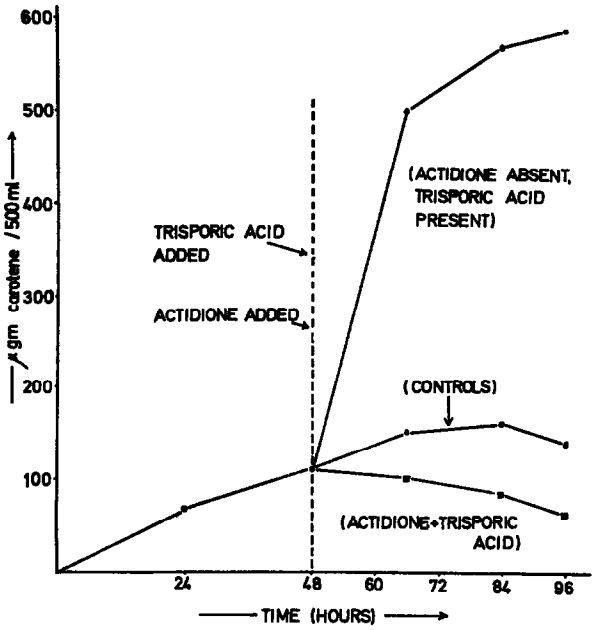


FIG. 1. THE EFFECT OF ACTIDIONE ON TRISPORIC ACID-STIMULATED CAROTENOGENESIS IN *B. trispora* (−) STRAIN.

48-hr cultures treated in the following manner and reincubated for a further 96 hr. A—actidione (50 μg/ml) and trisporic acid added; B—trisporic acid only added; C—no additions (Control).

metabolism of the organism. There was no change in the rate of oxygen uptake when actidione (final concentration 50 μg/ml medium) was added to mycelia respiring (in a Warburg manometer) in growth medium in the presence or absence of glucose (4%, w/v).

The results of the following experiment indicate that actidione has no effect on carotenogenesis *per se*. Ten flasks (100-ml conicals each containing 30 ml of medium) of 72-hr cultures

were treated with trisporic acid and incubated for a further 12 hr during which time carotene began to be rapidly synthesized. Each flask was then treated with 1 μC DL-[2-^{14}C]mevalonic acid and five were incubated for four hours (Group A); actidione (final concentration 50 μg/ml medium) was added to the remaining five before incubation for four hours (Group B). The results (Table 2) indicate that the incorporation of DL-[2-^{14}C]mevalonic acid into β-carotene is not affected by actidione. That is, once the enzymes for carotene synthesis have been formed under the influence of trisporic acid, actidione does not affect their activity.

TABLE 2. THE EFFECT OF ACTIDIONE ON INCORPORATION OF [2-^{14}C]MEVALONATE INTO β-CAROTENE BY *B. trispora*

Group No.	Amount of β-carotene isolated (μg)	Total activity (dis/min)	Specific activity (dis/min/μg)
A	109	1190	10·9
B	95	1290	13·6

72-hr cultures treated with trisporic acid and incubated for further 12 hr. Cultures divided into two groups: DL-[2-^{14}C]MVA added to A; [2-^{14}C]MVA + actidione added to B; both groups incubated for further 4 hr. For further details, see text.

Action of Chloramphenicol on Protein Synthesis and Carotenogenesis in B. trispora

Chloramphenicol at a high concentration (1 mg/ml medium) has some inhibitory effect on the trisporic acid stimulation of carotenogenesis in the (−) strain of *B. trispora* (Table 3). However, even at this high concentration, it does not inhibit protein synthesis in this strain as measured by the uptake of DL-[1-^{14}C]leucine into the mycelial protein by 72-hr cultures (Table 4). This means that the ribosomes of this organism are most probably insensitive to chloramphenicol,[4] and that the limited effect on carotenogenesis is a secondary effect not connected with protein synthesis.

TABLE 3. THE INFLUENCE OF CHLORAMPHENICOL ON CAROTENOGENESIS IN TRISPORIC ACID-TREATED (−) STRAIN OF *B. trispora*

Pigment (mg/500 ml)	Control	Chloramphenicol treated (1 mg/ml)
β-Carotene	1150	560
ζ-Carotene	120	80
γ-Carotene	25	15
Neurosporene	10	5
Lycopene	15	10

The Chloramphenicol was added 2 hr prior to addition of trisporic acid; cultural conditions as in Table 1.

[4] T. J. FRANKLIN, *Biochem. J.* **87**, 449 (1963).

TABLE 4. THE EFFECT OF CHLORAMPHENICOL ON INCORPORATION OF [^{14}C]-LEUCINE INTO PROTEIN BY *B. trispora* (−) STRAIN

Culture No.	Wt. of protein isolated (mg)	Total activity (counts/min)	Specific activity (counts/min/mg)
A1	21·6	11,660	540
A2	20·2	10,000	495
A3	23·7	9,290	390
B1	16·2	8,090	500
B2	20·5	9,250	450
B3	25·9	8,380	320

72-hr cultures resuspended in fresh medium with addition of DL-[1-^{14}C]leucine 0·5 μC/flask and incubated at 29° for 5 hr with shaking. Group A, control flasks; Group B, with chloramphenicol added (final concn. 1 mg/ml).

Dependence of Trisporic acid-stimulated Carotenogenesis on an Exogenous Nitrogen Source

From the experiments with actidione it can be concluded that trisporic acid affects carotenogenesis by stimulating synthesis of a specific enzyme. The dependence of the trisporic acid effect on protein synthesis was further demonstrated by the following experiment. Ten flasks (100 ml conicals each containing 30 ml medium) were inoculated with the (−) strain of *B. trispora* and incubated at 29° for 48 hr. They were then divided into two groups. In Group A the mycelia were washed with sterilized water and resuspended in a medium containing all normal components except the nitrogen source, L-asparagine. Group B mycelia were washed and resuspended in the complete medium. Trisporic acid was then added to both groups of flasks which were incubated for a further 48 hr. Visual examination at the end of this period clearly revealed that the Group B flasks had turned orange-yellow, as expected, but that the Group A flasks retained their original cream colour. Thus the absence of an exogenous source of nitrogen prevents trisporic acid from stimulating carotenogenesis, presumably because under these conditions new protein cannot be synthesized.

DISCUSSION

All the experiments reported in this paper point to the conclusion that trisporic acid acts on carotenogenesis by stimulating synthesis of an enzyme or enzymes in the biosynthetic sequence from acetyl-CoA to β-carotene. On modern views of regulation of protein synthesis, trisporic acid is probably acting as a derepressor of a gene regulating the synthesis of a specific enzyme. This conclusion is based on the fact that actidione, a specific inhibitor of protein synthesis (Table 1), nullifies the effect of trisporic acid when added together with this compound to cultures of the (−) strain (Fig. 1); it has no effect on respiration of the fungus and does not inhibit the incorporation of [2-^{14}C]mevalonic acid into β-carotene previously treated with trisporic acid (Table 2).

β-Ionone has a rather similar effect in stimulating carotenogenesis in *Phycomyces blakesleeanus* without itself being incorporated into the pigment. Reyes, Nakayama and Chichester (quoted by Ciegler[1]) concluded that β-ionone was acting as an enzyme stimulator rather than a derepressor because its effect was not nullified by chloramphenicol. However, our experiments with chloramphenicol (Table 3) showed that even at high concentrations (1 mg/ml compared with 50 μg/ml actidione) it was comparatively inactive in inhibiting trisporic acid-

stimulated carotenogenesis in (−) strain of *B. trispora* and completely inactive in inhibiting protein synthesis. These observations suggest that chloramphenicol is exerting a minor non-specific effect on carotene synthesis. The same explanation may possibly be the reason for the negative results with the closely related *P. blakesleeanus*. On the other hand, β-ionone appears to act differently from trisporic acid in *B. trispora* because it only stimulates carotenogenesis in mated cultures, and not in the (−) strain alone.[1]

At the genetic level the trisporic acid effect can be explained by assuming that two complementary genes I^+ and I^- exist in the (+) and (−) strains respectively. In the mated strain, I^+ and I^- interact to allow the production of trisporic acid. This then stimulates carotenogenesis in the (−) strain; its failure to stimulate the (+) strain significantly[1,7] can be explained on the assumption that this strain contains a suppressor (S), which is absent from the (−) strain.

EXPERIMENTAL

Organisms

The (+) [NRRL 2895 (A-9216)] and (−) [NRRL 2896 (A-9159)] strains of *Blakeslea trispora* were a gift from the Process Investigation Department (Fermentation), Glaxo Laboratories Ltd., Barnard Castle, Co. Durham.

Cultural Conditions

The organisms were maintained and cultured as described in the previous paper.[7]

Incorporation of DL-[1-^{14}C]*Leucine into Protein of* B. trispora

Cultures grown under standard conditions for 72 hr at 29° were centrifuged, and the mycelia resuspended in fresh standard medium. DL-[1-^{14}C]leucine, with or without a protein inhibitor, was added and the cultures incubated for a further 4 hr at 29° with shaking. The mycelial pads were collected by centrifugation and extracted three times with 0·2 N perchloric acid to remove free amino acids and other pool materials. Lipid materials were removed by soaking the mycelial pads in ethanol (twice) and ethanol–chloroform (1:1 v/v) and incubating at 35° for 15-min periods with gentle prodding from a glass rod. The pads were recovered by centrifugation, and after drying in a stream of air were steeped in N NaOH (50 ml) for 90 min at 37° with occasional stirring. After centrifugation, each mycelial pad was washed twice with 25 ml volumes of distilled water, and the three supernatants combined. Acidification was effected by dropwise addition of cold 5 N perchloric acid. The milk-white protein precipitate which was obtained flocculated after standing for 30 min and was collected by centrifugation, washed twice with distilled water, and finally suspended in 4 ml distilled water, ready for assay for radioactivity.

Trisporic Acid

No attempt was made to purify the trisporic acid, the crude extract being used for all experiments. Because of the instability of this compound, prior to each induction experiment a fresh supply was obtained from 5 l. of mated culture broth by extraction into ether. The acid residue on evaporation of the solvent was dissolved in 1 ml of ethanol, and 0·1 ml of this solution was added to each flask.

[7] D. M. THOMAS and T. W. GOODWIN, *Phytochem.* 6, 355 (1967).

Extraction of Lipid and Preparation of Unsaponifiable Material

Methods previously described in detail were used.[7] The labelled β-carotene was purified after column chromatography on magnesia-celite 5:2 (w/w) by thin-layer chromatography on alumina with benzene/light petroleum (3:7, v/v) as developer. The β-carotene zone was removed for spectrophotometric assay and measurement of radioactivity.

Radioassay

The mycelial protein was assayed in an I.D.L. windowless solid Scintillation Counter No. 6001, with scaler 1700/F, and the β-carotene in a Packard Tri-carb Liquid Scintillation Spectrometer No. 314EX.

Materials

DL-[1-^{14}C]leucine and [2-^{14}C]mevalonic acid were obtained from the Radiochemical Centre, Amersham. Chloromycetin (chloramphenicol, B.P.) from Parke, Davis Co. and Actidione from the Koch-Light Co.

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