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STUDIES ON CAROTENOGENESIS IN *BLAKESLEA TRISPORA*—I.

GENERAL OBSERVATIONS ON SYNTHESIS IN MATED AND UNMATED STRAINS

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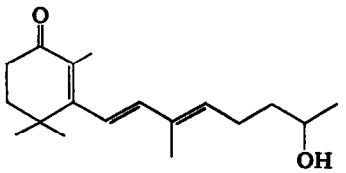
Abstract—Mated strains of *Blakeslea trispora* synthesized under our cultural conditions about 850 μg/g dry wt. of carotenes of which about one third is β-carotene, about one third is phytoene, about one sixth γ-carotene and the remainder consists of small amounts of phytofluene, ζ-carotene, lycopene with traces of β-zeacarotene and neurosporene. Unmated (+) and (−) strains synthesize about 30 and 50 μg/g dry wt. of β-carotene, and only traces of γ-carotene and lycopene. Diphenylamine strongly inhibits β-carotene synthesis in mated strains and greatly stimulates phytoene production (2·9 mg/g dry wt.). The main sterol in (+), (−) and mated strains is ergosterol; synthesis of ergosterol in the mated strain is about two times greater than in the unmated strains.

INTRODUCTION

IN 1956 Barnett *et al.*[1] showed that mated strains [(+) and (−) strains cultured together] of the heterothallic fungus *Choanephora cucurbita* synthesized some 15–20 times more β-carotene than either strain cultured alone. This observation has been extended to include other members of the family Choanephoraceae (Order, Phycomycetes), and β-carotene production by one, *Blakeslea trispora*, can be stimulated so greatly under appropriate cultural conditions that industrial production of the pigment is feasible (see review by Ciegler).[2] More recently Plempel,[3] Prieto *et al.*[4] and Sebek and Jager[5] showed that mated cultures of *B. trispora*, but not the (+) or (−) strain alone, produced a series of acids which when added to the culture medium stimulated carotenogenesis in the (−) strain.

Caglioti *et al.*[6] found that three factors were produced and elucidated the structure of the major component which they named trisporic acid C (I). Sebek and Jager[5] produced the



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[1] H. L. BARNETT, V. G. LILLY and R. F. KRAUSE, *Science* **123**, 141 (1956).
[2] A. CIEGLER, *Adv. Appl. Microbiol.* 7, 1 (1965).
[3] M. PLEMPEL, *Naturwiss.* **50**, 226 (1963).
[4] A. PRIETO, C. SHALLA, M. BIANCHI and G. BIFFI, *Chem. Ind. (London)* 551 (1964).
[5] O. SEBEK and H. JAGER, *Abstr. 148th Meet. Am. Chem. Soc.*, p. 90 (1964).
[6] L. CAGLIOTI, G. CAINELLI, B. CAMERION, R. MONDELLI, A. PRIETO, A. QUILICO, T. SALVATORI and A. SELVA, *Chim. Ind. (Milan)* **46**, 1 (1964).

labelled factor by culturing the mated strains in the presence of [U-^{14}C]glucose and showed that it is not itself incorporated into β-carotene. In the present investigation certain aspects of the effect of mating i.e. the addition of trisporic acid, in stimulating carotene and sterol synthesis in the (−) strain of *B. trispora* are reported. In a second communication,[7] the mechanism of action of trisporic acid is considered.

RESULTS AND DISCUSSION

Nature and Amount of Pigment Produced in the (−) Strain, (+) Strain, Mated Strains and (−) and (+) Strains Stimulated with Trisporic Acid

The nature and concentration of the various C-40 polyenes in the mated cultures of *Blakeslea trispora* are listed in Table 1. These observations, which are similar to those

TABLE 1. QUANTITATIVE DISTRIBUTION OF CAROTENOIDS IN 96 HR MATED CULTURES OF *Blakeslea trispora*

Fraction number	Description	Absorption maxima (nm) in petrol ether	$E^{1\%}_{1cm}$ value taken*	μg/g Dry wt.	% of total pigment	Identification
A	Colourless	278 287, 298	1100	265	32·84	Phytoene
B	Colourless (Fluoresces green in u.v. light)	322 347, 346	1200	40	4·95	Phytofluene
C	Orange yellow	430 452, 481	2500	268	33·20	β-Carotene
D	Lemon yellow	402 427, 452	2570	5	0·62	β-Zeacarotene
E	Pale yellow	378 398, 421	2270	18	2·23	ζ-Carotene
F	Orange red	440 461, 491	2720	136	16·85	γ-Carotene
G	Yellow	416 440, 470	2990	5	0·62	Neurosporene
H	Red	447 471, 501	3470	70	8·69	Lycopene

Unsaponifiable matter chromatographed on column of MgO/Celite (5:2) with light petroleum containing increasing concentrations of acetone as developer.

* Values taken from Davies.[8]

obtained when the (−) strain is treated with trisporic acid, contrast strongly both qualitatively and quantitatively with those observed in the unstimulated (+) and (−) strains and with the stimulated (+) strains. In the (−) and (+) cultures essentially only β-carotene is the major pigment present; smaller amounts of γ-carotene and lycopene are detectable but with the amount of extracts used in these experiments no phytoene or phytofluene have ever been detected. The amounts of β-carotene found are generally of the order of 50 μg/g dry wt. for the (−) strain and 30 μg/g dry wt. for the (+) strain. The mated strains produce some five

[7] D. M. THOMAS, R. C. HARRIS, J. T. O. KIRK and T. W. GOODWIN, *Phytochem.* 6, 361 (1967).

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

times more β-carotene than the (−) strain but the total polyene synthesis is some fifteen times greater. It is interesting that all the intermediates in the biosynthetic chain from phytoene to β-carotene, with the exception of neurosporene, which is only present in traces, are present in easily observable amounts. It is worth emphasizing the presence of β-zeacarotene which has recently been considered an intermediate in β-carotene biosynthesis.[9] These results are very similar to those briefly reported by Sebek and Jager.[5] The effect of trisporic acid on the (+) strain of *B. trispora* is insignificant compared with its effect on the (−) strain (Table 2).

TABLE 2. THE EFFECT OF TRISPORIC ACID ON CAROTENOGENESIS IN (+) AND (−) STRAINS OF *Blakeslea trispora*

Culture	Carotenoid concn. (μg/100 ml medium)				
	Phytoene	β-Carotene	ζ-Carotene	γ-Carotene	Lycopene
Untreated (−) strain	—	28	—	20	12
(−) strain plus trisporic acid	1760	2300	240	50	30
Untreated (+) strain	—	20	—	16	10
(+) strain plus trisporic acid	—	40	—	24	16

24-hr cultures treated with trisporic acid and incubated for a further 48 hr before harvesting. For further details see text.

A kinetic investigation of the formation of phytoene and β-carotene in the mated strains and of β-carotene in the (−) strain (Fig. 1) showed that in the (−) strain and in the mated strains, β-carotene reached its maximum after about 64 hr and then began to fall off slightly. On the other hand, phytoene, which is not present in detectable amounts in the (−) strain, increases throughout the experimental period of 120 hr in the mated strains.

It is clear that trisporic acid stimulated an enzyme, which in non-mated strains must be rate-limiting, which acts before phytoene in the biosynthetic sequence; because of the rapid synthesis of phytoene compared to that in unmated strains the enzymes concerned with the steps between phytoene and β-carotene becomes saturated and the intermediates not seen in normal strains appear in small amounts.

Effect of Mating on Sterol Synthesis

As the biosynthetic pathways of carotenoids and sterols share a number of common steps in the early stages it was important to see if mating had any effect on the nature and amount of sterol synthesized. It was found that the major sterol was the same in the (+) and (−) strains and in the mated strains. The following criteria indicated that it was ergosterol:

(i) positive tests for Δ^7-sterol with Liebermann-Burchard reagent, i.e. immediate pink flush followed by a change to a green colour; (ii) single peak with the same retention time as that of authentic ergosterol on gas–liquid chromatography on a QF.1 column; (iii) characteristic absorption maxima in ethanol at 272, 282 and 294 nm; (iv) similar R_f value to that of authentic ergosterol on Kieselgel G thin layer plates developed with $CHCl_3$; (v) same colour with both specimens when thin layer plates were sprayed with antimony trichloride in chloroform.

[9] T. W. GOODWIN, (1965a). In *Biosynthetic Pathways in Higher Plants* (Edited by J. B. PRIDHAM and T. SWAIN). Academic Press, New York (1965).

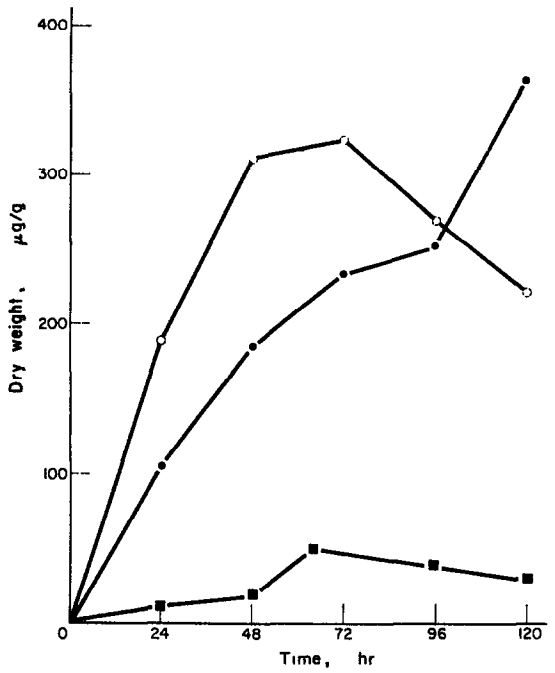

FIG. 1. TIME COURSE OF SYNTHESIS OF CAROTENOIDS BY *B. trispora*.
—●—● Phytoene in mated strains
—○—○— β-Carotene in mated strains
—■—■— β-Carotene in (−) strains.
(Standard medium; shake culture; 29°).

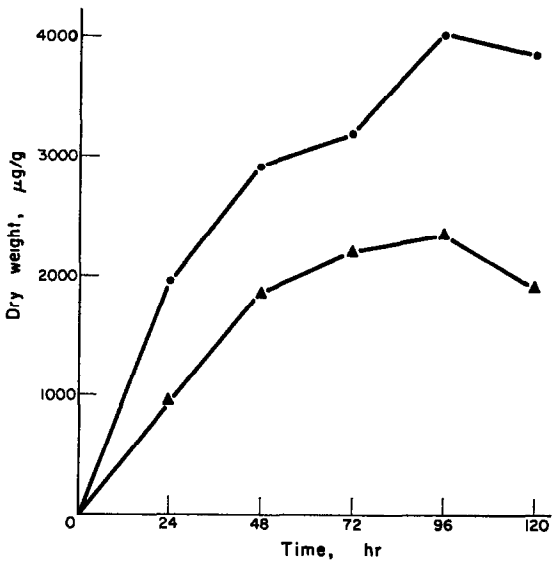

FIG. 2. TIME COURSE OF SYNTHESIS OF ERGOSTEROL IN *B. trispora*.
—●—●— Mated strains
—▲—▲— (−) Strain.

A kinetic experiment (Fig. 2) showed that over the period studied (24–120 hr) the concentration of ergosterol in the mated strains was some two times greater than in the (−) strain. Relatively this stimulation is less than that observed with the carotenes but it is much greater in absolute amount. No significant stimulation was observed with the (+) strain.

The pathway of sterol and carotenoid synthesis share common biosynthetic intermediates as far as farnesyl pyrophosphate, so, as ergosterol synthesis is also stimulated somewhat by trisporic acid, the enhanced carotene synthesis is not at the expense of sterol synthesis. Thus an enzyme acting before the conversion of farnesyl pyrophosphate into geranylgeranyl pyrophosphate may be concerned with the trisporic acid effect. This aspect of the problem is under active investigation.

Effect of Diphenylamine on Mated Cultures

Diphenylamine inhibits the synthesis of β-carotene in *Phycomyces blakesleeanus* and causes the accumulation of the precursor phytoene (see Goodwin[10]). An experiment was carried out to see if diphenylamine would inhibit the synthesis of β-carotene in mated strains

TABLE 3. THE INFLUENCE OF DIPHENYLAMINE ON CAROTENOID SYNTHESIS BY MATED STRAINS OF *B. trispora*

Polyene	Total (μg)	Concentration μg/g (dry wt.)	% of total polyene present
Phytoene	14200	2910	9952
Phytofluene	27·5	5·6	0·19
β-Carotene	20·0	4·1	0·14
ζ-Carotene	10·8	2·2	0·076
γ-Carotene	5·4	1·1	0·038
Lycopene	5·1	1·05	0·036

Normal cultural conditions; but with 0·3 ml of diphenylamine solution (0·572 g/100 ml in ethanol) added to each 100 ml of medium.

of *B. trispora* and cause a concomitant accumulation of phytoene. The results of an experiment recorded in Table 3, show that diphenylamine strongly inhibits the synthesis of β-carotene and related coloured carotenoids. It also causes the accumulation of phytoene so that it represents more than 99% of the total polyenes present. It should be noted that far more phytoene accumulates than can be accounted for by the drop in carotene synthesis. This has been observed previously in other organisms, e.g. *P. blakesleeanus*[9] and *Rhodospirillum rubrum*[10] and it confirms the view that diphenylamine may exert two functions, one inhibiting the dehydrogenation of phytoene and the second stimulating its synthesis. There is now good evidence for enzyme inhibition of phytoene dehydrogenation[11] but there is no evidence yet for a mechanism for the second effect.

EXPERIMENTAL

Organism

The (+) strain [NRRL 2875 (A 9216)] and (−) strain [NRRL 2896 (A 9159)] of *Blakeslea trispora* were a gift from the Process Investigation Department (Fermentation), Glaxo

[10] T. W. GOODWIN, *Chemistry and Biochemistry of Plant Pigments*. Academic Press, New York (1965).
[11] H. C. RILLING, *Arch. Biochem. Biophys.* **110**, 39 (1965).

Laboratories Ltd., Barnard Castle, Co. Durham. Both strains were maintained on agar slopes and plates consisting of per litre, glucose, 2·0 g; L-asparagine, 1·0 g; KH_2PO_4, 1·0 g; $MgSO_4$, $7H_2O$, 0·5 g; $FeCl_3$, 0·2 mg; $ZnSO_4$, 0·2 mg; thiamine, 1·0 mg; agar (Difco) 20 g. The pH was adjusted to 6·2 with N NaOH.

Culture

The organism was cultured in conical flasks in a gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 29°. The culture medium contained per litre, glucose 40 g; L-asparagine; 2 g; KH_2PO_4, 0·5 g; $MgSO_4$, $7H_2O$, 0·25 g; thiamine 0·5 mg. The pH was adjusted to 6·2 with N NaOH. Another culture was obtained by inoculating the medium with a small piece of mycelium obtained from agar slopes. After incubation, the culture was transferred aseptically into a blender (M.S.E. Ato-Mix) and homogenized at full speed for 30 sec. The homogenate was transferred aseptically into a sterile conical flask and stored at −4° until required. This preparation, which only remained viable for a few days, was used for inoculating experimental batches. A pipette (10 ml) with a sawn-off end was used to dispense the inoculum.

Extraction of Lipid and Preparation of Unsaponifiable Matter

The washed cultures were homogenized and the disrupted mycelia were either exhausted with solvent directly or freeze-dried to constant wt. before extraction. The lipid was saponified and the unsaponifiable material extracted by standard procedures.[12]

Separation and Determination of Pigments

The unsaponifiable matter, dissolved in light petroleum, was chromatographed on a column of magnesia : celite (5 : 2 w/w) and the various fractions up to and including β-carotene (Table 1) were eluted with light petroleum containing 0–5% (v/v) acetone. To obtain the pigments more strongly adsorbed than β-carotene, the column was extruded and the various bands separated manually. Each band was eluted with ether; the ethereal solution was filtered and taken to dryness under a stream of nitrogen. The residue was dissolved in light petroleum ready for further examination. The pigments were identified by their absorption spectra measured in a Perkin-Elmer Model 137 u.v. Recording Spectrophotometer and adsorptive behaviour relative to known compounds. The amounts present were determined from the $E^{1\%}_{1cm}$ values quoted in Table 1.

Gas–Liquid Chromatography

Separation was carried out on a glass column (120 cm × 4 mm) packed with Gas-Chrom P 80–100 mesh as solid support and Dow Corning QF as liquid component and argon (43 ml/min) as the mobile phase. Column temperature was 219° and voltage 1500 V. The apparatus was the Pye Argon Chromatograph No. 12265 coupled to a recording unit No. 12352. Cholestane was used as standard, and ergosterol, β-sitosterol and stigmasterol were reference compounds.

[12] T. W. GOODWIN, In *Modern Methods of Plant Analyses* (Edited by K. PAECH and M. V. TRACEY), Vol. 3, p. 272. Springer, Heidelberg (1955).