Effect of Light on Carotenoid and Riboflavin Production by the Fungus *Cephalosporium diospyri*

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SUMMARY

Carotenoids were produced in the mycelial form of *Cephalosporium diospyri* only after exposure to light of wavelength < 500 nm, and production occurred in three stages. The initial photoinduction required light and was temperature-independent. Both subsequent dark stages, consisting of a lag period and of actual synthesis of carotenoids, were temperature-dependent. All steps had an absolute requirement for oxygen. Filtrates from dark-grown cultures contained riboflavin, while lumichrome was present in filtrates from light-grown cultures. Intracellular riboflavin levels were the same in both. Concentrations of diphenylamine which inhibited carotenogenesis in light-grown cultures also inhibited extracellular production of riboflavin by dark-grown organisms. Several compounds with known photomimetic properties were tested on dark-grown mycelia, but pigmentation was induced only in plate cultures containing p-hydroxymercuribenzoate. These pigments had the appearance of carotenoids, but have not been chemically characterized.

INTRODUCTION

Recent reviews by Page (1968) and Carlile (1970) illustrate the diversity of fungal photoresponses. Some fungi produce pigments in response to light, the commonest being carotenoids. In some organisms, e.g. *Fusarium aquaeductum* (Eberhard, Rau & Zehender, 1961) light enhances carotenoid production, but in others there is an absolute requirement for light for pigmentation as in the mycelium of *Neurospora crassa* (Zalokar, 1954). The mechanism of photoinduction of carotenoids has been studied with *N. crassa* (Zalokar, 1954; 1955) and *F. aquaeductum* (Rau, 1967*a*, *b*; 1969) as well as two mycobacteria (Rilling, 1962, 1964; Batra & Rilling, 1964).

Discussion of the identity of the photoreceptors for many fungal photoresponses has centred on the relative merits of two major groups of compounds, flavins and carotenoids (e.g. Page, 1968; Carlile, 1970). However, the evidence obtained for and against a particular compound operating as the photoreceptor in fungi has been indirect and open to both practical and theoretical criticisms (Carlile, 1970). In carotenogenesis induced by blue-light, many reports have suggested the involvement of flavins (e.g. Rau, 1967*a*), and in bacteria that can respond to longer wavelengths, porphyrins (Burchard & Hendricks, 1969; Howes & Batra, 1970).

Cephalosporium diospyri Crandall has (except in its yeast phase – see below) an absolute requirement for light for carotenoid synthesis (Codner & Platt, 1959), and was thus suitable for further study of the mechanism of light-dependent carotenoid production.

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METHODS

Growth and maintenance of cultures. *Cephalosporium diospyri* (ATCC9066) described by Crandall (1945) was reclaimed from a freeze-dried culture from the Bath University culture collection and maintained in sterile soil at 4 °C. A primary inoculum was grown on Oxoid Malt Extract agar for 7 days at 25 °C. Spores obtained from these cultures by scraping the mycelial mat in 5 ml distilled water were used to inoculate seed flasks of a medium (GM) consisting of (% w/v): glucose, 7; glycine, 0.2; KH_2PO_4 , 0.15; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; and Oxoid yeast extract, 0.05. Fifty ml amounts in 250 ml conical flasks were used and the final pH after autoclaving was 4.8 to 5.0. After a week's growth the cultures were harvested aseptically by centrifugation, washed and finally resuspended in sterile distilled water. A uniform spore suspension was obtained by aseptic filtration of the suspended organisms through glass wool, and this inoculum adjusted so that a 1/10th dilution had an absorbance of 1.0 at 610 nm. All manipulations were carried out either under a Kodak red safe light, or in darkness. This spore suspension (1 ml) was used to inoculate experimental flasks.

Cultures were grown in orbital incubators (A. Gallenkamp & Co. Ltd, London) at 25 °C and 250 rev./min. Light-grown organisms were continually illuminated directly from above by four 40 W fluorescent tubes in a white metal box. The light path was 45 cm and yielded about 2.1×10^4 lux at the culture surfaces. The incubator used to grow cultures in complete darkness was covered with black polyethylene sheet. All comparative experiments were carried out from the same batch of inoculum. Mycelia were harvested as described above, washed, freeze-dried and stored in powder form under desiccant at -20 °C. Culture filtrates were stored at -20 °C.

During the course of this study, it was noticed that this fungus was able to grow as two distinct morphological forms: the normal floccose mycelial type and a single-celled yeast form. This yeast form produced pigments even when grown in the dark. They appear to be carotenoids since their synthesis was inhibited with diphenylamine (10^{-4} M). All inocula were checked for the presence of the yeast form by plating on malt-agar plates which were then incubated in the dark. Small pink colonies indicated its presence, and only inocula free from it were used for subsequent experiments.

Carotenoid estimation. *Cephalosporium diospyri* grown in GM medium for 7 days was harvested by centrifugation and washed $3 \times$ with M/60 phosphate buffer, pH 5.6, containing 2 % (w/v) glucose and 0.1 % (w/v) glycine. Finally, the fungus was suspended in 25 ml amounts of the buffer, to give about 1 mg dry wt/ml, in 250 ml conical flasks.

After incubation, 0.5 ml 40 % (w/v) trichloroacetic acid was added and the mycelia were harvested by centrifugation, washed and freeze-dried. Total carotenoid content of the mycelia was estimated after extracting them (50 mg dry wt) in 3×5 ml chloroform and methanol (1+2, v/v) by means of a Potter-Elvehjem homogenizer driven by a flexible rubber coupling on a small electric motor. Extracts were combined and the absorbance measured at 475 nm (the E_{max} of an extract). Carotenoid content was calculated by assuming a coefficient of extinction ($E_{1\text{cm}}^{1\%}$) of 3000. Values obtained by this technique can be used only comparatively. Results are expressed as μg carotenoid/g dry wt of fungus.

Riboflavin estimation. Total riboflavin was measured by the method of Strong (1955). Samples were read on an Aminco-Bowman Spectrophotofluorimeter (American Instrument Co. Inc., Silver Springs, Maryland, U.S.A.). Riboflavin content is expressed as $\mu\text{g/g}$ dry wt of fungus. Culture filtrates were adjusted to pH 4.5 and samples then analysed as above.

Chromatography of culture filtrates for flavins. Culture filtrates were concentrated by freeze drying to 1/10th their original volume and purified by the method of McNutt (1954).

Fig. 1. A growth curve of *Cephalosporium diospyri* grown at 25 °C in GM medium. Each determination was the average of at least six replicates. Total dry weight: light-grown mycelia, □—□; dark-grown mycelia, ■—■. Residual dry weight after hot water (100 °C) extraction of freeze-dried mycelia for 1 h: light-grown mycelia, ○—○; dark-grown mycelia, ●—●.

Further sample separation was by the paper-chromatography techniques of Kilgour, Felton & Huennkens (1957). Flavins were detected by viewing under ultraviolet radiation (254 nm).

Other analytical methods. Sterols were extracted by the method of Kieber, Payne & Appleton (1955) and estimated with the Liebermann–Burchard colorimetric method of Stoudt & Foster (1954).

Total carbohydrate was estimated as glucose equivalents by using the phenolsulphuric acid method of Dubois *et al.* (1956).

The presence of peroxides in culture media was tested for by the method of Hollomon (1966). Results were compared with those from freshly autoclaved GM medium.

RESULTS

Effect of light on the growth of Cephalosporium diospyri

Both total and residual dry weights (Taber & Siepmann, 1965) of illuminated cultures in GM medium (Fig. 1) were slightly lower (about 10 %) than those grown in the dark. The percentage of material extractable in hot water and the total carbohydrate in the extract were in close agreement for both light- and dark-grown cultures, indicating that their overall biosynthetic capacity was similar. Peroxide formation in illuminated culture medium may cause light to inhibit growth of fungi (e.g. Weinhold & Hendrix, 1963). However in no instance could peroxides be detected in filtrates from either light- or dark-grown cultures.

Pigment production

Mycelial cultures of *Cephalosporium diospyri* grown on a wide range of media were orange-pink, while those grown in darkness remained colourless. The influence of light in inducing carotenogenesis could not be transmitted from illuminated to adjacent dark-grown mycelium.

Fig. 2. Time curve for carotenoid production by *Cephalosporium diospyri* incubated in phosphate medium under continuous illumination at 25 °C. Total carotenoids were extracted and estimated as described previously. Total carotenoid, O—O; dry weight, ●—●.

Fig. 3

Fig. 4

Fig. 3. Time curve for production of carotenoids in mycelia of *Cephalosporium diospyri* incubated for 3 days in the dark then exposed to light in phosphate medium at 25 °C.

Fig. 4. Dependence of carotenogenesis on the duration of light exposure in *Cephalosporium diospyri*. Mycelia were incubated in the dark for 3 days in phosphate medium, exposed to light for the stated time, replaced in the dark and their carotenoid content estimated after a further 3 days.

Time studies on carotenogenesis

Mycelia incubated under continuous illumination in the buffer medium produced pigments as shown in Fig. 2. A lag period of about 24 h before onset of carotenogenesis was followed by a linear increase in carotenoid content to about 120 h. Increase in biomass had ceased after about 48 h. Thus in all further experiments mycelia were incubated initially for 72 h in the dark and then exposed to light. Carotenoid production then commenced, after a short lag of about 6 h, and increased linearly for about 72 h (Fig. 3).

Since carotenogenesis in *Cephalosporium diospyri* did not require continuous illumination, further studies were performed on the initial photoinduction step. Exposure to light for at

Fig. 5. Time curve for carotenogenesis in mycelia of *Cephalosporium diospyri* incubated in phosphate medium for 3 days in the dark, exposed to light for 90 min, then replaced in the dark at 25 °C.

Table 1. Requirement for aerobic conditions in carotenoid synthesis by *Cephalosporium diospyri*

Mycelia incubated for 3 days in the dark in phosphate medium were flushed with nitrogen for 15 min aseptically, the flasks tightly sealed with rubber stoppers, and the fungi then exposed to light for 90 min. Carotenoid content calculated after a further 72 h in the dark under the specified conditions.

Conditions of		Carotenoid content (µg/g dry wt)
Light induction	Dark incubation	
N ₂	N ₂	0
Air	Air	56.8
N ₂	Air	0
Air	N ₂	0

least 30 min was required to initiate pigmentation, and a near maximum response was obtained by 90 min exposure (Fig. 4). Carotenoid synthesis in mycelia exposed to light for 90 min, then replaced in the dark, showed a lag period before onset of pigmentation of about 6 h and continued for about 30 h (Fig. 5).

Requirements for initial photoinduction

Cephalosporium diospyri had an absolute requirement for oxygen in both the initial photoinduction step and the subsequent synthesis of carotenoid pigments (Table 1). Previous studies suggest that the primary effect of light is a photochemical oxidation and thus should be independent of temperature. This has been verified (Table 2) but the remainder of the process of carotenoid synthesis which is not light dependent cannot take place at low temperature. Furthermore, once exposed to light, the fungus could be stored at low temperature (−20 °C) for periods of up to 8 weeks without loss of ability to produce carotenoids (Table 3). The product of the proposed initial photo-oxidation step might well be responsible for this memory for light exposure.

Table 2. *Effect of temperature on the induction of carotenoids in Cephalosporium diospyri*

Mycelia incubated in phosphate medium for 3 days in the dark were exposed to light for 90 min at two temperatures. Subsequent incubation in the dark for a further 3 days. Mycelia exposed at -20°C were frozen as a film in the vessel and exposed to light under stationary conditions.

Temperature of		Carotenoid content ($\mu\text{g/g}$ dry wt)
Light induction	Dark incubation	
25	25	53.0
25	-20	0
-20	-20	0
-20	25	45.5

Table 3. *Memory for light exposure in Cephalosporium diospyri*

Mycelia incubated in phosphate medium for 3 days in the dark, were exposed to light for 90 min, then stored at -20°C for various times and finally incubated in the dark for a further 3 days before their carotenoid content was measured.

Time at -20°C (days)	Carotenoid content ($\mu\text{g/g}$ dry wt)
7	51.5
14	49.4
28	53.8
56	54.2

Quality of light effective for carotenogenesis

Only light $<$ about 500 nm was effective in the induction of carotenoids. This result is qualitatively similar to those for more detailed action spectra (Zalokar, 1955; Rau, 1967a; Howes & Batra, 1970), which have been viewed as strong supporting evidence for flavins as photoreceptor molecules. However, this unequivocal interpretation has been criticized (Hager, 1970; Hager & Perutz, 1970), and a serious reappraisal of action spectra and photoreceptor molecules is sought.

Presence of riboflavin in culture filtrates

Filtrates from dark-grown cultures of *Cephalosporium diospyri* in GM medium fluoresced more strongly than those from light-grown cultures. This was due to substantial amounts (up to $3.5 \mu\text{g/ml}$) of free riboflavin, identified by fluorescence spectra and chromatography. Lumichrome was detected as the major flavin component of light-grown culture filtrates.

Effect of light on riboflavin production

Light appeared to have little or no effect on the synthesis of riboflavin, since mycelia grown in the light immediately began to produce detectable amounts of riboflavin in the culture medium when placed in darkness (Fig. 6). Thus the difference in appearance of culture media for light- and dark-grown mycelia probably reflected not differences in synthetic capabilities, but subsequent photolysis of the riboflavin under continuous illumination. Carotenoid production in dark-grown mycelia subsequently exposed to light was visible after most of the riboflavin had been converted to lumichrome. Therefore the possible role of lumichrome in the photoinduction of carotenoids was considered. However, dark-

Fig. 6

Fig. 6. Subsequent fate of extracellular riboflavin produced in submerged culture of *Cephalosporium diospyri*. Cultures were grown for 5 days in the light or dark at 25 °C in GM medium, and then conditions reversed. Cultures grown in the dark then exposed to light, ○—○; cultures grown in the light then incubated in dark, ●—●.

Fig. 7

Fig. 7. Riboflavin content of mycelia of *Cephalosporium diospyri* grown in GM medium at 25 °C. Light-grown mycelia, ○—○; dark-grown mycelia, ●—●.

grown cells suspended in fresh GM medium containing lumichrome extracted from a 6-day-old light-grown culture of *Cephalosporium diospyri* did not produce any detectable carotenoid pigments on subsequent incubation in the dark.

Riboflavin content of mycelia

Mycelia grown under separate light and dark regimes were analysed for their riboflavin content (Fig. 7). There appeared to be no difference between the riboflavin contents of mycelia containing coloured carotenoids and of non-pigmented mycelia, as found by Zalokar (1957) for *Neurospora crassa*.

The absorption spectrum of a 75 % (v/v) methanolic extract from dark-grown mycelia, which was slightly yellow in colour, is shown in Fig. 8(a). This is very similar to that obtained by Trinci & Banbury (1969) from dark-grown cultures of *Aspergillus giganteus* and suggested by them to resemble chrysogenin, the anthraquinone pigment produced by *Penicillium chrysogenum* (Wolf, Kim & Jones, 1960). However, the fluorescence spectrum of the extract from *Cephalosporium diospyri* (Fig. 8b) possessed the characteristic emission spectrum for riboflavin, quite unlike the fluorescence spectrum of chrysogenin (Wolf *et al.* 1960), and chromatography revealed one major component corresponding again to riboflavin. It is possible that the pigment described by Trinci & Banbury (1969) was a flavin whose characteristic absorption spectrum was blurred with interfering material. In no instance was lumichrome detected in extracts from light-grown cultures.

Photomimetic compounds and pigmentation

SH group inhibitors. Parahydroxymercuribenzoate (PHMB), an SH-group inhibitor known to substitute for light in carotenogenesis in *Fusarium aquaeductum* (Rau, 1967b) was tested as a possible photomimetic compound with *Cephalosporium diospyri*. Colonies on malt-agar plates containing PHMB were grown at 25 °C in the dark. A non-diffusible orange-brown pigment was produced in the presence of a final concentration range of 5×10^{-4} to 5×10^{-5} M-PHMB. Similar results were obtained on using GM medium (solidified

Fig. 8. (a) Absorption spectrum of a 75 % (v/v) methanolic extract of mycelia of *Cephalosporium diospyri* grown in the dark in GM medium at 25 °C for 7 days. (b) Emission spectrum of the same extract. Excitation wavelength was 380 nm.

with 2 %, w/v, agar) containing the same concentration range. Only those parts of the mycelium in contact with the solid media (and hence the inducer) responded in this way. A range of the more commonly used -SH group inhibitors was tested and none found to be effective in the concentration range used, which suggested that -SH groups were not directly involved. This pigment has not been extracted in sufficient quantity to allow chemical characterization, but the similar findings of Valadon & Mummery (1971) with *Verticillium agaricinum* and parachloromercuribenzoate suggest that the induced pigment is a naphthoquinone.

Despite numerous attempts, no similar response to PHMB or other -SH group inhibitors was achieved in liquid shake culture of *Cephalosporium diospyri* under a wide range of cultural conditions. However, submerged cultures of mycelia grown in the presence of PHMB (5×10^{-4} M) in the dark in GM medium for 10 days were dark brown. When the logarithm of absorbance was plotted against wavelength, this pigment (extracted by refluxing with 0.5 N-NaOH for 30 min) read between 400 and 600 nm, giving a straight line with a negative slope. A value for this slope of 0.0025 compares favourably with the value quoted by Schaeffer (1953) for a similar plot, and taken by him to indicate the melanin-like characteristics of his pigment. No satisfactory explanation of this observation has been proposed,

Fig. 9. Effect of diphenylamine on carotenoid synthesis in *Cephalosporium diospyri* grown under continuous illumination for 7 days at 25 °C. Mycelia in GM medium, ●—●; mycelia in phosphate medium, ○—○.

but this pigment may be chemically similar to that produced in response to PHMB on solid medium.

Antimycin A. Antimycin A, shown by Batra (1967) to substitute for light in carotenogenesis in *Mycobacterium marinum*, was ineffective when added to *Cephalosporium diospyri* incubated under a wide range of cultural conditions in the dark.

Hydrogen peroxide. Under none of the cultural conditions tried with PHMB (and in conditions which gave good pigmentation in control cultures in the light) did hydrogen peroxide induce carotenoid production in *Cephalosporium diospyri*. Thus the results of Theimer & Rau (1970) with *Fusarium aquaeductuum* could not be reproduced with *C. diospyri*.

Inhibition of carotenoid production

The effect of diphenylamine on carotenoid production by *Cephalosporium diospyri* is shown in Fig. 9. Total inhibition of carotenogenesis in GM medium was achieved with 10^{-4} M-diphenylamine, at which concentration growth still occurred. Neither dithionite (2×10^{-3} M to 10^{-5} M) nor hydroxylamine (2×10^{-2} M to 10^{-3} M) inhibited carotenogenesis of *C. diospyri* grown in GM medium, in contrast to the findings of Theimer & Rau (1970) with *Fusarium aquaeductuum*.

Inhibition of riboflavin synthesis

Diphenylamine, which inhibits carotenogenesis in this fungus (Fig. 9), also inhibits the production of riboflavin in culture filtrates of dark-grown mycelia in the same range of concentration as those which inhibited carotenoid production in GM medium (Fig. 10). In the presence of 10^{-4} M-diphenylamine, riboflavin synthesis was almost completely inhibited whereas at 10^{-5} M there was little or no effect. Action of diphenylamine was on riboflavin synthesis, and not an alteration of already-formed pigment, since no detectable

Fig. 10. Effect of diphenylamine on the extracellular production of riboflavin by *Cephalosporium diospyri* grown in GM medium for 7 days at 25 °C. Light-grown culture filtrates, $\bigcirc$ — $\bigcirc$; dark-grown culture filtrates, $\bullet$ — $\bullet$.

qualitative or quantitative change occurred when a riboflavin solution (4 $\mu\text{g/ml}$) was incubated in the dark for 6 days in the presence of 10^{-4} M diphenylamine.

Effect of light on ergosterol content

The sterol content of *Cephalosporium diospyri* in GM medium was estimated to be 0.66 % (w/v) $\pm$ 0.09 for light-grown mycelia and 0.65 % (w/w) $\pm$ 0.07 for dark-grown mycelia. Therefore, although sharing a number of common biosynthetic steps with carotenoids, their production was affected little, if at all, by light.

DISCUSSION

The general features of light-induced carotenogenesis in *Cephalosporium diospyri* resemble those reported previously for *Neurospora crassa* (Zalokar, 1954), *Mycobacterium marinum* and *Mycobacterium* sp. (Rilling, 1964), *Fusarium aquaeductuum* (Rau, 1969) and *Verticillium agaricinum* (Valadon & Mummery, 1971). As with other species, the process could be subdivided into three stages: an initial irreversible photoinduction, a lag period, and appearance of detectable carotenoids. The photoinduction, unlike the two subsequent stages, was temperature-independent and required light.

The presence of a temperature-dependent lag phase following photoinduction would support the idea of a period of *de novo* protein synthesis (Rilling, 1962, 1964; Rau, 1967*b*). The proposal of a derepression mechanism (Rau, Lindemann & Rau-Hund, 1968; Batra, 1972) permitting expression of carotenogenic enzymes, although attractive, has not been shown conclusively to operate in any of the organisms studied so far. Rau (1967*b*) suggested that certain flavoproteins containing SH groups would be well suited to fulfil the dual role of photoreceptor and repressor in such a system, since light absorbed by the prosthetic group could readily energize the oxidation of SH groups in the protein moiety. His evidence was based on studies with PHMB, but since it now appears that the sites of action of PHMB and light are different (Theimer & Rau, 1969), as found earlier for antimycin A and *Mycobacterium marinum* (Batra, 1967), this suggestion loses some of its significance.

A more positive mechanism – the production of a photo-oxidized metabolite acting as an

enzyme inducer (Batra, 1972) or modifier of metabolism – receives some support from the study of Carlile (1962). A difficulty with this scheme has been the absence of any evidence that lumichrome occurs in natural photosensitive systems. Lumichrome was detected in filtrates from light-grown cultures of *Cephalosporium diospyri* in this study. In addition, when diphenylamine was used to inhibit carotenogenesis in this organism, production of riboflavin and hence of lumichrome was also inhibited. Both of these observations could be taken to implicate lumichrome in carotenoid production, especially since it is capable of acting as a photocatalyst (Berends, Posthuma, Sussenbach & Mager, 1966). However, attempts to substitute lumichrome for light in *C. diospyri* were unsuccessful. Furthermore lumichrome could not be detected in extracts of light-grown cells, although a pigment identical with riboflavin in all properties studied was present in both light- and dark-grown cells. *Cephalosporium diospyri* appears unusual among carotenoid-producing organisms studied to date in respect of the large amounts of extracellular riboflavin which can be detected in dark-grown culture filtrates. No evidence has been produced to suggest that light- and dark-grown cells of *C. diospyri* differ in their ability to synthesize riboflavin. Therefore it is most probable that lumichrome was produced only as a result of photolytic degradation of free riboflavin subsequent to secretion from the mycelia, and not intracellularly.

Photoinduction of carotenoids in *Cephalosporium diospyri* had an essential requirement for oxygen, supporting the observations of Howes, Batra & Blakely (1969) with *Mycobacterium* sp. and Valadon & Mummery (1971) with *Verticillium agaricinum*, but in contrast to those of Rau (1969) with both *Neurospora crassa* and *Fusarium aquaeductum* and of Trinci & Banbury (1969) with *Aspergillus giganteus*. Oxygen probably plays a direct role as a molecular participant in the photochemical reaction (Batra, 1972).

The significance of the findings on diphenylamine will be resolved only when more is known of its precise mode of action. The original belief (Olson & Knizley, 1962; Rilling 1965) that diphenylamine was a specific inhibitor of carotenogenesis is no longer tenable (Shanmugan & Berger, 1969), and the present findings would question other studies where its differential effect has been viewed as evidence in favour of riboflavin as a photoreceptor (e.g. Page, 1956).

Further differences between *Cephalosporium diospyri* and other similar systems were noted in attempts to mimic light chemically. All compounds previously reported as photo-mimetic had no similar action in this study. In addition, the failure of both dithionite and hydroxylamine to inhibit carotenoid production (Theimer & Rau, 1970) implies that fundamental differences in the physiology of light-induced carotenogenesis exists in the organisms examined so far. This may explain the present dispute over oxygen requirement for photoinduction. Therefore future investigations may discover a number of different photoreceptor mechanisms, especially if one bears in mind the diverse phylogenetic origins of fungi.

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