Pigments Induced by Organomercurial Compounds in *Cephalosporium diospyri*

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SUMMARY

The major pigments induced by parahydroxymercuribenzoate and parachloromercuribenzoate (both SH group inhibitors) in the fungus *Cephalosporium diospyri* have been characterized as carotenoids. Those present in both chemically-induced and light-induced cells include phytoene, β -carotene, γ -carotene, lycopene, torulene and neurosporaxanthin. Two other organomercurial compounds, phenyl mercuric acetate and phenyl mercuric chloride, were also found to induce pigment formation. These pigments have the absorption properties of carotenoids, but have not been fully characterized. A range of other SH group inhibitors tested failed to induce any pigment formation.

INTRODUCTION

Many heterotrophic micro-organisms respond to light by increased synthesis of carotenoid pigments (Batra, 1972). Rau (1967) first reported that parahydroxymercuribenzoate (PHMB) or parachloromercuribenzoate (PCMB) could substitute for light in carotenogenesis in *Fusarium aquaeductum*, and Batra (1967) showed that antimycin A was able to mimic light in carotenoid production in *Mycobacterium marinum*. More recently, hydrogen peroxide was reported to substitute for light in *F. aquaeductum* (Theimer & Rau, 1970). These compounds do not induce the same response in all light-sensitive fungi. Neither antimycin A nor hydrogen peroxide was effective in either *Verticillium agaricinum* (Valadon & Mummery, 1971*a*; Mummery & Valadon, 1973) or *Cephalosporium diospyri* (Seviour & Codner, 1973), although both fungi synthesize carotenoids in response to light. Furthermore, far from stimulating carotenoid production in the dark in *M. marinum*, PHMB inhibited normal synthesis in the light (Batra, Gleason & Jenkins, 1969).

The role of PCMB and PHMB in carotenoid synthesis in *C. diospyri* and *V. agaricinum* is not resolved. Seviour (1970) noticed that in the presence of these compounds, agar-grown colonies of *C. diospyri* synthesized a salmon-pink water-insoluble pigment in the dark. The colonies closely resembled those containing carotenoids synthesized under illumination, but the PCMB- and PHMB-induced pigments were not characterized. The assumption that this pigmentation was carotenoid in nature was criticized by Valadon & Mummery (1971*a*), because of observations that, under similar cultural conditions, *V. agaricinum* synthesized a pigment they suggested was a naphthaquinone. The present study was undertaken in an attempt to resolve this question by identifying the PCMB/PHMB-induced pigments in *C. diospyri* and comparing the pigment composition with that of light-treated cells, extending an earlier study on the identification of these carotenoids (Codner & Platt, 1959).

METHODS

Organism. *Cephalosporium diospyri* (ATCC9066), described by Crandall (1945), was reclaimed from a soil culture originally obtained from the University of Bath culture collection.

Growth conditions for pigment production. For the production of light-induced carotenoids, *C. diospyri* was grown in submerged liquid culture in 50 ml amounts of 2% (v/v) malt extract broth (Oxoid CM 57) in 250 ml Erlenmeyer flasks in a Gallenkamp orbital incubator (280 rev./min) at 25 °C, illuminated continuously from above with four 20 Watt fluorescent lights yielding approx. 5×10^3 lux at the surface of the cultures. After 10 days, mycelium was harvested by centrifugation, washed and freeze-dried.

Submerged liquid culture techniques could not be used for the production of PHMB/PCMB-induced pigments, since these compounds are ineffective under such conditions (Seviour & Codner, 1973). After preparation of malt extract agar (Oxoid CM 59) plates containing between 10^{-5} and 5×10^{-4} M-PCMB or PHMB, a sterile membrane filter (Gelman Metrical, 0.45 μ m, 47 mm diam) was placed on the agar, and the fungus inoculated on to this membrane, allowing the whole colony to be detached cleanly. After preliminary tests to ascertain the optimal concentrations of PCMB/PHMB to use for carotenoid production, about 300 plates were prepared for each compound, inoculated, and incubated in the dark at 25 °C for 21 days. The resulting colonies were bulked and freeze-dried for pigment analysis.

PHMB as its sodium salt and as a 0.05 M solution in glycylglycine buffer was supplied by Sigma, and PCMB as its sodium salt by Calbiochem.

Extraction of the pigments. Carotenoids were repeatedly extracted with acetone from light-grown cultures of *C. diospyri* until the extracts were colourless. The combined extracts were saponified and partitioned into epiphasic and hypophasic fractions essentially as described by Davies (1965). Sterols were removed by centrifugation after storing extracts at -20 °C. The chemically-induced pigments were extracted and analysed in the same manner at -20 °C. The chemically-induced pigments were extracted and analysed in the same manner as those from light-grown cultures.

Purification of epiphasic pigments. The total epiphasic fraction from light-, PCMB- and PHMB-induced mycelium was chromatographed on Whatman No. 1 paper impregnated with aluminium hydroxide equivalent to 7.5% (w/w) aluminium oxide (Whatman Chromedia AH 81 paper) as used by Valadon & Mummery (1971*b*), but run in ascending direction with *n*-hexane as developing solvent. The less polar components were resolved into one colourless zone and usually seven coloured zones (fractions 2 to 8), but separation of the small amounts of the several polar components was poor. All bands were eluted with acetone, with the exception of the slowest-moving pink zone in each extract. This was recovered with 5% (v/v) acetic acid in diethyl ether (Goodwin, 1952) and finally partitioned into *n*-hexane, where it was stored together with the other fractions under nitrogen at -15 °C. The absorption spectra given by each of these fractions showed that many were still mixtures, and so each was re-chromatographed on thin layers of silica gel G (Merck) developed with appropriate solvents. Fractions 2 to 8 were run with increasing concentrations of acetone (1 to 5%, v/v) and benzene (5 to 20%, v/v) in light petroleum, and the polar components with increasing concentrations of acetone (5 to 10%, v/v) in *n*-hexane. Separated components from these were again eluted with acetone, and finally transferred to *n*-hexane. In most cases each original fraction was resolved into more than one component. However, some of these were subsequently identified as being in more than one of the original

zones, as a result of the initial incomplete separation. Therefore, to clarify the presentation of results each final component will be referred to by suffix (e.g. original zone resolved into two components referred to as 2 a and 2 b, etc.).

Chromatographic separation of hypophasic pigments. Pigments were partitioned into benzene using a modification of the technique described by Rau & Zehender (1959), and finally dissolved in *n*-hexane. Individual components were separated by t.l.c. on cellulose adsorbent (Merck), developed with light petroleum (b.p. 40 to 60 °C), and eluted with acetone.

Determination of spectra. Absorption spectra were determined in various solvents with a Varian Techtron 635M recording spectrophotometer. Mass spectra were obtained by a direct-insertion probe method in a GEC-AEI MS902 instrument at 70 eV and a temperature gradient of 100 to 280 °C. As a high-resolution technique was involved, and only small quantities of pigments were available, results were recorded on a computer print-out showing both mass peak data and empirical formulae.

Quantification of pigments. Pigments were quantified (Davies, 1965) and each expressed as a percentage of the total. Values of $E_{1\text{cm}}^{1\%}$ for each were obtained from the literature (Goodwin, 1955; Davies, 1965), and an $E_{1\text{cm}}^{1\%}$ of 2500 used to calculate total pigment content.

RESULTS

Pigment production in response to PCMB and PHMB

PCMB and PHMB (in either aqueous or buffer solution) were only effective as pigment inducers over a very narrow concentration range. In both cases, maximal pigment production occurred at a final concentration of 5×10^{-4} M, but colony growth was severely inhibited. No pigment induction was noticed with either compound at concentrations lower than 5×10^{-5} M, which is higher than the minimal level required for *F. aqueductum* (Rau, 1967). To produce pigments for analysis a 2.5×10^{-4} M concentration was chosen.

Pigmentation of colonies was non-uniform, only those parts of the fungus in contact with the agar surfaces being coloured: consequently much less pigment was obtained per unit of tissue with these chemicals than with light, mainly because the non-pigmented areas of tissue were included in the sample analysed. Although PCMB and PHMB are in the same ionic form in solution (Sigma Technical Bulletin No. 300), the action of these two compounds on *C. diospyri* differed: PHMB produced a much greater inhibitory effect upon growth than did PCMB, and was less effective (approx. 50 %) in inducing pigment synthesis.

A general comparison between light- and chemically-induced total pigments

Pigments of both light- and chemically-induced cultures showed certain common features. They behaved identically during the saponification and solvent-partitioning procedures, and absorption spectra of crude total pigment extracts from light- or chemically-induced cells all showed the three- or four-peak spectra characteristic of carotenoids (Table 1). Hypochromic shifts in benzene and carbon disulphide were apparent for all three extracts, and such shifts are also characteristic of carotenoids (Davies, 1965). These preliminary results suggested that the chemically-induced pigments were carotenoid in nature, and the major individual components were characterized further.

Table 1. Absorption maxima of saponified total pigment extracts of light-, PCMB- and PHMB-induced cells of *C. diospyri* in a number of solvents

	Absorption maxima			
	<i>n</i> -Hexane	Light petroleum	Carbon disulphide	Benzene
Light	(515), 479.5, 454, (424)	(515), 479, 454, (424)	(558), 513, 488, (460)	(539.5), 495.5, (478)
PCMB	(515), 479, 454, (424)	(515), 479, 454, (427)	(557), 513, 487, (458)	(539), 495, (470)
PHMB	(515), 478.5, 454, (426)	(515), 478, 454, (426)	(555), 511, 486, (460)	(539), 494, (471)

(), Shoulder present at indicated absorption maxima.

Table 2. Epiphasic pigments which have been identified from light- PCMB- and PHMB-induced cells of *C. diospyri*

Absorption spectra in <i>n</i> -hexane					
Pigment	$\lambda_{\max}$ absorption			Literature values	Reference
	Light-induced pigments	PCMB-induced pigments	PMBH-induced pigments		
Phytoene	297.5(s), 286, 275.5(s)	297.5(s), 286, 275.5(s)	297.5(s), 286, 275.5	298(s), 286, 275(s)	Harding <i>et al.</i> (1969)
β -Carotene	477, 448, 425(s)	477, 448.5, 425(s)	477, 448, 426(s)	477, 448, 424(s)	Kushwana <i>et al.</i> (1972)
γ -Carotene	489.5, 458.5, 436	489.5, 458.5, 436	489, 458, 437	490, 459, 434	Harding <i>et al.</i> (1969)
Lycopene	503, 472, 448	504, 472, 446	502, 472, 446	505, 472, 446	Davies (1965)
Torulene	517.5, 483.5, 458	517.5, 483, 458	518, 483.5, 458	518, 484, 460	Davies (1965)
Neurospora-xanthin	504(s), 476, 449.5	504, 475, 451	—	505(s), 477, 450(s)	Harding <i>et al.</i> (1969)
			(s), Shoulder present.		

(s), Shoulder present.

Pigment composition of C. diospyri

Carotenoids identified from light-grown cells will be dealt with consecutively, and comparisons with chemically-induced pigments made where possible. The $\lambda_{\max}$ for all fractions identified are summarized in Table 2.

Phytoene. The colourless fractions 1 obtained from paper chromatography of all three treatments were not purified further. All had very similar absorption spectra, and $\lambda_{\max}$ which corresponded to published values for phytoene (Harding, Huang & Mitchell, 1969). As with most separated components, mass spectral analysis was not possible with chemically-induced fractions owing to insufficient recovery of pigment. However, a spectrum for light fraction 1 showed a molecular ion peak at m/e 544 (mol. wt 544), necessary for authentic phytoene (empirical formula $C_{40}H_{64}$). The fragmentation pattern was also very similar to published spectra of phytoene (e.g. Kushwaha, Pugh, Kramer & Kates, 1972). However, no fluorescent bands or absorption spectra characteristic of phytofluene were detectable in any of the colourless fractions.

β -Carotene. Fractions 2a from all three treatments gave $\lambda_{\max}$ showing close agreement with published values for β -carotene (Kushwaha *et al.* 1972). Mass spectral analysis of light-induced pigment 2a showed a parent ion at m/e 536 ($C_{40}H_{56}$, mol. wt 536). Characteristic ion peaks at m/e 444 (M-92) and m/e 430 (M-106) are consistent with β -carotene (Kushwaha *et al.* 1972). The mass spectrum of the PCMB-induced fraction 2a also contained the molecular ion (M^+) peak at 536, although in this case, due to the limited quantities of pigment, only an incomplete fragmentation pattern was obtained.

γ -Carotene. Absorption spectra of light 4a, PCMB 3b and PHMB 4a fractions had $\lambda_{\max}$ suggesting γ -carotene. When these three fractions were co-chromatographed on silica gel G in light petroleum (b.p. 40 to 60 °C) containing increasing concentrations of acetone, they failed to separate, providing further evidence of their similarity.

Lycopene. From $\lambda_{\max}$ values of absorption spectra of light 5b, PCMB 5a and PHMB 5a, these fractions were identified as lycopene (Davies, 1965). Again, co-chromatography using increasing concentrations of acetone in light petroleum failed to separate them. No mass spectral data could be obtained.

Torulene. Values of $\lambda_{\max}$ for the three 7 and 8 fractions agreed closely with published values for torulene (Davies, 1965). In addition, a mass spectrum of the light-induced sample showed a parent M^+ peak at 534, representing an empirical formula of $C_{40}H_{54}$ which corresponds to torulene. No mass spectral data were obtainable from the chemically-induced pigments. However, co-chromatography of all samples using increasing concentrations of benzene in light petroleum failed to produce any further separation.

Neurosporaxanthin. The hypophasic pigments were resolved into two major components on silica gel G. In both light- and PCMB-induced cell extracts, the faster (pink) band had $\lambda_{\max}$ at 504, 476 and 450 nm in *n*-hexane. These values agree well with published data for neurosporaxanthin (Harding *et al.* 1969). No well-defined spectrum was obtained with the corresponding PHMB fraction, so it was not identified. The slower-running yellow fraction present in all samples gave a poor absorption spectrum and remains unknown.

Results (Table 3) show that the relative proportions of identified carotenoids were very similar between both light- and chemically-induced cells.

Other epiphasic pigments. In addition to the major pigments discussed above, *C. diospyri* contained a number of other carotenoids. In most cases, they were present in trace amounts and were not obtained spectroscopically pure. These have not been identified. However, in a few instances spectra were obtained, and from these it was possible tentatively to identify

Table 3. Amounts of the major carotenoids present in light-, PCMB- and PHMB-induced cells of *C. diospyri*

Pigment	$E_{1\text{cm}}^{1\%}$	Reference	Percentage of total extract		
			Light	PCMB	PHMB
Phytoene	1250	Davies (1965)	14.90	10.60	14.90
β -Carotene	2580	Goodwin (1955)	27.30	23.00	23.40
γ -Carotene	3100	Davies (1965)	4.45	2.47	2.32
Lycopene	3450	Davies (1965)	0.75	0.98	1.90
Torulene	2780	Davies (1965)	21.30	26.10	11.70
Neurosporanganthin	1715	Harding <i>et al.</i> (1969)	16.80	15.90	—

the following carotenoids in both light- and chemically-induced cells: ζ -carotene (λ_{max} in *n*-hexane at 376, 399 and 425 nm), running just behind β -carotene on thin layers of silica gel G; neurosporene (λ_{max} at 416, 439 and 466 nm), running between γ -carotene and lycopene; and a pigment with λ_{max} at 450, 423 and 400 nm which is similar to published values for α -zeacarotene, but with different chromatographic behaviour, running behind lycopene on silica gel G (Davies, 1965).

Further evidence for the carotenoid nature of the chemically-induced pigments

Diphenylamine (DPA), which inhibits light-induced carotenoid production in *C. diospyri* (Seviour & Codner, 1973), also inhibited the chemically-induced pigment synthesis. This was illustrated using 'lawn sown' plates of *C. diospyri* in which two wells had been cut, one containing 10^{-2} M-DPA, the other 5×10^{-3} M-PCMB or -PHMB. After dark incubation, pigmentation occurred as a narrow ring around the wells containing PCMB or PHMB, except on the side adjacent to the well containing DPA, where pigmentation was inhibited.

Other chemical inducers of pigmentation

Because the inhibitory properties of PCMB and PHMB might implicate SH groups in the induction process (Rau, 1967), a wide range of other SH group inhibitors, including both organic and inorganic mercury-containing compounds, were tested. In addition, parahydroxybenzoate (PHB) and 3,4-dihydroxybenzoate (DHB), both possible intermediates in microbial degradation of PCMB or PHMB (Furukawa & Tonomura, 1971), were tried. Of all the compounds tested, only phenylmercuric chloride (PMC) and phenylmercuric acetate (PMA) induced detectable amounts of pink pigment in *C. diospyri*, and then only when grown on solid medium. The most effective concentration for both compounds was 2.5×10^{-4} M, on malt extract agar. Neither compound has previously been reported as having photomimetic properties. However, the pigments appeared to be carotenoid, and an absorption spectrum of extracts from PMC-induced cells showed a typical carotenoid three-peak spectrum between 400 and 500 nm. Absorption maxima occurred at different wavelengths in crude extracts of light- and PCMB/PHMB-induced cells (λ_{max} in *n*-hexane at 428, 460 and 483 nm), suggesting significant differences in their carotenoid composition. Because only small quantities of pigment were available, further characterization was not attempted.

DISCUSSION

A combination of analytical techniques has provided convincing evidence that the pigments induced in *Cephalosporium diospyri* by PCMB and PHMB are carotenoids, identical to those produced in response to light in this organism. None of the data obtained supported claims

that these pigments might be naphthaquinones (Valadon & Mummery, 1971a). Although only the major pigment components were identified, *C. diospyri* has a very similar carotenoid composition to other light-inducible fungi such as *Neurospora crassa* (Harding *et al.* 1969), *Verticillium agaricinum* (Valadon & Mummery, 1971a) and *Fusarium aquaeductuum* (Bindl, Lang & Rau, 1970). Quantitative studies (Table 3) have shown that in both light- and chemically-induced cells of *C. diospyri*, the carotenoids present in largest amounts are those which are considered to be end products in the biosynthetic pathway (Goodwin, 1971). Pigments thought to be intermediates were present in much smaller amounts. This suggests that the various carotenoids, regardless of the nature of the inducer, are synthesized via the same biosynthetic pathway which occurs in other tissues.

The observation that PMA and PMC also induce pigment synthesis in *C. diospyri* is interesting. These pigments have the spectral characteristics of carotenoids and, by this criterion, are seen to be quite different from the pigment 1, 4, 5, 8 tetrahydroxyanthraquinone produced in response to PMA in a mercury-resistant strain of *Pyrenophora avenae* (Greenaway, 1971). With this finding, some pattern begins to emerge concerning the structural features required of compounds for them to induce pigment production in *C. diospyri*. All four effective chemicals are aromatic organomercurial compounds. By contrast, several other aliphatic mercury compounds were ineffective, as were a number of inorganic mercury compounds. Furthermore, as neither PHB nor DHB induced pigmentation, the possibility that a phenyl or benzoate group may be solely responsible, either directly or after metabolic conversion, can probably be discounted. Although a search for further inducers among organomercurial compounds of this type may prove rewarding, the failure of other structurally similar organomercurial compounds tested for photomimetic effect (e.g. para-hydroxymercuriphenyl sulphonate) implies that other factors may be significant. Of these, membrane permeability is likely to be important, since it is known that organic and inorganic mercurial compounds differ in their ability to cross cell membranes (Scott, Knight, Settlemyre & Brierly, 1970; Selwyn, 1972).

Further understanding of the mechanism of induction by these chemicals will probably have to await the development of a more suitable experimental system than agar-grown cultures of *C. diospyri*. However, this organism, which produces no coloured carotenoids in the dark, is likely to prove a more informative organism for resolving light-regulated carotenoid synthesis than are fungi which produce small quantities of carotenoids even before exposure to light.

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