Studies in Carotenogenesis

17. THE CAROTENOIDS PRODUCED BY DIFFERENT STRAINS OF *MYCOBACTERIUM PHLEI*

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Chargaff (1930) first demonstrated the presence of carotenoids in *Mycobacterium phlei*, and since then various strains of this organism have been investigated by Chargaff & Dieryck (1932), Ingraham & Steenbock (1935); Takeda & Ohta (1939, 1940); Turian (1950*a*, *b*, *c*, 1951, 1953) and Turian & Haxo (1952). The carotenoids reported to be present include α -, β - and γ -carotenes, a 'lycopene-like' pigment, cryptoxanthin, a 'cryptoxanthin-like' pigment, zeaxanthin, and an acidic carotenoid, chrysophlein. The acidic carotenoid astaxanthin is said to be produced by various species of Mycobacteriaceae, excluding *Myco. phlei*, when grown on hydrocarbon oils but not when grown on agar (Haas & Bushnell, 1944). Leprotene, a pigment with an absorption spectrum similar to that of β -carotene but with a greater adsorptive power and without vitamin A activity, has also been isolated. The name derives from the fact that it was first isolated from an organism considered to be *Myco. leprae* (Grundmann & Takeda, 1937), but later shown to be a strain of *Myco. phlei* (Takeda & Ohta, 1939). It has also been found in *Myco. tuberculosis* (Takeda & Ohta, 1944) and *Myco. brughoghe* et *adant* (Lederer, personal communication) and would appear to be a characteristic pigment of the Mycobacteriaceae. Recently, however, it has been found in an unidentified organism which does not appear to be a typical *Myco. phlei* (Goodwin & Jamikorn, 1956).

Ingraham & Steenbock (1935) first studied the factors controlling pigment synthesis in *Myco. phlei*, and found that replacement of glucose by glycerol or addition of either alcohols (particularly isopropanol) or glycols to the glucose media increased pigmentation. This was due mainly to the synthesis of an unidentified pigment which was similar to phthiocol isolated from *Myco. tuberculosis* by Anderson & Newman (1933); however, there was at the same time increased production of carotenoids. In contrast to the carotenoids, which reached their maximal levels before the cells began to autolyse, this pigment appeared to be an end-product of metabolism for it continued to be formed long after autolysis had set in. Later Darzins (1939) observed qualitatively that addi-

tion of olive oil increased, whilst exposure to the vapours of pine oil decreased pigmentation in *Myco. phlei*.

More recently, Turian (1950*b*) found that carotenogenesis could be separated from growth in *Myco. phlei*; ferric ions stimulated both growth and carotenogenesis equally and manganous ions specifically stimulated carotenogenesis. Developing the original observations of Kharasch, Conway & Bloom (1936) that chromogenesis in many bacteria and fungi was inhibited by diphenylamine, Turian (1950*c*) showed that this substance inhibits carotenogenesis in *Myco. phlei* without affecting growth; the inhibition was reversed by ferric ions (0.1 mg./100 ml.) but by no other cation tested. Turian (1951) further observed that phenol (1:2000-1:5000) also inhibited carotenogenesis but less effectively than did diphenylamine; resorcinol was two to three times less active than diphenylamine, whilst α -naphthylamine, thiourea, potassium cyanide and salicylaldehyde were almost inactive. Dinitrophenol, on the other hand, stimulated carotenogenesis. Turian & Haxo (1952) showed that inhibition of carotenogenesis in *Myco. phlei* by diphenylamine is accompanied by a stimulation of synthesis of the related colourless polyene, phytofluene, and a partly saturated carotene reminiscent of flavacene (Tischer, 1938, 1939).

Turian (1953) has recently reported that the incubation temperature has an important effect on carotenogenesis in *Myco. phlei*. At 30° very little chrysophlein was produced although the neutral carotenoids were formed in considerable amounts; at 37° the situation was reversed.

The present paper reports an investigation into the carotenoids elaborated by three strains of *Myco. phlei* and some factors controlling their synthesis.

EXPERIMENTAL

Cultures. The strains of *Myco. phlei* investigated were Crottin and Fleole, kindly supplied by Dr A. Lutz (Strasbourg), and Haudroy (Lausanne), kindly supplied by Dr G. Turian (Lausanne). In this paper, the latter will be called Turian's strain for convenience.

Culture conditions and media. The standard solid medium used, on which cultures were also maintained, contained (all w/v) peptone, 1%; glucose, 2%; glycerol, 2%; agar, 1.8%. The four strains were subcultured monthly.

The standard liquid medium used contained (per l.): asparagine, 5.0 g., Na_2HPO_4 , 4.0 g., KH_2PO_4 , 3.0 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g., Na citrate, H_2O , 2.5 g., ferrous ammonium sulphate, 0.01 g., glucose, 20 g. and thiamine 0.4 mg. All the constituents of the media, except ferrous ammonium sulphate, were sterilized together by autoclaving (15 lb./sq.in./15 min.); the latter was sterilized separately by filtration and added to the autoclaved medium. The medium (100 ml.) was dispensed in 250 ml. Erlenmeyer flasks and incubated in the dark at 37°.

Analytical methods. Dry-weight determinations were carried out on the centrifuged cell mass according to the method described for the fungus *Phycomyces blakesleeanus* (Garton, Goodwin & Lijinsky, 1951). The carotenoids were also extracted according to the method of Garton *et al.* (1951) and determined as 'total carotenoids' by measuring the *E* value of the extract in light petroleum (light petroleum b.p. 40–60° was used throughout this investigation) at 450 m μ . and assuming the $E_{1\%}^{1\text{cm}}$ (450 m μ .) for the mixed pigments to be 2500.

Separation of the pigments. The pigment extract was first saponified according to the method of Goodwin & Morton (1946) in order to remove the considerable quantities of lipid which were present and which would probably have interfered with the chromatographic separation. The resulting unsaponifiable extract was then chromatographed on active alumina (P. Spence and Co., Widnes, Grade 'O'), or alumina deactivated with methanol (Goodwin & Srisukh, 1949), or an appropriate mixture of the two, or CaCO_3 (analytical grade; British Drug Houses Ltd.). The chromatographic methods followed were those of Goodwin (1952a, 1953) and Goodwin, Jamikorn & Willmer (1953).

Production of the leprotene-iodine adduct was carried out by the method of Kuhn & Lederer (1931).

RESULTS AND DISCUSSION

Pigments present in the various strains

In order to obtain maximal amounts of pigments for chromatographic analysis, the various strains were cultured on the standard solid medium for 10 days. The same pigments are also produced in the same concentrations on the standard liquid medium; but as growth is heavier on the solid medium, the yield of pigments is consequently much greater.

As the three strains produced the same pigments a typical chromatogram is recorded for only one strain (Fleole). The first Zone (A) of the first separation (Table 1) on deactivated alumina consisted of a mixture of pigments and was therefore rechromatographed on a mixture (4:1) of activated and deactivated alumina (Table 2). A1 and A2 had properties identical with β -carotene and ζ -carotene respectively, and this was confirmed by comparison in mixed chromatograms with authentic specimens of these pigments obtained from *Phycomyces blakesleeanus* (Goodwin, 1952a). A4 was similarly shown to be lycopene by comparison with an authentic specimen from the petals of *Calendula officinalis* (Goodwin, 1954). Although A3 was not obtained in crystalline form, it is almost certainly leprotene; it has an absorption spectrum very similar to that of β -carotene, but is adsorbed more strongly on a column and, on mixed chromatography with an authentic specimen of β -carotene, the two components could easily be separated. The original investigators of leprotene (Grundmann & Takeda, 1937; Takeda & Ohta, 1939) stated that its absorption spectrum was the same as that of β -carotene. By means of the more precise spectrophotometric methods now available it has been found that the absorption spectrum of leprotene is shifted slightly to longer wavelengths compared with that of β -carotene. This is demonstrated in light petroleum in Fig. 1. The absorption

Table 1. *Chromatographic separation of the carotenoids of Mycobacterium phlei strain Fleole*

Adsorbent deactivated alumina; developer light petroleum containing varying amounts of diethyl ether; zones in order of increasing adsorptive power.

Zone	Description	Absorption maximalight petroleum (m μ .)	Concn. of ether (% v/v) required for elution
A	Yellow	—	1
B	Yellow	448–452	10–20
C	Brownish yellow	428, 452	25–50
D	Brown trace	430–440*	50
E	Brown trace	428*	80–100

* Very ill-defined maxima.

Table 2. *Separation of fraction A (Table 1) of carotenoids of Mycobacterium phlei strain Fleole*

Adsorbent activated + deactivated alumina (4:1); developer light petroleum containing varying amounts of diethyl ether; pigments in order of increasing adsorptive power.

Zone	Description	Absorption maxima (m μ .) (in light petroleum)	Concn. of ether (% v/v) required for elution	Identification
A1	Yellow-brown	425, 450, 475	5	β -Carotene
A2	Lemon yellow	380, 401, 422	5–10	ζ -Carotene
A3	Brownish	428, 452, 479	10–15	Leprotene
A4	Pink	443, 470, 500	25	Lycopene

spectrum of leprotene in various other solvents is given in Fig. 2, because the full curves have not previously been recorded in detail; the exact values of the absorption maxima are given in Table 3. The iodine adduct of leprotene was formed, using the method of Kuhn & Lederer (1931) for β -carotene, and then decomposed with $\text{Na}_2\text{S}_2\text{O}_3$. Chromatography of the regenerated pigment on a mixture of activated and deactivated

and zeaxanthin, respectively. The absorption spectra of the two pigments are compared in Fig. 3 with that of zeaxanthin (the spectrum of cryptoxanthin is indistinguishable from that of zeaxanthin). It is apparent that these pigments cannot be either zeaxanthin or cryptoxanthin. Recently Turian (1953) has reported a pigment 'related to cryptoxanthin' in his strain of *Myco. phlei*; it has properties identical with those of pigment *B*.

Fig. 1. Comparison of the absorption spectra of β -carotene and leprotene in light petroleum. β -Carotene, ---; leprotene, —.

Fig. 2. Absorption spectrum of leprotene in various solvents. Ethanol, —; benzene, ---; carbon disulphide, ····; —•—, inflexion.

alumina (4:1) revealed two zones; the major zone was unchanged leprotene (65–70% of the total), whilst the second pigment was slightly less strongly adsorbed and had its absorption maxima shifted to slightly lower wavelengths compared with leprotene (426, 450 and 475 $\text{m}\mu$. in light petroleum). It appears to be a *cis*-isomer of leprotene. No pigment which might have corresponded with dehydro- β -carotene was detected. This reaction, therefore, represents a further means of differentiating leprotene from β -carotene, for the latter when treated with iodine is converted into a mixture of stereoisomers of dehydro- β -carotene (*isocarotene*) (Kuhn & Lederer, 1931; Zechmeister & Wallcave, 1953).

From their adsorptive properties and the positions of their main maxima fractions *B* and *C* (Table 1) are almost certainly the pigments considered by previous workers to be cryptoxanthin

Table 3. Absorption spectra maxima of leprotene in various solvents

Solvent	Absorption maxima ($\text{m}\mu$)
Light petroleum (b.p. 40–60°)	~428, 452, 479
Carbon disulphide	~455, 483, 512
Benzene	~438, 464, 492
Ethanol	~428, 452, 480

~ Denotes inflexion.

The possibility that pigments *B* and *C* were *cis*-isomers of cryptoxanthin and zeaxanthin respectively appears to be ruled out because, although *cis*-isomers of cryptoxanthin and zeaxanthin have the sharpness of their longest wave-band reduced, it is never lost completely as occurs with pigments *B* and *C*, in which it appears only as an inflexion. The absorption spectra were, however, not examined for a possible *cis*-peak in the

region 320–340 $m\mu$. (Zechmeister, 1944, 1954). In the case of pigment *C*, the intensity of absorption in the region 350–370 $m\mu$. relative to that at the main maximum is much greater than normally encountered in a carotenoid; the reason for this is obscure, but it occurred in all preparations (see Figs. 3 and 4). Because of the essential single-banded nature of the absorption spectra of *B* and *C*, they may be ketonic carotenoids (Goodwin, 1952*b*). At the moment they do not appear to be identifiable with any previously described carotenoids. Zones *D* and *E* (Table 1) appeared to be traces of oxidative artifacts and were not examined further.

Turian (1953) has reported the presence of γ -carotene in his strain of *Myco. phlei*. Only once out

Fig. 3. Absorption spectra in light petroleum of: Pigment *B*, —; pigment *C*, ---; zeaxanthin, •—•.

of numerous analyses was γ -carotene detected in our cultures of his strain; on that occasion it was present only in minute traces.

In the majority of cultures of strain Fleole and the strain used by Turian phytofluene was detected in traces. It was identified by its absorption spectrum, its position on a chromatographic column (below β -carotene), its bright green fluorescence in ultraviolet light, and by a mixed chromatogram with an authentic specimen from *P. blakesleeanus* (Goodwin, 1952*a*). Phytofluene was never observed in strain Crottin.

Chrysophlein

Turian (1950*a, b*) described chrysophlein as an acidic carotenoid present in his strain of *Myco. phlei*; he adopted the following procedure to obtain this pigment:

The dried bacteria were extracted with acetone, diluted 1:3 with 2.5% (w/v) aqueous KOH and shaken with light petroleum, which removed carotenes and esterified xanthophylls. The residual pigment, chrysophlein, was then extracted from the aqueous layer with ethyl ether or, after acidification, with light petroleum. Chrysophlein was considered an acidic carotenoid on the assumption that in the presence of KOH it formed a salt which was soluble in ethyl ether but insoluble in light petroleum; acidification liberated the free pigment which was soluble in light petroleum as well as in ethyl ether.

Fig. 4. Absorption spectrum in light petroleum of 'chrysophlein', —; fraction *A* from chrysophlein (compare pigment *B*, Fig. 3), ---; fraction *B* from chrysophlein (compare pigment *C*, Fig. 3), •—•.

Attempts have been made to identify chrysophlein with one of the pigments, just described, which were isolated from *Myco. phlei* by more conventional methods. By following exactly the instructions of Turian (1950*b*), specimens of chrysophlein were obtained from Turian's strain and chromatographed on deactivated alumina. Two fractions were separated: the first (*A*) moved off the column with 20% (v/v) ethyl ether in light petroleum and the second (*B*) moved off when the concentration of ether was raised to 50% (v/v). The absorption spectra of the two fractions

Table 4. Separation of polyenes of fraction A Table 1 from diphenylamine cultures of the three strains of *Mycobacterium phlei*

Adsorbent, activated + deactivated alumina (4:1); developer, light petroleum containing varying amounts of ethyl ether; polyenes in order of increasing adsorptive power.

Zone	Description	Absorption maxima in light petroleum (m μ .)	Concn. of ether (% v/v) required for elution	Provisional identification
A	Colourless, no fluorescence in u.v. light	282	1-2	Phytoene
B	Colourless, green fluorescence in u.v. light	332, 348, 368	3-5	Phytofluene
C	Brownish	428, 450, 475	5	β -Carotene
D	Light yellow	424, 456	10	Pigment X*
E	Lemon-yellow	380, 404, 424	10	ζ -Carotene
F	Khaki	441.5, 470	15	Neurosporene
G	Brown	429, 452, 478	15-20	Leprotene
H	Red	445, 470, 501	20-25	Lycopene

* This pigment (Goodwin & Osman, 1953) is reminiscent of flavacene (Tischer, 1938).

Table 5. Quantitative pigment distribution in the various strains of *Mycobacterium phlei* 7-day cultures on standard solid medium; temp. 37°.

Pigment	Strain					
	Fleole		Crottin		Turian	
	Concn. (μ g./g. dry wt.)	% of total pigments	Concn. (μ g./g. dry wt.)	% of total pigments	Concn. (μ g./g. dry wt.)	% of total pigments
Phytofluene	Trace	—	0	—	Trace	—
β -Carotene	12.7	8.7	5.9	9.6	5.5	5.2
ζ -Carotene	Trace	—	0*	—	Trace	—
Leprotene	82.5	57.3	28.4	46.5	51.8	49.3
Lycopene	12.7	8.9	3.0	4.9	1.8	1.7
Pigment B	28.2	19.6	8.8	14.6	27.6	26.3
Pigment C	7.9†	5.5	14.9†	24.4	18.3†	17.5
Total	144		61		106	

* Occasionally detected in traces.

† These values represent upper limits because of the possible presence of oxidative artifacts.

are given in Fig. 4. It is clear that these pigments are identical with pigments B and C isolated from the bacteria by the conventional methods (see Table 1 and Fig. 3); this was confirmed by carrying out mixed chromatograms of the chrysophlein fractions with pigments B and C. Turian also found his chrysophlein separated into two fractions, which he considered to be the free pigment and its salt. Acidic carotenoids, e.g. astacin, would, however, have remained in the saponifiable portion of our extract, whilst B and C were found in the unsaponifiable portion (Table 1), being extracted along with the neutral carotenoids, β -carotene, leprotene, etc. It is difficult to accept the idea that the potassium salt of chrysophlein is insoluble in light petroleum but soluble in ethyl ether; potassium salts of fatty acids and acidic carotenoids are completely insoluble in ethyl ether. The observations of Turian (1950a), which we have confirmed, that under certain conditions chrysophlein is extracted from an aqueous medium by ethyl ether but not by light petroleum may eventually be explained on grounds of partition.

Action of diphenylamine

Chromatography of the mixture of pigments produced when the three strains were grown on the standard solid medium containing diphenylamine (1:35 000) indicated the synthesis of polyenes additional to those present in normal cultures. The separations obtained were the same in all cases (Table 4). The additional polyenes identified were those already found in diphenylamine cultures of *P. blakesleeanus* (Goodwin, 1952a) and *Rhodospirillum rubrum* (Goodwin & Osman, 1954), namely phytoene (in traces), phytofluene, ζ -carotene, neurosporene and pigment X. The latter is reminiscent of flavacene (Goodwin & Osman, 1953; Turian & Haxo, 1954), first obtained from the blue-green alga *Aphanomenizon flos-aquae* (Tischer, 1938), but their identity has not yet been demonstrated.

Quantitative distribution of pigments

The amounts of the various components produced on the standard solid medium under the same experimental conditions by the three strains

Table 6. *Relative amounts of polyenes synthesized by diphenylamine cultures of three strains of Mycobacterium phlei*

Standard solid medium + diphenylamine (1:35 000); 7-day cultures; temp. 37°.

	Percentage of total pigments present		
	Fleole	Crottin	Turian
Phytofluene	18.7	5.9	9.5
β -Carotene	2.8	0.8	2.3
Pigment X	16.8	3.4	6.7
ζ -Carotene	3.5	1.4	1.0
Neurosporene	Trace	0	4.4
Leprotene	34.0	23.6	24.1
Lycopene	6.4	15.5	5.4
Strongly adsorbed carotenoids (pigments B and C)	17.8	49.4	46.6

inhibition in that chromatography of the extracts showed that reduction of the synthesis of leprotene, etc., was not accompanied by an augmented synthesis of the more saturated polyenes (phytofluene, etc.). Contrary to the observations of Turian, however, it was not possible to overcome the phenol and diphenylamine inhibitions by Fe^{2+} (Table 7).

SUMMARY

1. Three strains of *Mycobacterium phlei* produce the same carotenoids in the same relative amounts; they are β -carotene, ζ -carotene, leprotene, lycopene and two unidentifiable xanthophylls (B and C); these last are the pigments previously considered to be cryptoxanthin and zeaxanthin. Turian's

Table 7. *Effect of diphenylamine and phenol on growth and carotenogenesis in Mycobacterium phlei*

Strain Turian; standard liquid medium; amounts produced in 100 ml. medium in 250 ml. Erlenmeyer flasks; temp. 37°; each value represents the mean of two flasks.

Inhibitor	6-day cultures			10-day cultures		
	Total dry wt. (mg.)	Carotenoids		Total dry wt. (mg.)	Carotenoids	
		Amount ($\mu\text{g.}$)	Concn. (p.p.m.)		Amount ($\mu\text{g.}$)	Concn. (p.p.m.)
A None (control)	358	8.4	23	355	12.5	35
B Diphenylamine (1:35 000)	218	2.6	11	224	5.7	23
C B + FeSO_4 (1 mg./100 ml.)	250	2.9	12	216	4.8	22
D Phenol (1:2500)	53	0.6	12	102	2.4	21
E D + FeSO_4 (1 mg./100 ml.)	55	0.75	14	103	2.4	21

are recorded in Table 5. It will be seen that, although the distribution of the component pigments is essentially the same in all strains, strain Crottin produced less total pigment than do the other two strains; this is quite obvious visually, the Crottin strain being yellow and the others orange-red. The same pigments are produced in the same amounts when glucose is replaced as the carbon source by glycerol, or when the solid medium is replaced by the standard liquid medium.

In the presence of diphenylamine, the relative amounts of the more unsaturated polyenes are reduced in all strains because of the appearance of the more saturated polyenes (Table 6). The total amounts present were determined only in the case of Turian's strain (Table 7), when considerable reduction was demonstrated. In the other strains the amounts were also obviously reduced because the colour of the diphenylamine cultures of Fleole were yellow-orange and of Crottin pale yellow compared with the deep orange-red and deep yellow of the normal cultures.

In agreement with Turian's observations (1951), phenol was also found to inhibit carotenogenesis in his strain to a greater extent than growth. Phenol inhibition differs, however, from diphenylamine

chrysophlein appears to be a mixture of the xanthophylls B and C.

2. The relative amounts of the pigments present in each strain have been determined.

3. Diphenylamine stimulates the synthesis of the more saturated polyenes (phytofluene, etc.), whilst inhibiting that of the more unsaturated polyene (β -carotene, leprotene, etc.). Phenol inhibits synthesis of β -carotene, etc., but does not stimulate synthesis of the phytofluene series. Fe^{2+} does not reverse the effect of diphenylamine or phenol.

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Studies in Carotenogenesis

18. CAROTENOID PRODUCTION BY A STRONGLY CHROMOGENIC BACTERIUM ISOLATED FROM BUTTER

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The nature of the carotenoid pigments and the factors controlling their production have been investigated in a strongly chromogenic bacterium isolated from butter by Dr A. Lutz (Strasbourg). It was originally designated *Mycobacterium phlei* strain Pelligrino, but Dr Ruth Gordon (Rutgers University, N.J.), who recently kindly examined the strain, reported that it is not a typical *Myc. phlei*. 'It is not acid-fast but resembles strains coming to us as *Nocardia* spp.' As Topley & Wilson (see Wilson & Miles, 1946) do not recognize the genus *Nocardia*, and as Dr Gordon is 'inclined to agree with them', the difficult taxonomic problem of its complete identification remains unsolved and there does not appear any immediate possibility of its solution. A culture of this organism has been lodged with the DSIR National Collection of Industrial Bacteria, Teddington, Middlesex.

In spite of this difficulty of identification, the results of our investigation are now presented mainly because of their relevance to the general problem of carotenogenesis, but also because the information may eventually help to identify the organism.

EXPERIMENTAL

Culture. The culture of the unidentified organism was kindly supplied by Dr A. Lutz, Strasbourg. In this paper it will be called Pelligrino.

Culture conditions and media. The standard solid medium and standard liquid medium used were those described in the previous paper (Goodwin & Jamikorn, 1956). All media were normally sterilized by autoclaving (15 lb./sq.in./15 min.), although the iron salts used in an attempt to reverse the effect of diphenylamine were sterilized by filtration. Washed suspensions were prepared as described previously for *Rhodospirillum rubrum* (Goodwin & Osman, 1954).

Analytical methods. The methods used for determination of dry weight lipid and total carotenoids and for chromatographically separating and identifying the pigments were those described in the previous paper (Goodwin & Jamikorn, 1956).

RESULTS

Pigments present

Pigment extracts from Pelligrino cultured on the standard solid medium for 10 days were first chromatographed on deactivated alumina. Fraction A ran straight through the column and was