The author wishes to thank Dr R. R. Porter for his valuable advice on the use of fluorodinitrobenzene and for generous gifts of materials, Dr F. O. Howitt of the British Cotton Industry Research Association for supplies of silk fibroin and Mr A. V. Schalley for expert experimental assistance.

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Studies in Carotenogenesis

3. IDENTIFICATION OF THE MINOR POLYENE COMPONENTS OF THE FUNGUS *PHYCOMYCES BLAKESLEEANUS* AND A STUDY OF THEIR SYNTHESIS UNDER VARIOUS CULTURAL CONDITIONS

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Zechmeister & Sandoval (1945, 1946) described phytofluene, a colourless polyene* showing a bluish-green fluorescence in ultraviolet light, which accompanies carotenoids* in the parts of higher plants, such as fruit and flower petals, which contain little, if any, chlorophyll. It has also been shown to occur in three classes of fungi: in the Schizomycete *Rhodotorula rubra* (Bonner, Sandoval, Tang & Zechmeister, 1946), in the Ascomycete *Neurospora crassa* (Haxo, 1949) and in the Basidiomycete *Cantharellus cinnabarinus* (Haxo, 1951). Phytofluene is more saturated than the carotenoids, containing only seven ethylenic bonds, five of which are conjugated. Porter & Lincoln (1950) consider that it is probably dodecahydrolycopene. If, as first suggested by Bonner *et al.* (1946), phytofluene is an intermediate in the biogenesis of carotenoids, it is of interest to see if it is produced by *Phycomyces blakesleeanus*, the fungus at present in use in this laboratory to study carotenogenesis. The possibility that polyenes, such as phytoene (Porter & Lincoln, 1950), which are even more saturated than phytofluene, also occur has been investigated.

In addition to the already well established occurrence in *Phycomyces* of large amounts of β-carotene and traces of α-carotene (Schopfer, 1935; Karrer & Krause-Voith, 1948; Bernhard & Albrecht, 1948; Garton, Goodwin & Lijinsky, 1951), the present investigation has shown that very small amounts of four other carotenes also occur; Bernhard & Albrecht (1948) had previously stated that traces of lycopene were present and whilst the work now reported was in progress Schopfer & Grob (1950) stated that five carotenoids exist in their strain of *Phycomyces*, but they did not discuss identification.

As colourless polyenes were found in *Phycomyces*, it was considered important to determine whether

* In this paper the term polyene refers to all the C_{40} compounds discussed, whilst the term carotenoid is used when reference is made to the coloured members of the series.

fungi which do not produce carotenoids can synthesize these related more saturated compounds. Two such fungi were examined: *Ascobolus furfuraceous*, which produces a pure white mycelium although Zopf (1890) reported that *A. pulcherrimus* synthesized lipochromes, and *Nectria cinnabarina*, which produces carotenoids only in its fruiting bodies (Kohl, 1902), but not in its mycelium when cultured on liquid media (Goodwin, 1951*a*).

When the minor polyene components of *Phycomyces* had been identified, experiments were carried out to find out how variations in cultural conditions which alter the amount of β-carotene synthesized (Garton *et al.* 1951; Goodwin & Lijinsky, 1951) affected the synthesis of the other components. The effect of the presence in the culture medium of small amounts (1/40,000) of diphenylamine was also studied, for Kharasch, Conway & Bloom (1936) noted that it inhibited chromogenesis in a number of bacteria and fungi. Turian (1950) has recently shown that in the case of *Mycobacterium phlei* it is the synthesis of carotenoids that is inhibited by diphenylamine.

A short account of a part of this work has already appeared (Goodwin, 1951*b*).

EXPERIMENTAL

Cultural conditions. Phycomyces blakesleeanus (+ and − strains) was grown under the conditions previously described in detail (Garton *et al.* 1951). In order to obtain large growths, penicillin pots containing 250 ml. of medium were used instead of the 8 oz. medicine bottles containing 15 ml. of medium used in the previous investigation. *Ascobolus furfuraceous*, *Nectria cinnabarina* and *Neurospora crassa* were cultured under the same conditions except that the medium was fortified by the addition of 0·2% (w/v) of Marmite.

Extraction and separation of carotenoids. Throughout this investigation the ether used was always freshly redistilled and the light petroleum of b.p. 40–60°. The lipid fraction was extracted from mycelia with ether according to the method of Garton *et al.* (1951) and the ether removed *in vacuo* at room temperature. The ease with which carotenoids undergo *cis-trans* isomerization on heating makes it advisable to use as little heat as possible, especially when, as here, some of the constituents readily produce numerous isomers even on slight warming.

The lipid fraction was then dissolved in a small volume (about 10 ml.) of light petroleum and chromatographed on a column of alumina. Pilot experiments showed that the lipid fraction did not need to be saponified before chromatography, but that it was necessary to carry out the separation in four stages. In each case the adsorbent was either activated or deactivated alumina or a mixture of the two; the active alumina used was Spence Grade '0', whilst the deactivated material was obtained by stirring Grade '0' alumina with an excess of methanol, allowing it to stand for 1–2 hr., removing the solvent at the pump and drying overnight at 30–40°.

Separation 1. Approximately 1·5 mg. of polyenes were fractionated on a column (15 × 1·5 cm.) containing a 4:1 (w/w) mixture of activated and deactivated alumina. The resulting chromatogram is described in Table 1.

Table 1. *First separation of* Phycomyces *polyenes on a mixture of* 4:1 *(w/w) activated and deactivated alumina, using light petroleum containing* 20% *(v/v) of ether as developer*

(The zones are numbered in order of decreasing adsorptive power.)

Zone no.	Description	Absorption maxima (in light petroleum) (mμ.)
1	Colourless; slight blue fluorescence	288, 274, ~265
2	Orange-yellow	500, 467, 437, 426, ~410
3	Orange-red. Major zone	480, 450, 423
4	Colourless; bright bluish-green fluorescence	367, 348, 331
5	Narrow; yellow-brown	446, 475
6	Colourless; blue fluorescence	340, ~280, ~240, 221
7	Colourless; non-fluorescent	296, 284, 276, 265

In additon to the zones described in Table 1 a few cultures (1–2% of total number examined) contained an ether-soluble orange-red pigment which was very strongly adsorbed at the top of the column, and which could only be eluted with ether containing 5% (v/v) of glacial acetic acid.

Separation 2. Fraction 2 (Table 1) (10–20 μg.) was chromatographed on a column (10 × 1 cm.) containing a 1:4 (w/w) mixture of activated and deactivated alumina. Table 2 shows the development of the chromatogram.

Table 2. *Resolution of fraction* 2 *(Table* 1*) on a mixture of* 1:4 *(w/w) activated and deactivated alumina, using light petroleum containing* 3–5% *(v/v) ether as developer*

(Zones are numbered in order of decreasing adsorptive power.)

Zone no.	Description	Absorption maxima (in light petroleum) (mμ.)
2*A*	Reddish-pink	505, 470, 445
2*B*	Brownish-yellow	470, 440, 416
2*C*	Pink	490, 459, 434
2*D*	Lemon-yellow	451, 423, 400, 377, 362

Separation 3. The combined fractions 6 (Table 1) from a number of experiments (representing only a very small amount of material) were chromatographed on a column (10 × 1 cm.) of active alumina. The chromatogram which developed is described in Table 3.

Separation 4. Fraction 7 (Table 1) was rechromatographed on a column (15 × 1·5 cm.) of activated alumina using 5% (v/v) of ether as developer. Absence of any property (e.g. colour, fluorescence in ultraviolet light) which would allow the development of the chromatogram to be followed easily, made it necessary to run the chromatogram 'blind'; numerous fractions were collected in an automatic fraction collector and examined spectroscopically. The first fractions (15 × 5 ml.) contained mainly material with an absorption maximum at 255 mμ. The best of these were combined as fraction 7*A*, but even in these a small amount

Table 3. *Resolution of fraction* 6 (*Table* 1) *on activated alumina using light petroleum containing* 5 % (*v*/*v*) *of ether as developer*

(Zones are numbered in order of decreasing adsorptive power.)

Zone no.	Description	Absorption maxima (in light petroleum) (mμ.)
6*A*	Bright-blue fluorescence	~270, ~230
6*B*	Trace of green fluorescence	
6*C*	Sky-blue fluorescence	~280, ~270, ~240, 220
6*D*	Bright-blue fluorescence (major zone)	341, 326

Table 4. *Wavelengths and* $E^{1\%}_{1\,cm.}$ *values* (*in light petroleum*) *used in the quantitative determination of* Phycomyces *polyenes*

Polyene	Wavelength (mμ.)	$E^{1\%}_{1\,cm.}$	Reference
Phytoene	285	1100*	Porter & Lincoln (1950)
Phytofluene	348	1200	Zechmeister & Sandoval (1946)
α-Carotene	443	2700†	Zechmeister (1944)
β-Carotene	450	2590†	Zechmeister (1944)
γ-Carotene	459	2760†	Zechmeister (1944)
Lycopene	469	3460†	Zechmeister (1944)
ζ-Carotene	422	2500*	Porter & Lincoln (1950)
Neurosporene	439	2990	Haxo (1949)

* Calculated from data reported by Porter & Lincoln (1950).
† Calculated from graphs.

of material absorbing light maximally at 285 mμ. was present. The slower moving fraction (7*B*) contained the 285 mμ. material which also exhibited subsidiary absorption peaks at 272 and 296 mμ.

Examination of fractions

Fraction 1 (Table 1) contained mainly ergosterol (Bernhard & Albrecht, 1948) and was discarded.

Fractions 2*A–D* (Table 2). After further chromatographic purification the adsorption and spectral properties of these pigments indicated that they were probably lycopene (2*A*), neurosporene (2*B*), γ-carotene (2*C*) and ζ-carotene (2*D*). In the case of the first three they were compared spectroscopically and in mixed chromatograms with authentic specimens of the suspected pigment. Lycopene was obtained from tomatoes (e.g. Karrer & Jucker, 1949) and neurosporene and γ-carotene from *Neurospora crassa* (Haxo, 1949). In the case of ζ-carotene, no authentic sample could be obtained. It did not occur in various samples of commercial tomatoes examined (confirming the observation of Porter & Lincoln (1950) on American commercial varieties) and none could be detected in one sample of yellow maize (kindly given by J. Bibby and Son Ltd.), although it has been reported to be present in traces in some samples (White, Brunson & Zscheile, 1942).

Fraction 3 (Table 1) was β-carotene, already identified.

Fraction 4 (Table 1) corresponded with phytofluene and was compared with an authentic specimen of this polyene obtained from tomatoes according to the original method (Zechmeister & Sandoval, 1945, 1946).

Fraction 5 (Table 1) was α-carotene, already identified.

Fractions 6*A–D* (Tables 1 and 3). Little could be done with the traces available of this series of fluorescent materials, except to note that they are unsaponifiable and to record their absorption spectra (Table 3).

Fractions 7*A*, *B* (*separation* 4). Fraction 7*A* which could not be completely separated from 7*B* was not examined further. The properties of fraction 7*B* were compared with those reported for phytoene (Porter & Zscheile, 1946). An authentic specimen of phytoene could not be obtained for direct comparison. The observations of Porter & Lincoln (1950) that phytoene does not occur in ordinary commercial tomatoes was confirmed; they obtained their specimen of phytoene from specially bred crosses.

Quantitative experiments

In the experiments carried out to determine the effect of changes in cultural conditions on the synthesis of the various polyenes produced by *Phycomyces*, the separated components were dissolved in light petroleum and the amounts present determined spectrophotometrically in a Beckman instrument using the data recorded in Table 4.

In the case of phytofluene, a single chromatographic separation did not completely remove small amounts of material which absorbed light generally in the region 320–360 mμ. Using the Morton & Stubbs (1946) correction procedure, this irrelevant absorption could be corrected for by using the following equation:

$$E_{348\,m\mu.}\ (\text{corr.}) = [2E_{348\,m\mu.}\ (\text{obs.}) - E_{338m\mu.}\ (\text{obs.}) - E_{358\,m\mu.}\ (\text{obs.})]/0{\cdot}942.$$

When the amounts of lycopene, neurosporene, γ-carotene and ζ-carotene present were not sufficient to make a quantitative separation practicable they were determined together by measuring the extinction at 450 mμ. of the mixed extract in light petroleum and using an $E^{1\%}_{1\,cm.}$ value of 2500 to calculate amounts. The least accurate determinations are those for phytoene, in cultures not containing diphenylamine accurate allowance could not be made for the presence of the 255 mμ. material; the values recorded

probably represent an overestimate of the phytoene content of these cultures.

In cultures containing diphenylamine, the phytoene fractions are contaminated with diphenylamine which absorbs in the same spectral region as phytoene and thus causes analytical difficulties.

RESULTS

Identification of polyenes

The presence of lycopene, neurosporene, γ-carotene and phytofluene (fractions 2*A*, 2*B*, 2*C* and 4, respectively) in the (+) and (−) strains of *P.*

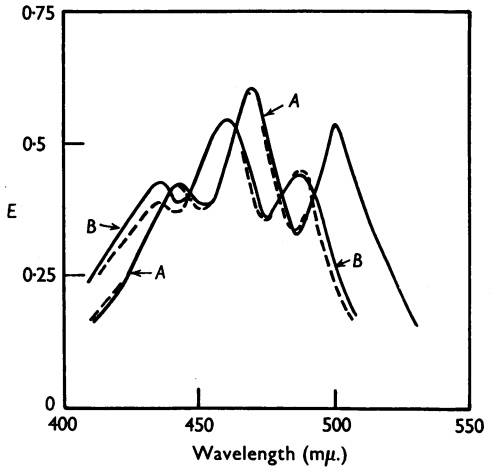

Fig. 1. The absorption spectra in light petroleum (b.p. 40–60°) of (*A*) lycopene and (*B*) γ-carotene. ——, Ex *Phycomyces*; - - - - -, an authentic specimen. In each case the *E* values of the two specimens at the wavelength of maximal absorption have been made equal.

blakesleeanus has been demonstrated. In each case the spectra of the suspected and authentic pigment had the same absorption maxima and, with but very minor deviations in the case of γ-carotene and neurosporene, identical shapes (Figs. 1 and 2). Further, mixed chromatograms of the suspected and authentic pigment on two adsorbents using appropriate developing solvents (Table 5) produced single zones, with no signs of separation. The other polyenes which were obtained chromatographically pure are almost certainly ζ-carotene and phytoene (fractions 2*D*, Table 2; and fraction 7, Table 1). In neither case was final proof of this established because authentic specimens were not available for direct comparison, but all other properties pointed to the identity suggested.

The pigment considered to be ζ-carotene (fraction 2*D*, Table 2) had the following properties identical with those described for ζ-carotene by Nash & Zscheile (1945) and Nash, Quackenbush & Porter (1948): (*a*) it is adsorbed on an alumina column as a lemon-yellow zone just above β-carotene and just below γ-carotene; and (*b*) it has absorption maxima at 362, 377, 400 and 423 mμ. (the relative intensities at these wavelengths agreeing with those shown by ζ-carotene; see Fig. 2). The only other known

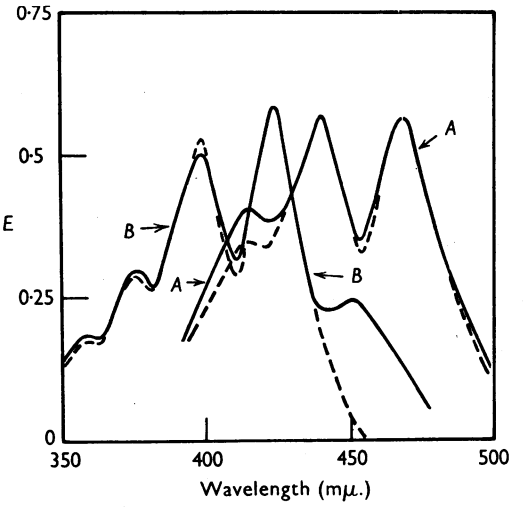

Fig. 2. The absorption spectra in light petroleum (b.p. 40–60°) of (*A*) neurosporene and (*B*) ζ-carotene. ——, Ex *Phycomyces*; - - - - -, an authentic specimen in the case of neurosporene; redrawn from the curve (in *iso*octane) of Nash & Zscheile (1945) in the case of ζ-carotene. In each case the *E* values of the two specimens at the wavelength of maximal absorption have been made equal.

carotenoid with a somewhat similar spectrum is aurochrome (Karrer & Rutschmann, 1942), but this pigment is very much more strongly adsorbed than is ζ-carotene (Nash *et al.* 1948). The one difference noted between the pigment of Nash *et al.* (1948) and the *Phycomyces* pigment is that the latter always showed a small additional absorption band at 451 mμ. This band was not recorded by Nash & Zscheile (1945), but it does not appear to be due to an impurity, for repeated chromatography on different adsorbents not only failed to eliminate it but did not alter its *E* value relative to those of the main bands.

Fraction 7*B* (Table 1) thought to be phytoene had the following properties, identical with those reported for phytoene (Porter & Zscheile, 1946): (*a*) it passes down an alumina column just in front of phytofluene; (*b*) it is colourless and does not fluoresce in ultraviolet light except in very concentrated solutions when it fluoresces faintly blue; and (*c*) it exhibits an absorption spectrum with its maxima in light petroleum located at 272, 285 and 296 mμ. The shape of the absorption spectrum was, however, not identical with that of phytoene, owing to the presence of small amounts of fraction

Table 5. *Adsorbents and solvent mixtures used in mixed chromatograms for the identification of the polyenes of* Phycomyces

Polyene	Adsorbent 1	Developer 1 (mixtures of light petroleum and ether*)	Adsorbent 2	Developer 2 (mixtures of light petroleum and ether)
Lycopene	Al_2O_3 weakened (see p. 551)	25:1	$CaCO_3$ (analytical grade)	25:1
γ-Carotene		100:3		100:3
Neurosporene		100:3		100:3
Phytofluene		50:1	$Ca(OH)_2$ (analytical grade)	25:1

* All concentrations are v/v.

7*A* which it was never possible to remove completely. This, combined with the fact that authentic phytoene was not available for comparison (the observations of Porter & Lincoln (1950) that no phytoene occurs in commercial tomatoes was confirmed), precluded the unequivocal identification of fraction 7*B* as phytoene. It will therefore be referred to as the 'phytoene-like fraction'.

It was thought for a long time that the presence of diphenylamine in the culture medium greatly stimulated the synthesis of phytoene by *Phycomyces* (Goodwin, 1951 *b*). Very recently, however, it has been found that the phytoene fractions from diphenylamine-treated cultures were contaminated with traces of diphenylamine itself. It happens by coincidence that the spectral and chromatographic properties of diphenylamine agree very closely with those reported for phytoene. It is, therefore, unwise to discuss the effect of diphenylamine on phytoene production by *Phycomyces*, until this unexpected analytical difficulty has been overcome. Work is in progress on this problem.

Unidentified compounds

Fractions 6*A–D* (Table 3). This group of four fluorescent compounds occurred only in very small amounts and it was not possible to examine them in any detail. They could not be identified with any known polyenes. Fractions 6*A–C* exhibited very indeterminate spectra (Table 3), whilst the major fraction 6*D* possessed a more well-defined spectrum with maxima in the near ultraviolet (Fig. 3).

Fraction 7*A* (separation 4). This material, which runs on a column slightly faster than phytoene, could not be obtained free from phytoene and has not been identified.

The strongly adsorbed orange-red pigment. This pigment, which was occasionally encountered strongly adsorbed at the top of an alumina column, could only be eluted with ether containing 5% acetic acid. It is not a carotenoid for it is extractable from an ethereal solution with aqueous sodium carbonate (5% w/v) to give a bright-green colour which rapidly changes to a deep orange; further it exhibits a spectrum quite different from that of any known carotenoid (see Fig. 3); its sporadic appearance precluded a complete investigation.

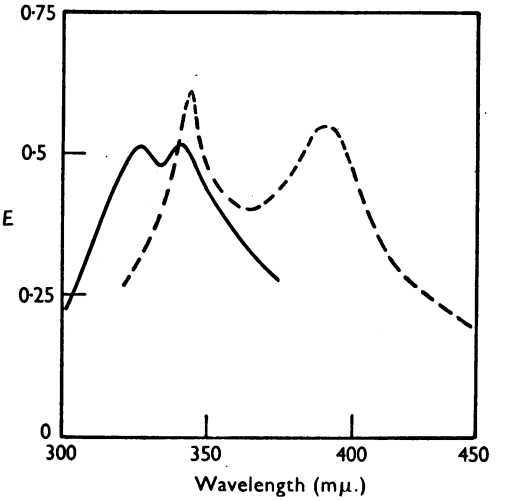

Fig. 3. The absorption spectra of (*a*) blue fluorescent material (fraction 6*D*) adsorbed on an alumina column just above phytoene, in light petroleum (b.p. 40–60°) (——); (*b*) a red pigment, very strongly adsorbed on alumina, which occurred only in 1–2% of the mycelia examined, in ether (- - - - -).

Quantitative experiments

In the experiments described in this section the (−) strain of *Phycomyces* was used unless otherwise stated. The relative amounts of the constituent polyenes (just described) were determined in a large number of mycelia cultured for 9 days on a standard medium (Garton *et al.* 1951) and the mean results obtained are collected in Table 6. Mycelia which were analysed at different stages of growth (from 3 to 22 days) showed no significant deviations from the values quoted in Table 6, and the results are therefore not recorded here. It will be seen from Table 6 that the carotenoids identified in the present work are very minor components, for, calculated on the basis of the pigments present, β-carotene represents about 95% of the total.

Table 6. *Quantitative distribution of the polyene components of the* Phycomyces (−*strain*) *when cultured for* 9 *days on a standard medium* (3 % (*w*/*v*) *glucose*, 0·2 % (*w*/*v*) *asparagine*.)

(Mean values of a large number of experiments.)

Polyene	Amount present (as % of total polyenes)
Phytoene-like fraction	13·5
Phytofluene	1·9
α-Carotene	1·9
β-Carotene	80·3
γ-Carotene	0·85
ζ-Carotene	0·35
Neurosporene	0·60
Lycopene	0·60

The production of the various polyenes was determined under the cultural conditions known to reduce (Garton *et al.* 1951) and increase (Goodwin & Lijinsky, 1951) the amount of β-carotene produced. The results, recorded in Table 7, indicate that, although the absolute amounts of the various components can vary with cultural conditions, their relative amounts show no significant variations on any of the media examined. Similarly, no changes in relative amounts are observed when *Phycomyces* was grown in the dark or when the (+) strain was used in place of the (−) strain (Table 8).

It was found that diphenylamine, when added to culture media in a concentration of 1/30,000–1/40,000, reduces the growth rate of *Phycomyces* considerably, but the final growth and lipid production are only reduced by, at most, 20 %. The surface of mature mycelia in contact with the medium is, however, colourless or slightly lemon-green with a marked green fluorescence in ultra-violet light instead of the usual bright orange-

Table 7. *Production of the polyene components by the* (−) *strain of* Phycomyces *cultured for* 9 *days on different media in the light*

(The salt and aneurin concentrations are unchanged throughout. The amounts are those produced in one penicillin pot containing 250 ml. of medium.)

	Medium used											
	3 %* glucose, 0·2 % L-asparagine		3 % xylose, 0·2 % L-asparagine		3 % fructose, 0·2 % L-asparagine		1 % glucose, 0·2 % L-asparagine		1 % glucose, 0·1 % L-leucine, 0·1 % L-asparagine		1 % glucose, 0·1 % L-valine, 0·1 % L-asparagine	
	Amounts produced											
Polyene	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total
Phytoene-like fraction	105	13·5	50	13·5	32	11·0	13	11·8	42	14·6	32	20·5
Phytofluene	15	1·9	7	1·9	6	2·1	1	0·9	6	2·1	3	1·9
α-Carotene	15	1·9	6	1·6	8	2·8	4	3·6	7	2·3	3	1·9
β-Carotene	626	80·3	300	80·7	238	82·0	89	81·0	228	79·3	113	72·5
γ- and ζ-Carotene, Neurosporene, Lycopene †	19	2·4	9	2·4	6	2·1	3	2·7	5	1·7	5	3·2
Total	**780**	—	**372**	—	**290**	—	**110**	—	**288**	—	**156**	—

* All concentrations are w/v. † Measured together as a single fraction.

Table 8. *Relative production of polyenes by the* (−) *strain of* Phycomyces *cultured for* 9 *days on the standard medium in the light and in the dark and by the* (+) *strain in the light*

(The amounts are those produced in one penicillin pot containing 250 ml. of medium.)

	Amounts produced					
	(−) strain light		(−) strain dark		(+) strain light	
Polyene	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total
Phytoene-like fraction	105	13·5	81	15·7	81	17·4
Phytofluene	15	1·9	14	2·7	15	3·2
α-Carotene	15	1·9	12	2·3	10	2·1
β-Carotene	626	80·3	388	75·2	354	76·0
γ- and ζ-Carotenes, Neurosporene, Lycopene *	19	2·4	21	4·1	6	1·3
Total	**780**	—	**516**	—	**466**	—

* Measured together as a single fraction.

yellow with little or no fluorescence. Quantitative analyses of the polyenes extracted from mycelia grown on media containing diphenylamine showed that, in all the cultures examined, the distribution had also undergone a profound change. Table 9 gives typical values for the polyene distribution in *Phycomyces* growing on media containing diphenylamine and shows that the synthesis of the most unsaturated polyenes (α-, β- and γ-carotenes and lycopene) has been almost completely inhibited whilst that of the more saturated compounds has been correspondingly increased. In these cultures the contribution of β-carotene to the total amount of polyenes present falls from 75 to 80 % to between 6·8 and 21 %, whilst lycopene and γ-carotene disappear completely from the mycelia. The percentage of phytofluene, on the other hand, increases from about 3 to over 50 % and ζ-carotene undergoes a similar increase (of the order of five to seven times); neurosporene is also increased but to a somewhat lesser extent. The effect on phytoene remains to be settled (see p. 554).

An examination of the two fungi *Ascobolus furfuraceous* and *Nectria cinnabarina*, which under the cultural conditions employed in this study, do not produce carotenoids, showed that they also do not produce colourless polyenes. When cultured on media containing diphenylamine, growth rate was reduced considerably, but the final amount of growth only slightly. The effect of the presence of diphenylamine in the culture media on the unsaponifiable matter produced by either fungus was, in contrast to *Phycomyces*, not appreciable. This is demonstrated in Fig. 4 which shows that the absorption spectra of the crude lipid fraction of *Ascobolus* grown on media with and without the addition of diphenylamine are indistinguishable over the range 220–310 mμ. No appreciable light absorption occurred in either fraction above 310 mμ. The absorption bands noted in Fig. 4 are probably due to ergosterol.

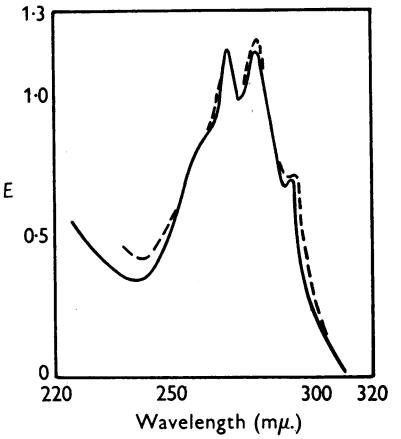

Fig. 4. The absorption spectrum of the crude lipid extract of *Ascobolus furfuraceous* cultured on a standard medium (Garton *et al.* 1951) + Marmite (0·2 % w/v), with and without the addition of diphenylamine (1/35,000). ——, Without diphenylamine; - - - - -, with diphenylamine.

Table 9. *Effect of the addition of diphenylamine* (1/40,000) *on the relative production of polyenes by* (+) *and* (−) *strains of* Phycomyces *grown for* 9 *days on various media in the light and the dark*

(The amounts are those produced in one penicillin pot containing 250 ml. The aneurin and salt concentrations of the media remained constant.)

	Medium and strain used											
	(−) strain 3 % glucose, 0·2 % L-asparagine		(−) strain 3 % glucose, 0·2 % L-asparagine		(+) strain 3 % glucose, 0·2 % L-leucine		(−) strain 1 % glucose, 0·2 % L-asparagine		(−) strain 1 % glucose, 0·1 % L-valine, 0·1 % L-asparagine		(−) strain 1 % glucose, 0·1 % L-leucine, 0·1 % L-asparagine	
	Amounts produced											
	In light		In dark		In light		In light		In light		In light	
Polyene	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total
Phytofluene	70	58·8	109	64·9	38	87·1	24	54·6	16	55·1	20	71·4
α-Carotene	0	0	0	0	0	0	0	0	0	0	0	0
β-Carotene	25	21·0	15	8·9			3	6·8	6	20·7	3	10·7
ζ-Carotene	17	14·3	34	20·2			11	25·0	7	24·2	5	17·9
γ-Carotene	0	0	0	0	11*	12·9	0	0	0	0	0	0
Neurosporene	7	5·9	10	6·0			6	13·6	Trace	—	Trace	—
Lycopene	0	0	0	0			0	0	0	0	0	0
Total	**119**	—	**168**	—	**49**	—	**44**	—	**29**	—	**28**	—

* Measured as a single fraction.

DISCUSSION

The demonstration of the presence in *Phycomyces* of small amounts of the polyenes γ- and ζ-carotene, neurosporene, lycopene, phytofluene and phytoene is of interest from the point of view of the general distribution of carotenoids in fungi as well as from that of carotenogenesis in fungi. From the former viewpoint the present investigation extends to the Phycomycetes the presence of lycopene, neurosporene and phytofluene, which have been noted previously only in the other classes (see Goodwin, 1952, for full details). γ-Carotene, on the other hand, has already been found in the male gametangia of the sexual forms of various species of the aquatic Phycomycete, *Allomyces* (Emerson & Fox, 1940), as well as in the other classes. ζ-Carotene is not so widely distributed as are the other polyene components, for it has not been previously reported as such in any other fungus; but it now appears certain (Haxo, 1949) that the pigment occurring in one of the *Rhodotorula rubra* mutants of Bonner *et al.* (1946) is ζ-carotene. This is the first time that phytoene has been observed in a fungus, but it may be widely distributed for Goodwin (1951*a*) has recently observed it in the Basidiomycete *Dacromyces stillatus*.

The series of polyenes present in *Phycomyces* is very similar to that present in tomato fruit, the main difference being that the major pigment in *Phycomyces* is β-carotene whilst that in tomatoes is lycopene. The very great similarity between the two series of polyenes does, however, strongly suggest that the biosynthetic route may be the same in both. It is instructive, therefore, to consider the mechanism postulated by Porter & Lincoln (1950) in tomatoes as a possible route in *Phycomyces*.

ζ-Carotene is octahydrolycopene (Nash *et al.* 1948) and Porter (unpublished work quoted by Porter & Lincoln, 1950) considers that neurosporene, phytofluene and phytoene are tetra-, dodeca-, and hexadeca-hydrolycopene, respectively. Porter & Lincoln (1950) also state that tetrahydrophytoene (eicosahydrolycopene) exists in tomatoes. As little information regarding its properties is as yet available, it has not been possible to state whether or not it is present in *Phycomyces*.

From breeding studies Porter & Lincoln (1950) conclude that lycopene is the parent $C_{40}H_{50}$ carotenoid and that it is synthesized from the highly saturated compound tetrahydrophytoene, via phytoene, phytofluene, ζ-carotene and neurosporene, by the stepwise removal of four hydrogen atoms; α-, β- and γ-carotenes, etc. are then produced by the isomerization of lycopene. It is not considered likely that lycopene is the first product and that the more saturated polyenes are produced from it by reduction.

The possibility that, in bacteria, the most unsaturated polyenes are also produced from their more saturated derivatives has been envisaged by Turian (1950) who found that diphenylamine inhibited carotenogenesis in *Mycobacterium phlei*. He postulated that the inhibition occurred at the point in the chain where phytofluene or a related compound was converted into the more unsaturated polyenes, although he did not produce any experimental evidence in support of this. In fact, it has only recently been shown that phytofluene occurs in this bacterium (Goodwin, 1951*a*).

The experiments recorded here (Table 9) show conclusively that diphenylamine inhibits the production of the most unsaturated polyenes (α-, β- and γ-carotene and lycopene) and stimulates the synthesis of more saturated ones (phytofluene, ζ-carotene, neurosporene and possibly phytoene). This appears at first sight to confirm the hypotheses of Porter & Lincoln (1950) and Turian (1950). Although this interpretation of these results may be correct, it must be emphasized that another interpretation is possible. Instead of being produced stepwise one from the other, the series of polyenes could be produced in parallel syntheses from a common precursor, thus:

COMMON PRECURSOR
- →Phytoene
- →Phytofluene
- →ζ-Carotene
- →Neurosporene

} Unaffected by diphenylamine

- →α-Carotene
- →β-Carotene
- →γ-Carotene
- →Lycopene

} Blocked by diphenylamine

If the synthesis of the fully unsaturated polyenes is blocked by diphenylamine then more of the common precursor is available for the synthesis of the more saturated derivatives, which will then accumulate in the mycelium.

Tables 7 and 8 show that variations in cultural conditions which alter the β-carotene production by *Phycomyces* (Garton *et al.* 1951; Goodwin & Lijinsky, 1951) also alter to the same extent the relative amounts of the other components synthesized. The reduced synthesis of β-carotene in the dark is also reflected in the other components, and this is also the case with the (+) strain (Table 8). Thus there is no indication from these results that the decreased β-carotene production observed under certain conditions is due to the failure to convert a related

polyene into this pigment. Similarly, increased β-carotene synthesis is never at the expense of another polyene component.

The coloured components of the polyene series identified in *Phycomyces*, α-, γ- and ζ-carotenes, neurosporene and lycopene, represent only 5% of the total pigments present. As their proportion does not vary under varying cultural conditions not involving the presence of diphenylamine in the medium, no sensible errors are introduced by assuming, as Garton *et al.* (1951) and Goodwin & Lijinsky (1951) have done, that the unchromatographed pigment is β-carotene.

It is interesting to note that the hyphae of the cultures of *Phycomyces* produced in the presence of diphenylamine are still strongly positively phototropic, in spite of the fact that the β-carotene content is reduced by about thirty times. This suggests that the view of Galston (1950) that phototropic bending may be mediated through riboflavin and not β-carotene may be correct, although the small amount of β-carotene still present in 'diphenylamine cultures' may still be sufficient for this purpose. Obviously the use of these 'diphenylamine cultures' should be of great help to the plant physiologists in settling this problem.

SUMMARY

1. Phytofluene, γ-carotene, ζ-carotene, neurosporene and lycopene occur in *Phycomyces blakesleeanus*, in addition to α- and β-carotene already recorded. They represent 2·0, 0·85, 0·35, 0·6 and 0·6% respectively, of the total polyenes present. Phytoene is also probably present.

2. A red pigment, not a carotenoid, occurs in about 2% of the mycelia examined; the reason for its sporadic appearance is unknown.

3. Unidentified unsaponifiable materials include a substance with an absorption band at 255 mμ., associated with phytoene, and a small group of four fluorescent substances adsorbed on alumina between phytoene and phytofluene.

4. Variations in the cultural conditions which alter the amount of β-carotene synthesized affect the synthesis of the other component polyenes equally.

5. Addition of diphenylamine (1/40,000) to the media almost completely inhibits the production of the most unsaturated carotenoids (α-, β- and γ-carotene and lycopene), whilst stimulating the production of the more saturated components phytofluene, ζ-carotene, neurosporene and possibly phytoene.

6. *Ascobolus furfuraceous* and *Nectria cinnabarina* when cultured on liquid media do not synthesize polyenes. Diphenylamine has no effect on the unsaponifiable fraction of these fungi.

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