Studies in Carotenogenesis

30. THE PROBLEM OF LYCOPERSENE FORMATION IN *NEUROSPORA CRASSA*

By B. H. DAVIES, D. JONES AND T. W. GOODWIN

Department of Agricultural Biochemistry, University College of Wales, Aberystwyth, Wales

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There is evidence that the first tetraterpene formed in carotenoid biosynthesis is not lycopersene, the C_{40} analogue of squalene, but phytoene (15,15'-dehydrolycopersene). Davies, Goodwin & Mercer (1961) failed to find lycopersene in any carotenogenic system they examined, and it was suggested that lycopersene plays no part in carotenoid biosynthesis (Davies, 1961, 1962; Goodwin, 1961).

Mercer, Davies & Goodwin (1963) studied the incorporation of several labelled substrates into higher plant terpenes, but found no evidence for lycopersene being a carotenoid precursor. Anderson & Porter (1962) found that labelled terpenyl pyrophosphates are incorporated into phytoene by tomato and carrot plastids, but no radioactive lycopersene was detected.

In fungi, however, although lycopersene is absent from *Phycomyces blakesleeanus* (B. H. Davies, unpublished work), Grob, Kirschner & Lynen (1961) reported that a system from *Neurospora crassa* could synthesize radioactive lycopersene from [^{14}C]geranylgeranyl pyrophosphate. Grob & Boschetti (1962) reported that the two major components of a phytoene-free fraction of the total lipid from *N. crassa*, which had been cultured in the presence of diphenylamine, were squalene and lycopersene. The apparent difference between this and other carotenogenic organisms led to the present investigation into the alleged formation of lycopersene in *N. crassa*.

Carotenoid biosynthesis is partially inhibited by diphenylamine in *N. crassa*, as in many other carotenogenic micro-organisms (see Goodwin, 1959). This inhibition results in the accumulation of the more saturated C_{40} polyenes, phytoene, phytofluene, θ -carotene and neurosporene (Turian & Haxo, 1952; Turian, 1957; Zalokar, 1957). If lycopersene were concerned in carotenoid biosynthesis, it would probably accumulate along with phytoene, etc. in diphenylamine-inhibited cultures.

EXPERIMENTAL

Organism. Two strains of *Neurospora crassa* were used in these investigations. One was isolated from bread and provided by Dr I. M. Wilson, Department of Botany, University College of Wales, Aberystwyth; the other was a wild type (A 10336) provided by Professor E. C. Grob,

Institut für organische Chemie der Universität, Bern, Switzerland.

Cultural conditions. The fungus was maintained on Oxoid Czapek-Dox agar slopes in the light at room temperature. Inoculation of liquid cultures was carried out with a spore suspension prepared from such a slope.

The organism was grown for 6 days in shake cultures in the light (4×100 w tungsten lamps, 2 ft. away) at 37° . The formation of large aggregates was prevented by the addition to the culture medium of Tween 80, which also promotes carotenogenesis (Krzeminski & Quackenbush, 1960*a, b*). The medium used was similar to that of Mitchell & Houlahan (1946), as adapted for maximal carotene production by Krzeminski & Quackenbush (1960*a, b*). Its composition, per l., was: sucrose, 20 g.; ammonium tartrate, 5 g.; NH_4NO_3 , 5.5 g.; KH_2PO_4 , 1 g.; $MgSO_4 \cdot 7H_2O$, 0.5 g.; $CaCl_2$, 0.1 g.; $FeCl_3$, 5 mg.; $ZnSO_4 \cdot 7H_2O$, 2 mg.; biotin, 50 μg .; Tween 80, 8 g. The final pH of the culture medium was 5.0. Production of fully unsaturated carotenoids was inhibited by the addition of 5 ml. of ethanolic 0.25% diphenylamine/l. of medium.

Grob & Boschetti (1962) cultured *N. crassa* statically in the dark at 29° for 6 days. Their culture medium was essentially the same except that the detergent was omitted, and glucose replaced sucrose as the carbon source (E. C. Grob & A. Boschetti, personal communication).

Materials. The source of materials and the purification of solvents are described in the preceding paper (Mercer *et al.* 1963).

Extraction of the unsaponifiable material. The lightly pigmented cultures of *N. crassa* were filtered through cheese-cloth under suction and washed several times with water to remove traces of diphenylamine and detergent. The mycelia were then ground under redistilled acetone with washed silver sand until all the pigment had been extracted. The solid residue was filtered off and the lipids were transferred from acetone to diethyl ether by the addition of an equal volume of diethyl ether and then water, dropwise, until two layers formed. Any emulsions formed by traces of the detergent were broken by the addition of solid NaCl. The diethyl ether was removed under reduced pressure and the lipid residue was then saponified by boiling for 10 min. with ethanolic KOH. The solution was allowed to cool, diluted with 2 vol. of water and extracted three times with diethyl ether. The ethereal extracts were bulked and washed with water until the washings were no longer alkaline to phenolphthalein. The pale-yellow solution was dried over anhydrous Na_2SO_4 and the solvent evaporated under N_2 . The unsaponifiable material was a yellow oil.

Chromatography of the unsaponifiable material. Samples of the unsaponifiable material (usually about 100 mg.) were dissolved in light petroleum ($40-60^\circ$) and chromatographed on a 20 g. alumina column. The acid-washed alumina

(Woelm) was deactivated with water to Brockmann grade II, made up in a slurry with light petroleum and added in this form to a 1.5 cm. diam. glass column. After the alumina particles had settled, the column was washed with 100 ml. of light petroleum to remove hydrocarbon impurities. The sample of the unsaponifiable material was added to the column in solution in light petroleum, and the same solvent was used to develop the chromatogram. Fractions (5 ml.) were collected and were evaporated to dryness under a stream of N_2 before subsequent analysis.

Thin-layer chromatography of terpenoid hydrocarbons. Small samples of the unsaponifiable material and hydrocarbon fractions from the alumina column were chromatographed on thin layers of silica gel G (Merck) supported on glass plates (20 cm. $\times$ 20 cm. or 50 cm. $\times$ 20 cm.); light petroleum (b.p. 40–60°) was used to develop the chromatograms. There was a slight variation in R_F values, although a saturation chamber was employed (Stahl, 1959; Davies, 1963), but authentic samples of phytoene, lycopersene and squalene were always chromatographed on the same plate. After the solvent had run 10 cm., the positions of the hydrocarbons were revealed.

Two methods of staining were employed: either iodine vapour (Davies *et al.* 1961) or $SbCl_3$ (Grob & Boschetti, 1962). When a plate was stained with iodine, it was kept in an airtight chamber in which a few crystals of iodine were dropped on to hot sand in a crucible. The terpenoid (unsaturated) hydrocarbons were revealed as brown spots against a white background; the use of too much iodine resulted in the overall staining of the plate. The hydrocarbons can also be revealed by spraying the plate with a saturated solution of $SbCl_3$ in $CHCl_3$ and heating the plate at 110° for 15–20 min. Staining with iodine is the more sensitive method, since as little as 0.05 μ g. of squalene, lycopersene or phytoene can be detected (Davies *et al.* 1961), but all the hydrocarbons stain yellow or brown, according to the amount present. Antimony trichloride, though less sensitive, has the advantage that different colour reactions are given with different hydrocarbons (see Table 1). The sensitivity of this method, however, can be increased by viewing the plate after staining under long-wave (366 m μ) u.v. light. The stained hydrocarbons are detected by their orange fluorescence. As little as 1 μ g. of squalene, lycopersene or phytoene can be detected in this way.

A fuller account of some of these methods has been given by Mercer *et al.* (1963).

Determination of hydrocarbons. The very small quantities of hydrocarbons can only be measured by putting the above-mentioned colour reactions with iodine and $SbCl_3$ on a quantitative basis. Since fading occurs, both on the plate and in solution, elution of the coloured spots followed by spectrophotometric determination has proved inaccurate (E. I. Mercer & K. J. Treharne, unpublished work). A greater accuracy (within 10%) has been achieved by direct visual comparison of the stain intensities of spots with those of standard quantities run on the same plate.

Determination of radioactivity. Measurements of ^{14}C activity were made with a Nuclear Measurements Corp. (Chicago, Ill., U.S.A.) PCC-10A proportional counter (efficiency 35%) coupled to a Panax (Redhill, Surrey) 100C E.H.T. and fast scaler unit. All counts were made at infinite thinness.

Spectroscopic examination of polyenes. Absorption spectra were recorded with a Unicam SP. 500 spectrophotometer. Samples were dissolved in redistilled light petroleum (b.p. 40–60°).

RESULTS

Impurities in solvents. Light petroleum (AnalaR) must be purified by redistillation, because the residue left after distillation contains components that run with R_F values 0.7, 0.4, 0.3, 0.15 and 0.0 on the thin layer of silica gel 'G' and stain with iodine. Synthetic lycopersene also runs with R_F 0.3, but it can be distinguished from the petroleum impurity by examination under ultra-violet light, when the impurity fluoresces; lycopersene does not. While lycopersene gives a pink colour in its antimony trichloride reaction, the petroleum impurity stains grey-brown. Similar impurities could be detected in the AnalaR diethyl ether and acetone, so these, too, were redistilled before use.

Before the unsaponifiable material was applied to the alumina column, the column was pre-flushed with 100 ml. of light petroleum to remove a material that runs with the same R_F as squalene on a silica-gel-G plate. Another impurity had to be removed from the sodium chloride and anhydrous sodium sulphate before these could be used in the analytical procedure. This material ran with R_F about 0.2 and stained blue-green with iodine vapour.

Terpenoid hydrocarbons in diphenylamine-inhibited cultures of *N. crassa*. Cultures of *N. crassa* were grown in the presence of diphenylamine (12.5 mg./l.) in the light at 37°. The unsaponifiable material was isolated from 100 g. of mycelia (fresh wt.) and chromatographed on a 20 g. alumina column (Brockmann grade II), with redistilled light petroleum (b.p. 40–60°) as the developing solvent. Twenty fractions, each of 5 ml., were collected and evaporated to dryness, and each was chromatographed on a thin layer of silica gel G. No lycopersene could be detected (Fig. 1), even by viewing the antimony trichloride-sprayed chromatogram under long-wave u.v. radiation. The concentration of the squalene in the eluate was maximal after 30 ml. of light petroleum had run through the column, and the phytoene concentration was maximal after 70 ml. A control analysis of a mixture of squalene, lycopersene and phytoene showed that, if lycopersene were present, its concentration in the eluate should reach a peak value after 50 ml. of light petroleum had run through the column.

The more sensitive iodine-staining method failed to reveal lycopersene, even when the unsaponifiable material from larger quantities (200–300 g.) of fresh mycelium was analysed. Several compounds other than those already described, however, could

Fig. 1. Squalene (○) and phytoene (●) obtained from the unsaponifiable fraction of 100 g. of *N. crassa* grown in the presence of diphenylamine, separated on an alumina column (20 g., Brockmann grade II) with light petroleum.

be detected in the unsaponifiable material by thin-layer chromatography followed by staining with iodine or antimony trichloride. The R_F values of these materials on a fresh silica-gel-G plate are shown in Table 1.

These results are representative not only of analyses carried out on cultures grown under the conditions described above, but also of those of shake or static cultures grown in the light or in darkness over the temperature range 29–37°. The only variations are in the amounts of θ -carotene, phytofluene and phytoene, which are slightly increased in cultures grown in the light. There was no detectable difference in the hydrocarbon contents of the two strains of *N. crassa* that were examined.

*Incorporation of DL-[2- 14 C]mevalonate into terpenoid hydrocarbons by diphenylamine-inhibited *N. crassa*.* *N. crassa* was grown in the presence of diphenylamine and 20 μ C of [2- 14 C]mevalonic acid in the light, and the unsaponifiable material was isolated. The 14 C activity of this was assayed and then the fraction was chromatographed on an alumina column, as previously described, and the first 200 ml. of the light-petroleum eluate was collected. This was evaporated to dryness, leaving a yellow, oily residue. The visible absorption maxima in light petroleum indicated that the yellow colour was due to the presence of θ -carotene (357, 375, 396.5 and 420 m μ). Phytofluene was

Table 1. *Thin-layer chromatography of the unsaponifiable fraction from Neurospora crassa*

Materials present in the fraction of unsaponifiable material of *N. crassa* were eluted with light petroleum (200 ml.) from a 20 g. Brockmann grade II alumina column, chromatographed on a thin layer of silica gel G with light petroleum and stained with antimony trichloride. (Synthetic lycopersene stains pink and has R_F 0.3.)

Colour with SbCl ₃	Nature (if known)	R_F
Yellow	Waxy material	1.0
Purple	—	0.8
Grey-blue	—	0.75
Pink	Squalene	0.4
Brown	Phytoene	0.2
Dark grey-brown	Phytofluene	0.1
Dark grey	θ -Carotene	0

Table 2. *Labelling of components of the unsaponifiable fraction from Neurospora crassa*

Radioactivity was measured in components of the unsaponifiable fraction from *N. crassa* grown in the presence of diphenylamine and DL-[2- 14 C]mevalonic acid (17 μ moles, 20 μ C)

Fraction	Total activity (counts/min.)
Total unsaponifiable fraction	3.5×10^5
Light-petroleum eluate from alumina column	1.8×10^4
Fractions from thin-layer chromatography:	
(1) Solvent-front fraction (waxy material)	90
(2) (Stains purple with SbCl ₃)	150
(3) (Stains grey with SbCl ₃)	100
(4) Squalene	5000
(5) Synthetic carrier lycopersene	5
(6) Phytoene	5000
(7) Phytofluene	650
(8) θ -Carotene fraction (at origin)	6000

also present, as indicated by its fluorescence and its absorption maxima at 331, 348 and 367 m μ . Carrier synthetic lycopersene was added to this fraction, and the mixture chromatographed on a thin layer of silica gel G (20 cm. $\times$ 20 cm.). A vertical strip, 1 cm. wide, was removed from the layer with transparent adhesive tape, and this was treated with iodine vapour to reveal the positions of the components. When this was replaced on the plate, the appropriate sections of the adsorbent could be scraped off and extracted with ethanol. The residue from each zone was extracted three times with 5 ml. quantities of ethanol; the three extracts were pooled and evaporated to dryness. The 14 C activity of each of the hydrocarbons was then determined. The results are shown in Table 2.

DISCUSSION

An analysis of the less polar constituents of the unsaponifiable fraction of two strains of *N. crassa*, cultured under conditions of diphenylamine inhi-

bition, failed to reveal the presence of lycopersene in this organism, although squalene, phytoene, phytofluene and θ -carotene, and other unidentified hydrocarbons less polar than squalene, are present in appreciable amounts. The incorporation of [^{14}C]mevalonate into squalene and phytoene was demonstrated, and although there was slight incorporation of radioactivity into certain other hydrocarbons, no radioactive lycopersene was detected. It seems clear, from these results therefore that lycopersene plays no obvious part in carotenoid biosynthesis in *N. crassa*.

This conclusion is at variance with that of Grob & Boschetti (1962), who obtained a positive identification of squalene and lycopersene as components of a lipid fraction of diphenylamine-inhibited *N. crassa*. It is difficult to see where this discrepancy has arisen. The possibility of a strain difference has been eliminated since the results presented above refer not only to our own strain, but also to the wild type (A 10336) that was used by Grob & Boschetti (1962). In their method for detecting lycopersene, these workers did not saponify their lipid extract, so the possibility existed that a saponifiable compound which ran with the same R_f , or was broken down to a compound which ran with the same R_f as lycopersene, was present in this total lipid fraction. The application of our method of analysis to the total lipid, however, has failed to reveal any material with R_f similar to that of lycopersene. We had previously shown that lycopersene is not destroyed by our saponification techniques (Mercer *et al.* 1963).

Anderson & Porter (1962) and Chichester and his co-workers (C. O. Chichester, personal communication) have failed to find any evidence for the participation of lycopersene in carotenoid biosynthesis in tomato plastids and preparations from *P. blakesleeianus* respectively. However, Grob *et al.* (1961) reported the synthesis of lycopersene *in vitro* from [^{14}C]geranylgeranyl pyrophosphate in a cell-free system from *N. crassa* in the presence of NADPH. Although NADPH is the hydrogen donor concerned with the synthesis of squalene from farnesyl pyrophosphate (Goodman & Popják, 1960), NADP $^{+}$ is the cofactor concerned in the synthesis of phytoene, and NADPH is inhibitory (Anderson & Porter, 1962). If lycopersene is indeed formed in the *N. crassa* system, then its formation would appear to take place under somewhat artificial conditions. The separation of squalene and lycopersene by Grob *et al.* (1961) was carried out with a reversed-phase paper-chromatography system; this is much less efficient than the thin-layer method now in use.

The results described above indicate that, in tetraterpene formation, there is no difference between fungi and other carotenogenic organisms

such as higher plants and photosynthetic bacteria (Davies *et al.* 1961; Mercer *et al.* 1963). In all cases, phytoene appears to be the first C_{40} compound formed in carotenoid biosynthesis.

SUMMARY

1. A detailed analysis of the less polar hydrocarbons of the unsaponifiable fraction of *Neurospora crassa*, cultured in the presence of diphenylamine, has revealed the presence of squalene, phytoene, phytofluene and θ -carotene. Several unidentified hydrocarbons, less polar than squalene, have also been detected, but lycopersene, the C_{40} analogue of squalene, was absent.

2. [^{14}C]Mevalonic acid was significantly incorporated into squalene and phytoene and other tetraterpenes more unsaturated than phytoene by *N. crassa* grown in the presence of diphenylamine. Labelled lycopersene could not be detected.

3. These results indicate that in *N. crassa*, as in other carotenogenic organisms, phytoene, and not lycopersene, is the first C_{40} compound formed in carotenoid biosynthesis.

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