Phytochemistry, 1975, Vol. 14, pp. 463–469. Pergamon Press. Printed in England.

CAROTENE BIOSYNTHESIS BY CELL EXTRACTS OF MUTANTS OF *PHYCOMYCES BLAKESLEEANUS*

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(*Received* 23 *June* 1974)

Key Word Index—*Phycomyces blakesleeanus*; Mucoraceae; fungi; mutants; carotene biosynthesis; MVA-[2-^{14}C].

Abstract—Cell extracts capable of converting MVA-[2-^{14}C] into isoprenoids were obtained from the yellow C115-*mad*-107(−) and red C9-*carR*21(−) mutants of *Phycomyces blakesleeanus*. Neither air nor light was essential for carotene biosynthesis. The specific activities of the terpenoid-synthesizing enzymes varied with the age of the cultures although the formation of lycopene (ψ,ψ-carotene) in the C9 and of β-carotene (β,β-carotene) in the C115 mutants, respectively, followed the increase in the dry weight yield of the cultures. The significance of these results to the biosynthesis of carotenes and to the classification of these compounds as secondary metabolites is discussed.

INTRODUCTION

Studies on a number of fungi have figured prominently over the last 20 yr in the literature of carotenoid biosynthesis. Although the overall pathway of carotene formation in these organisms is reasonably well defined as a result of *in vivo* investigations (Scheme 1), a number of problems remain unsolved. These include the problem of the nature of the acyclic precursor for the formation of the bicyclic β-carotene (**7**; β,β-carotene), that of the mechanism of control of carotene biosynthesis and the question of cofactor requirements for the desaturation of the acyclic carotenes (**2**–**6**). Solutions to such problems necessitate the development of cell-free systems similar to those obtained from higher plant tissues [1, 2] and capable of forming carotenes *in vitro*. Chichester and his collaborators, using a cell-free system from wild-type *Phycomyces blakesleeanus*, have obtained indirect evidence of the participation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) in carotene formation [3], and, by direct incorporation experiments in essentially the same type of system, have demonstrated that farnesyl pyrophosphate (FPP) [4] and geranylgeranyl pyrophosphate (GGPP) [5] are converted into carotenes. Cell-free systems from other fungi have yet to be documented.

With these considerations in mind, we initiated studies into the development of a cell extract capable of converting labelled mevalonic acid (MVA) into carotenes. The organisms chosen were mutant strains of *P. blakesleeanus*, obtained by *N*-methyl-*N*-nitroso-*N'*-nitroguanidine treatment of vegetative spores of the wild-type [6]. These mutants differ from the wild-type (NRRL 1555) in their ability to form carotenes; the yellow C115-*mad*-107(−) mutant accumulates exclusively β-carotene while the red C9-*carR*21(−) contains predominantly acyclic carotenes, especially lycopene (**6**; ψ,ψ-carotene) [7, 8]. The present publication reports the ability of cell extracts of these fungi to biosynthesize carotenes from MVA-[2-^{14}C] both in the presence and absence of light and oxygen. In addition, variations in the specific activities of the isoprenoid-synthesizing enzymes with the age of the fungal cultures are described.

RESULTS

Incorporation of MVA-[2-^{14}C] into the terpenoids of the C9 and C115 mutants

Preliminary experiments, designed to investigate the effect of light and oxygen on the formation of isoprenoids, were successful in incorporating radioactivity from MVA-[2-^{14}C] into the terpenoids under all the experimental conditions used (Table 1). A boiled enzyme control failed to incorporate radioactivity into any of the fractions. The

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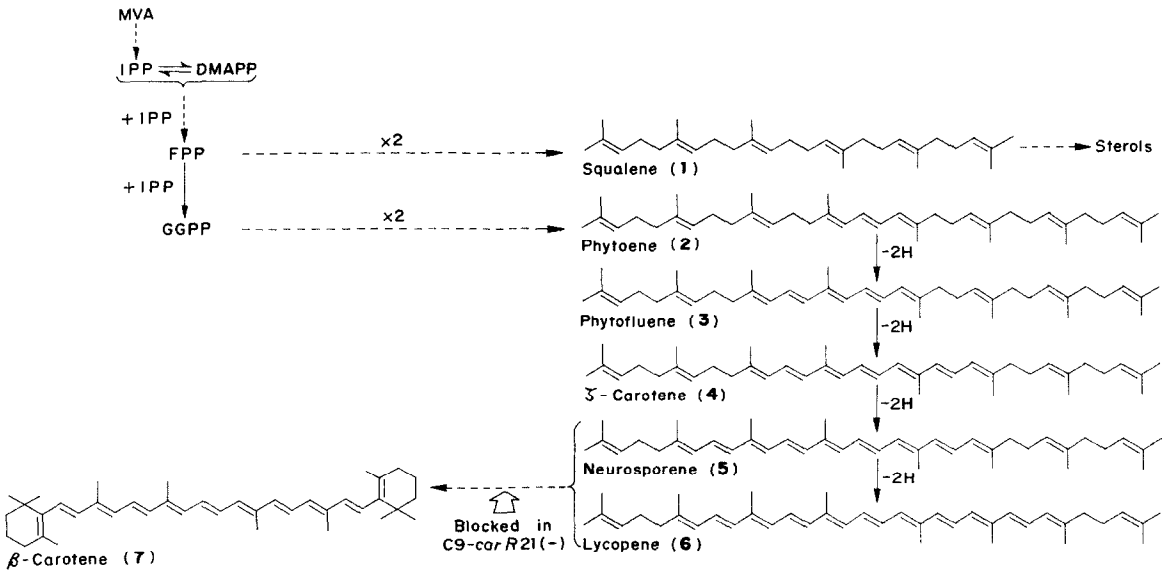

Scheme 1. Formation of carotenes from mevalonic acid (MVA). ——→ Direct reactions; - - - → two or more reactions.

radioactivity (dpm) of each compound reflects the total incorporation into that compound less that percentage which was not recovered during the particularly rigorous purification procedures. These losses varied slightly among the individual carotenes but were all within the range 20–25%.

Neither oxygen nor light was essential for the conversion of MVA-[2-^{14}C] into the carotenes, although aerobic conditions favoured the formation of the triterpenoids (squalene and sterols) of the C9 mutant and the sterols of the C115 mutant. There was a negligible difference between the formation of β-carotene in the light by the C115 mutant under aerobic and anaerobic conditions (incorporations of 12·6 and 12·3%, respectively), while maximum formation of lycopene by an extract from the C9 mutant took place under dark, anaerobic conditions.

All subsequent incubations of MVA-[2-^{14}C] with the extract from the C9 mutant showed incorporations similar to those presented in Table 1, but later investigations using a C115 extract revealed

Table 1. Percentage incorporation* of MVA-[2-^{14}C] into the terpenoids of C115-*mad*-107(−) and C9-*carR*21(−) extracts

Organism	C115-*mad*-107(−)†				C9-*carR*21(−)‡			
Incubation conditions§	Light O_2	Dark O_2	Light	Dark	Light O_2	Dark O_2	Light	Dark
Unsaponifiable lipid	58·9	36·5	47·1	48·7	27·4	27·6	25·1	28·1
Squalene	0·06	1·62	0	0·48	9·1	6·9	3·7	3·2
Phytoene	0	0	0	0	3·1	2·0	1·2	0·55
β-Carotene	12·6	11·4	12·3	7·9	—	—	—	
Lycopene	—	—	—	—	2·5	2·2	1·6	4·6
Sterols¶	3·9	2·3	0·32	0·15	0·71	1·1	0·51	0·17
Total carotenes (as % of unsaponifiable lipid radioactivity)	21·4	31·2	26·1	16·8	9·0	8·0	6·4	16·3

* Based on one isomer of DL-MVA-[2-^{14}C].
† Incubation mixtures (1 ml) contained 18·0 mg protein.
‡ Incubation mixtures (1 ml) contained 25·8 mg protein.
§ Full details given in text.
¶ Ergosterol and lanosterol.

Table 2. Incorporation of radioactivity from MVA-[2-^{14}C] into the terpenoids of samples of C115-*mad*-107(−) extract incubated for different times

Fraction	Percentage incorporations* in incubation of time (hr): 0·25	0·50	0·75	1·0	1·5	2·0	2·5	3·0	3·5	4·0
Unsaponifiable lipid	4·1	9·6	15·4	20·0	19·1	19·0	19·3	19·7	19·2	19·0
Phytoene	0·56	0·92	1·43	2·58	2·35	2·13	2·12	2·11	2·05	2·13
Squalene	2·45	4·65	5·63	6·76	8·06	8·06	8·03	8·05	7·95	7·61
β-Carotene	0	0·07	0·08	0·09	0·10	0·12	0·10	0·09	0·09	0·08

* Percentage incorporations based on one isomer of DL-MVA-[2-^{14}C]; incubation mixtures (1 ml) contained 15·0 mg protein; other conditions are described in the text.

some incorporation of radioactivity into both phytoene (**2**; 7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) and squalene (**1**) at the expense of β-carotene formation. This trend is reflected in the kinetics of the conversion of MVA-[2-^{14}C] into β-carotene, phytoene, squalene and the total unsaponifiable lipid fraction (Table 2).

These incorporation patterns show the reactions to be essentially complete after 1–2 hr of incubation; further incubation did not significantly alter the percentage incorporations. Addition of more radioactive substrate after 2 hr failed to increase the total radioactivity incorporated into any fraction, indicating the inactivity of the enzyme extract at this stage. The turbidity of the mixtures increased after a 2–3 hr period of incubation, so denaturation of the protein probably accounts for the loss of enzymic activity.

The effect of growth on carotene formation

Studies on the growth characteristics of the fungal mutants revealed a variation in the activity of the terpenoid-synthesizing enzymes with the time of harvesting the fungal cultures (Figs. 1 and 2).

For each mutant, 52 liquid shake cultures (each consisting of 100 ml medium in a 250 ml Erlenmeyer flask) were inoculated with identical volumes of a standard spore suspension ($9·8 \times 10^6$ and $4·6 \times 10^6$ spores/100 ml medium for the C9 and C115 mutants respectively) and were incubated in the light at 24°. The contents of four flasks, chosen at random, were harvested at 6-hr intervals from 18–84 hr after inoculation and at 96 hr. There was a lag period of 18–24 hr before growth commenced and the germination of the spores was accompanied by a fall in the pH of the culture medium from 5·0 to a minimum of 3·8, 48 hr after inoculation. Growth continued until 78–84 hr after inoculation, when the dry weight yield levelled off and the pH of the medium became steady at 4·4. The increases in the lycopene content of the C9 and in the β-carotene content of the C115 cultures followed the growth curves, each carotene reaching a maximum some 72–84 hr after inoculation. The yield of total lipid also paralleled the growth curves of both strains. Microscopic examinations of the oldest (96 hr) cultures showed that the carotenes were contained in discrete oil droplets within the fungal mycelia. While analyses of the C115 cultures detected no phytoene, those

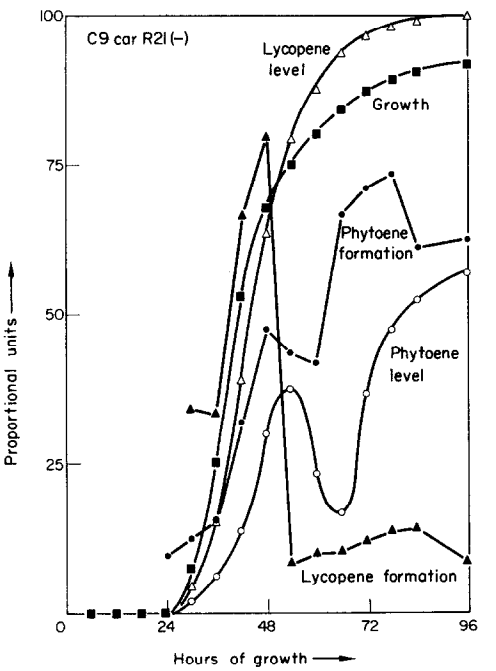

Fig. 1. Growth and carotene formation in the C9-*carR*21(−) strain of *Phycomyces blakesleeanus*. ■—Growth (100 ≡ 700 mg dry wt/100 ml); △—lycopene concentration (100 ≡ 1500 μg/g dry wt); ○—phytoene concentration (100 ≡ 1500 μg/g dry wt); ▲—MVA-[2-^{14}C] incorporation into lycopene (100 ≡ 250 dpm in lycopene/mg protein); ●—MVA-[2-^{14}C] incorporation into phytoene (100 ≡ 3000 dpm in phytoene/mg protein). Incubation conditions for enzyme assays are described in the text.

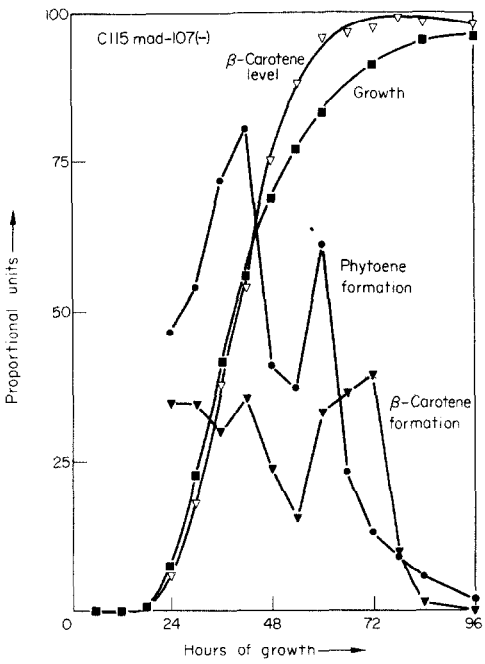

Fig. 2. Growth and carotene formation in the C115-*mad*-107(−) strain of *Phycomyces blakesleeanus*. ■—Growth (100 ≡ 750 mg dry wt/100 ml); ▽—β-carotene concentration (100 ≡ 2500 μg/g dry wt); ▼—MVA-[2-^{14}C] incorporation into β-carotene (100 ≡ 250 dpm in β-carotene/mg protein); ●—MVA-[2-^{14}C] incorporation into phytoene (100 ≡ 3000 dpm in phytoene/mg protein). Incubation conditions for enzyme assays are described in the text.

of the C9 samples showed that the concentrations of phytoene (**2**), phytofluene (**3**; 7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene) and ζ-carotene (**4**; 7,8,7′,8′-tetrahydro-ψ,ψ-carotene) increased up to 54 hr, decreased, and then rose again to reach maximum values some 84 hr after inoculation (only the phytoene levels are shown in Fig. 1).

Although phytoene was not detected in the C115 cultures, the incubation of MVA-[2-^{14}C] under the standard conditions with protein extracts from this mutant, and the subsequent addition and reisolation of carrier phytoene, β-carotene and squalene, resulted in radioactivity appearing in all three compounds. The incorporation of MVA-[2-^{14}C] into both phytoene and β-carotene (and into squalene) occurred in two distinct phases, with the cell extract showing maximal enzyme activity at 42 and 60–72 hr after inoculation. In the latter phase, maximum incorporation into phytoene preceded that into β-carotene by some 12 hr.

In the case of the C9 mutant, two biosynthetic phases were again apparent, although radioactivity from MVA-[2-^{14}C] was incorporated into lycopene with maximum efficiency only during the first period, the highest specific activity of the enzymes being at 42 hr after inoculation. While phytoene was formed by the cell extract during both phases, the higher amounts were produced during the period 66–78 hr after inoculation, thus accounting for the accumulation of phytoene observed on analysis of the carotenes.

DISCUSSION

The anaerobic formation of β-carotene from MVA-[2-^{14}C] by an extract of the C115 mutant of *P. blakesleeanus* confirms previous reports, from studies on higher plants [9, 10] and on a *Flavobacterium* sp. (Strain 0147) [11], that the synthesis of this carotene does not require oxygen. The formation of β-carotene by higher plant systems, however, does have an absolute requirement for light [10], in contrast to the formation of β-carotene in the C115 strain of *P. blakesleeanus* and of lycopene in the C9 mutant (Table 1). The cultures of *P. blakesleeanus* used for the preparation of the enzyme extracts, however, were grown in the light; it may be that some of the enzymes concerned in the formation of lycopene and β-carotene (or part of an enzyme multiplex) are photoinduced, as appears to be the case in *Neurospora crassa* [12]. The incorporations into the respective carotenes indicate that light and oxygen interact and affect the biosynthesis of the carotenes in differing ways: maximum incorporation into lycopene was obtained under dark anaerobic conditions, while the same environment apparently had an inhibitory effect on the formation of β-carotene from MVA-[2-^{14}C], which was stimulated by illumination. This aspect needs a more detailed investigation of the enzymes involved in the conversions. Although the effect of light on other fungal cell-free systems capable of metabolizing MVA has yet to be reported, a number of moulds have either a partial or an absolute requirement for light in order to synthesize carotenoids *in vivo* [12, 13]. Liquid cultures of the C115 and C9 mutants have been found to contain 15 and 22% less total carotenes, respectively, in the dark than under illuminated conditions [14], while carotene formation in static

cultures of the wild-type *P. blakesleeanus* is, apparently, even more affected by light [15, 16].

The rate of incorporation of MVA-[2-^{14}C] into the carotenes, squalene and the unsaponifiable lipid fraction (Table 2) was significantly greater than those previously reported for MVA-[2-^{14}C] in a cell-free system of the wild-type strain of *P. blakesleeanus* [3] and for the incorporation of GGPP-[^{14}C] into the carotenes of the C9 mutant [5]. This is probably due to the fact that the protein concentrations of the extracts used in the present study were particularly high (up to 80 mg/ml when a small volume of buffer was used to extract the lyophilized mycelia).

Although the basic incubation mixture contained both pyridine and flavin nucleotides, no requirement for these cofactors could be demonstrated since the cell extracts were denatured by prolonged dialysis, as they were on storage [17]. Consequently, previous reports that the wild-type strain requires ATP, NADP, NADPH, NADH and Mn^{2+} for the conversion of MVA-[2-^{14}C] into the carotenes [3], while no such cofactors are needed for the incorporation of GGPP-[^{14}C] into the carotenes of the C9 mutant [5] could not be verified.

The growth curves of both mutants are typically fungal, as are the changes in pH of the culture medium during growth [18]. The latter are a useful guide to the stage of fungal growth. It is generally considered that carotenoids are secondary metabolites in the sense that they are only produced in the later stages of mycelial growth and their synthesis is suppressed while the cells are actively multiplying [19, 20]. The presence of intracellular carotenes in both the C9 and C115 mutants and the activity of the terpenoid-synthesizing enzymes throughout the growth of these cultures (Figs. 1 and 2) cases some doubt on this means of classification of the carotenoids as secondary metabolites. Previous investigations on the presence of carotenoids in fungal mycelia have yielded conflicting results: a major portion of the carotenes was formed only during the stationary phase of growth (idiophase [2]) of statically-grown cultures of wild-type *P. blakesleeanus* [22], while the same strain was found to contain β-carotene throughout its growth in liquid culture [23, 24]. This anomaly may reflect a difference in the roles of the carotenoids under these different cultural conditions.

The incorporation of radioactivity by the C9 mutant into phytoene rather than into lycopene in the later stages of growth might be consistent with a feedback inhibition, mediated by a high concentration of lycopene, of the enzyme which dehydrogenates phytoene (Fig. 1). This situation, apparently, does not occur in the C115 strain; the formation of β-carotene from MVA-[2-^{14}C] can occur right up to the stationary phase of growth, and high concentrations of β-carotene do not inhibit phytoene metabolism (Fig. 2). The appearance of β-carotene in lipid droplets at late stages of growth, comparable with those found in other organisms [25] may reflect the removal of this carotene from the site of its synthesis, and hence the simultaneous loss of feedback control. The incorporation of radioactivity into phytoene is not reflected in the appearance of phytoene in the mycelia of the C115 mutant at any stage of its growth (Table 2; Fig. 2). This may indicate an alteration in the kinetics of the enzymes responsible for phytoene synthesis and metabolism on extraction of the proteins from the lyophilized mycelia. Such a change could cause an accumulation of phytoene *in vitro*.

This novel method of preparing a fungal extract which metabolizes MVA-[2-^{14}C] provides an opportunity for studying numerous problems of carotenoid biosynthesis using these, and other, mutant strains of *P. blakesleeanus* and other carotenogenic fungi. Investigations into a number of these aspects will be reported in future publications.

EXPERIMENTAL

Solvents. All the solvents used were of AR grade. Light petrol. (b.p. 40–60° and 60–80°), C_6H_6 and Et_2O were dried over Na–Pb alloy and redistilled before use, the Et_2O from reduced Fe powder.

Radiochemical. DL-Mevalonic acid-[2-^{14}C] lactone (10·3 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. It was converted into the Na salt of the acid prior to use by the addition of the requisite quantity of aq. NaOH solution.

Organisms and culture conditions. The mutant strains, C9-*carR*21(−) and C115-*mad*-107(−), of *Phycomyces blakesleeanus* were kindly provided by Prof. M. Delbrück, Division of Biology, California Institute of Technology, Pasadena, Calif., U.S.A. The naming of these mutants follows the recommendations of the Caltech Group on Phycomyces Genetics Nomenclature [26]. Growth and maintenance conditions for these moulds were the same as those described for the C5-*car*-10(−) strain [27]. Dry wt determinations were made on lyophilized samples.

Spore counts. An aliquot (1 ml) of the spore suspension was diluted with 9 ml particle-free solution ("Isoton"; Coulter Electronics Ltd., Dunstable, Beds., U.K.) and the number of particles in the suspension was estimated using a Coulter Counter (Coulter Electronics Ltd., St. Albans, Herts., U.K.) using a 100 μm aperture, an aperture current setting of 7 and a threshold value of 22. Spore numbers were calculated from the mean of duplicate readings, corrected for background and for coincidence.

Preparation of cell extract. Mycelia were harvested 54–60 hr after inoculation (or after the appropriate growth period in the 96 hr culture experiments), washed with deionized water and squeezed to hand dryness. After freezing at −20°, the samples were lyophilized. The dehydrated mycelia were then rubbed through a 40-mesh sieve at room temperature. Three vols. of 0·2 M Tris–HCl buffer pH 8·0 were added to the fine powder, and the resulting paste was centrifuged at 10000 *g* for 30 min. Both of these operations were carried out at 4°. The supernatant was decanted and its protein concentration measured by the biuret method [28]. Once prepared, the cell extract was added to the incubation mixtures as soon as possible. Storage for one week at either 4° or −20° resulted in a pronounced loss of enzymic activity of the extract from the C9 mutant [17].

Incubation conditions. Incubations (1 ml) were carried out either aerobically or anaerobically in 15 ml Thunberg tubes for 3 hr (unless stated otherwise) at 24° and in a shaker water bath. Anaerobic conditions were obtained by the successive evacuation (oil vacuum pump) and purging of the tubes with argon. Tubes were shown to remain anaerobic for at least 3 hr by the continuous colourless appearance of reduced methyl viologen. The basic incubation mixture contained: DL-MVA-[2-^{14}C], 1 μCi (97 nmol); ATP, 10 μmol; GSH, 20 μmol; $MgCl_2.6H_2O$, 4 μmol; $MnCl_2.4H_2O$, 6 μmol; NAD, NADP, FAD, NADH and NADPH, 1 μmol each; cell extract, 0·4 ml; 0·2 M Tris–HCl buffer pH 8·0 to 1 ml. Any variations in these conditions are given in the Results section. FAD was obtained from the Sigma London Chemical Co. Ltd. while all other cofactors were from the Boehringer Corporation Ltd.

Extraction of radioactive compounds. EtOH (8 ml) and aq. KOH soln (60% w/v, 1 ml) were added to each incubate and the mixture was saponified at room temp. overnight. After the addition of the appropriate unlabelled carriers (50 μg each; carotenes were isolated from the mutant strains of *P. blakesleeanus* and squalene was obtained from the Eastman Kodak Co., Rochester, N.Y., U.S.A.), the unsaponifiable lipids were extracted by standard procedures [29].

Extraction of lipids from mycelia. Washed mycelia, suspended in Me_2CO, were homogenized with an Ultra-Turrax homogenizer. The mixture was filtered, the residue resuspended in Et_2O and homogenization repeated until the ethereal extract was colourless. The total lipid fraction (determined by wt) was recovered from the bulked organic extract and the unsaponifiable fraction isolated by methods already described [29].

Purification of polyenes. Preliminary separations of the carotenes and squalene from other unsaponifiable lipids were carried out on columns of aluminium oxide (Woelm neutral, Brockmann activity grade III) which were developed with light petrol. (P; b.p. 40–60°) containing increasing concentrations (v/v) of Et_2O (E). The fraction eluted by light petrol. alone contained squalene and phytoene, while the other carotenes were eluted by stepwise fractionation with concentrations of E/P from 0·25% (β-carotene) to 5% (lycopene). Finally, the sterols were eluted with 15% E/P.

Unlabelled carotenes, apart from phytoene, were further purified on columns of MgO (chromatographic grade)–Celite 545 (1:1) eluted with light petrol. (b.p. 60–80°) containing increasing concentrations of Me_2CO (up to 10%). Phytofluene, β-carotene and ζ-carotene were eluted from the column in this manner, while neurosporene (**5**; 7,8-dihydro-ψ,ψ-carotene) and lycopene were obtained by excision and extraction of the extruded adsorbent. The identity and purity of these pigments were confirmed by their chromatographic behaviour and by comparison of their absorption spectra with those previously documented [29].

Radioactive polyenes were purified to constant specific activity on various TLCs which were developed in dark saturation chambers [30]. Squalene and phytoene were separated on activated thin layers of Silica Gel G (Merck; 0·25 mm) using light petrol. (b.p. 60–80°); their R_fs were 0·38 and 0·20 respectively. These compounds were visualized by spraying with a solution of Rhodamine 6G in acetone (1% w/v) and viewing the plate under UV light. If necessary, the phytoene was further purified on thin layers of $AgNO_3$-impregnated Silica Gel G (3% w/w) using 30% (v/v) E/P as the developing solvent (R_f phytoene 0·35–0·40). Other radioactive carotenes were chromatographed on activated thin layers of MgO–Silica Gel G (1:1, w/w) with a solvent consisting of light petrol. (b.p. 60–80°) and C_6H_6 (1:1). The lycopene (R_f 0·06) and β-carotene (R_f 0·90) samples were rechromatographed on activated thin layers of MgO, using light petrol.–C_6H_6 (3:1, v/v) for β-carotene (R_f 0·25–0·30) and light petrol.–C_6H_6 (1:9 v/v) for lycopene R_f 0·10). The carotenes attained constant specific activities as a result of these procedures. Ergosterol and lanosterol were separated and purified on activated thin layers of Silica Gel G by developing with $CHCl_3$.

Crystallization of β-carotene-[^{14}C] and lycopene-[^{14}C]. These carotenes were crystallized in the presence of additional authentic β-carotene or lycopene. Each carotene was dissolved in the minimum vol. of C_6H_6, excess MeOH was added, and the solution was stored at −20° for 12 hr. Crystals were collected by filtration, washed with cold light petrol. (b.p. 40–60°) and their specific activity estimated. Subsequent recrystallizations from the concentrated mother liquor yielded samples of similar specific activities, thus confirming the radiochemical purity of the labelled carotenes.

Quantitative determination of carotenes. The carotenes were dissolved in light petrol. (b.p. 40–60°) to yield solutions of known volume and were estimated spectrophotometrically using standard $E^{1\%}_{1cm}$ values [29].

Radioassay. Scintillation fluid (10 ml), containing (per litre of toluene) 2,5-diphenyloxazole (PPO; 3·0 g) and 1,4-di-[2-(4-methyl-5-phenyloxazolyl)] C_6H_6 (dimethyl-POPOP; 0·5 g) was added to each sample prior to assay for radioactivity in an NE8310 Automatic Liquid Scintillation Spectrometer (Nuclear Enterprises Ltd., Edinburgh 11, Scotland). Corrections for colour quenching were carried out by the external standard (^{137}Cs) channels ratio method, using the procedure described elsewhere [31].

Acknowledgements—Our thanks are due to Prof. M. Delbrück for gifts of the fungal mutants and to the Science Research Council for a Research Grant and a Research Studentship (P.M.B.).

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