Carotenes and Retinal in Phycomyces Mutants[1]

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Received April 22, 1968.

Abstract. Three different types of β-carotene mutants of *Phycomyces* have been studied. In 2 mutants (Type I) β-carotene is still the principal carotene but scaled down or up relative to wild type. The carotene mixture of 2 mutants (Type II) consists mainly of phytoene and phytofluene. In Type III (2 mutants) β-carotene is replaced by lycopene.

The examination of the mutants reveals that the receptor pigment is very likely neither β-carotene nor retinal. Transmission spectra through the growing zone of live sporangiophores of 1 of these mutants which contains less than one-thousandth of the β-carotene content of wild type show that the receptor pigment extinction is less than 0.003 at its maximum.

The sporangiophore (spph) of *Phycomyces blakesleeanus* is a single cell with a growing zone which is able to respond to light (3, 13). Absorption spectra through the growing zone *in vivo* demonstrate that β-carotene is the principal pigment absorbing in the 400 to 500 mμ region (17). The similarity between the β-carotene absorption spectrum and the visible part of the action spectrum for the phototropic and the light growth response suggests that β-carotene might be involved in the primary light receptor system (1, 4). Action spectra similar to those of *Phycomyces* have also been found for phototropism in *Avena* coleoptiles (1), for initiation of synthesis of colored carotenes in *Neurospora crassa* (16), *Fusarium aquaeductuum* (11) and *Mycobacterium* (12), and for oxygen uptake in *Chlorella* (8, 9) suggesting that we are dealing with a light receptor system widely distributed among plants. Since the action spectra show a slightly greater resemblance to some known flavoproteins than to carotenes, it has been mostly assumed in the recent literature that the receptor pigment is a flavoprotein. Several β-carotene deficient mutants of *Phycomyces* were therefore isolated to examine the function of β-carotene in more detail. In this paper we report on the carotene composition in spphs of these mutants.

Retinal attached to a protein is known to be the photopigment of the eye in vertebrates, insects, and cephalopods. Absorption spectra of retinal-protein complexes also are reasonably similar to the action spectra of *Phycomyces*. Therefore, several experiments were done to investigate the presence and possible function of retinal in *Phycomyces*.

Materials and Methods

Isolation of Mutants[3]. Vegetative spores of *Phycomyces blakesleeanus* (strain NRRL 1555) at a concentration of 10^7 spores/ml were treated with 0.5 mg/ml of N-methyl-N-nitroso-N'-nitroguanidine in 0.2 M acetate buffer at pH 5 for 80 minutes at 22°. The suspension was shaken occasionally to prevent the sedimentation of spores. The spores were washed and seeded at a concentration of 50 viable spores per petri dish on agar plates containing glucose-asparagine medium (17) supplemented with 0.1 % yeast extract. The first crop of sporangiophores (spph) was plucked and discarded, and the covers of the petri dishes were removed. The second crop of sporangiophores was screened in stage III, since in this stage the white sporangia of the albino mutants are easily detected. The wild type sporangiophores around white sporangia were plucked and the spores of the albino mutants were harvested the next day.

The mutant R1 was found by chance. The mutant Ph 107 was isolated during a search for non-phototropic mutants. The bending mechanism of this mutant seems to be disturbed. At present it is not clear whether this is due to its high β-carotene level or to a second mutation.

Culture Conditions. Spphs of *Phycomyces* were normally grown on mashed potatoes in 17 cm $\times$ 25 cm stainless steel trays covered with aluminum foil. The foil was removed after 3 to 4 days, when the first stage I spphs had appeared. The spphs were grown in normal room light intensity, with 60 to 80 % relative humidity, and at 22 to 25°: they were plucked after another day when they were 5 to 7 cm long.

A glucose-asparagine medium was used for experiments with diphenylamine (DPA) and was prepared as described previously (17).

Extraction and Separation of Carotenes and Retinal. Plucked spphs (10 g) were extracted with about 30 ml of distilled methanol and 30 ml of petroleum ether, boiling point 30° to 60° (PE30/60) on a shaking machine at 5° for 1 day. In this and in all the following steps O_2 was removed thoroughly by washing with N_2. The extraction with PE30/60 was repeated 2 or 3 times. Only a small portion of the carotenes of the spores was extracted under these conditions. The collected PE fractions were evaporated to dryness and dissolved in a small volume of hexane, cooled with dry ice, centrifuged, and the sediment discarded. Most of the phospholipids and ergosterol were removed by this procedure. The supernatant containing the carotenes was adsorbed on a 40 g MgO/Celite (1:1 w/w) column or a 20 g Al_2O_3 column. Magnesia (Fisher Scientific Company) and Celite (Research Specialties Company) were dried at 180° for 4 hours. The compounds were eluted with a mixture of PE30/60 and PE60/70 (1:1) to which an increasing concentration of acetone was added. The phytoene content was separately determined using a column of neutral alumina, Grade IV (Bio-Rad). The compounds were eluted with the petroleum ether mixture containing 1 % ethanol.

The carotenes were determined spectrophotometrically with a Cary Model 15 Recording Spectrophotometer. The compounds were further identified by comparing their adsorption to the columns with those of the β-carotene precursors of wild type grown in the presence of DPA (2, 5).

Retinal isolated with the carotenes was separated from them after the cooling step by a 60 tube Counter Current Distribution train. The solvent system consisted of *n*-hexane and 2 % aqueous methanol. The fractions containing retinal were evaporated to dryness, dissolved in a small volume of methanol, and adsorbed on a thin-layer chromatography (TLC) plate of Silica Gel G (E. Merck A.G.), impregnated with liquid paraffin (15). CH_3OH saturated with liquid paraffin was used as solvent. Retinal was determined quantitatively by its color reaction with rhodanine (15).

The contents of the spphs were squeezed out under red light, by grinding with sand using a mortar and pestle. A rough fractionation of the contents of dark adapted (for 30 min) spphs was obtained by sedimentation in pH 7, 0.05 M tris buffer plus 0.1 % bovine serum albumin and 0.25 M sucrose. By low and high speed centrifugation (1000 × g, 10 min and 35,000 × g, 45 min) 3 fractions were obtained: 1) spores and larger aggregates; 2) mitochondria and nuclei, and 3) yellow scum. These fractions and the cell wall fraction were extracted under red light with either PE30/60 or 1 % digitonin solution pH 7 and PE30/60. The digitonin solutions were then exposed to light and again extracted with PE30/60. The PE30/60 extractions were investigated for their retinal content. These experiments are more difficult to interpret than an immediate extraction of spphs with methanol and petroleum ether, since O_2 was not excluded during the centrifugal fractionation and β-carotene is easily oxidized to retinal and other oxidation products.

In Vivo Absorption Measurements. Absorption measurements through the growing zone of single spphs were carried out as in previous studies using a Cary Model 15 recording spectrophotometer and a microscope attachment on loan from the Applied Physics Corporation, Monrovia, California (17). Spphs were grown on potato dextrose agar at light intensities of about 1 μw/cm².

Table I. *Carotene Concentration and Absorption Maxima of Wild Type and Mutants*
The absorption maxima were determined in *n*-hexane and are given in mμ.

	Wild type	Type I		Type II		Type III	
		Ph 107	Alb. 5	Alb. 1	Alb.10	Alb. 12	R1
		μg carotene/g dry wt sporangiophore					
Phytoene 276, 286, 296			<1	320	400	<1	70
Phytofluene 330, 348, 367			0.1	10	2	0.1	20
ζ-Carotene 376, 399, 425			<0.05	1	<0.1	<0.05	10
Neurosporene 415, 439, 469			<0.05	<0.1	<0.05	<0.1	0.5
Lycopene 445, 471, 503			<0.05	<0.05	<0.05	2	700
β-Zeacarotene 403, 426, 452			<0.05	0.4	<0.1	<0.1	<0.5
γ-Carotene 436, 461, 492			<0.05	<0.05	<0.05	0.3	0.5
β-Carotene 451, 480	220	1100	2.5	<0.1	0.1(?)	0.15(?)	0.4

Results

The Classes of Mutants. Table I summarizes the carotene contents of the spphs of some of our mutants. For wild type the principal carotene is β-carotene. We have not made a study of the minor components in wild-type spphs but smaller amounts of other carotenes, including lycopene, have been detected by studying the contents of the mycelium (5).

In table I the mutants are arranged as 3 types.

In Type I (2 mutants) the pattern of carotenes obtained is the same as in wild type but the quantity is scaled up or down.

In Type II (2 mutants) phytoene is found in very high amounts, somewhat higher even than the amount of β-carotene, the normal end product, in wild type. Thus there occurs in this type of mutant a net increase in the total amount of carotenes synthesized but only a very small fraction of this production gets beyond phytoene. One of these 2 mutants, albino 10, contains in addition an unidentified compound with absorption maxima at 395 and 420 mμ.

In Type III lycopene is the principal carotene found. The red colored mutant (R1) has a lycopene content about 3 times higher than that of β-carotene in wild type. In albino 12 the relative concentrations of various carotenes are similar to those in R1 but the absolute concentrations are scaled down by a factor of several hundred. The relation between these 2 mutants is in this respect similar to the relation between albino 5 and wild type.

The question marks attached to the values of β-carotene in albino 10 and albino 12 refer not to the amounts stated but to the fact that, since the fractions are so small, it could not be established unambiguously whether α- or β-carotene was present.

Retinal Contents of spphs. Analysis of spphs directly extracted with methanol and petroleum ether shows that wild type contains very small quantities of retinal, between 0.005 and 0.05 μg retinal/g dry weight spph, about 10,000 times less than its content of β-carotene. The retinal found seemed to be associated in some way with β-carotene since in cell fractions the quantities found paralleled the β-carotene content of the fraction. Attempts to find retinal protein complexes which liberate retinal upon exposure to light gave negative results. However, no retinal was found in the β-carotene deficient mutant albino 5 and in wild type grown with DPA. Tests of the photosensitivity of albino 5 and of wild type grown with DPA show that both of them had a sensitivity threshold close to that of wild type. It is therefore very likely that the retinal found is a spurious oxidation product of β-carotene and that it is not involved in the process mediating light sensitivity.

Transmission Spectra. Transmission spectra of the growing zones of live spphs, of wild type and of wild type grown with DPA have been reported previously (17). They show for wild type the absorption maxima of β-carotene and for wild type plus DPA the corresponding maxima of the earlier members of the biosynthetic sequence.

It was of special interest to supplement these measurements with spectra of the mutant lowest in its content of carotenoids, *i.e.* albino 10, since here the absorption of light by the receptor pigment might stand out. Three such spectra are shown in figure 1. They show a general rise in extinction in the region 550 to 400 mμ, undoubtedly due to scattering. The only peak or shoulder that can be discerned as superimposed upon this general rise is a peak at 410 mμ. This peak is certainly due to the cytochromes. Inspection of these spectra shows that peaks corresponding to the action spectrum (480–455 mμ) must correspond to an optical density smaller than 0.003/spph.

Discussion

Biosynthetic Network. The pattern of carotene distribution in the mutants of *Phycomyces* is similar to that found in mutants of other organisms, such as *Chlorella, Neurospora,* and tomato (7). There never seems to occur a complete block of any step but some of the steps of the presumed biosynthetic pathway may be strongly throttled and lycopene may replace the cyclized end products. The failure to find complete blocks may in some cases be due to the fact that complete blocks at an early stage of the biosynthetic pathway are lethal. For some of the later stages the failure to find a complete block may be due to the fact that the successive steps of dehydrogenation or of cyclization are so similar that
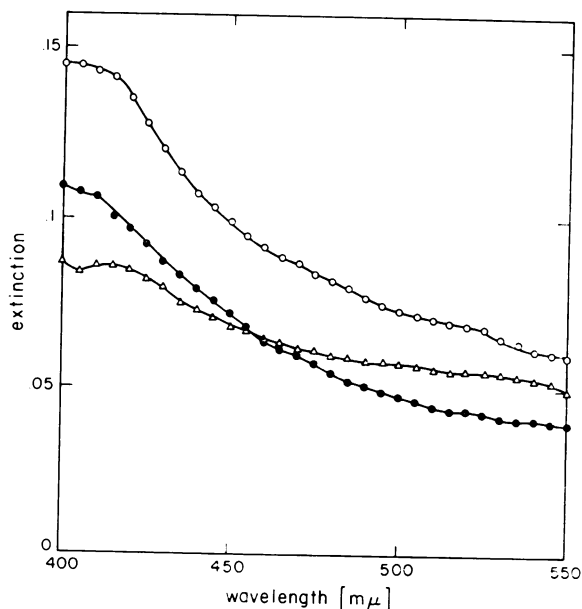

FIG. 1. *In vivo* absorption spectra through the growing zone of 3 different spphs of albino 10.

even though the enzyme specific for a particular step may be completely blocked an enzyme for a similar step may substitute for it, though poorly. Figure 2 shows the probable biosynthetic pathway of the carotenes (6, 10). Steps 1, 2, 3. and 5 symbolize dehydrogenations, steps 4 and 6 β-ionone cyclizations.

In the discussion of this biosynthetic network it should be borne in mind that the section we are concerned with, starting with the first C_{40} compound, phytoene, consists of water insoluble substrates. All of these reactions therefore involve enzymes that are presumably membrane bound.
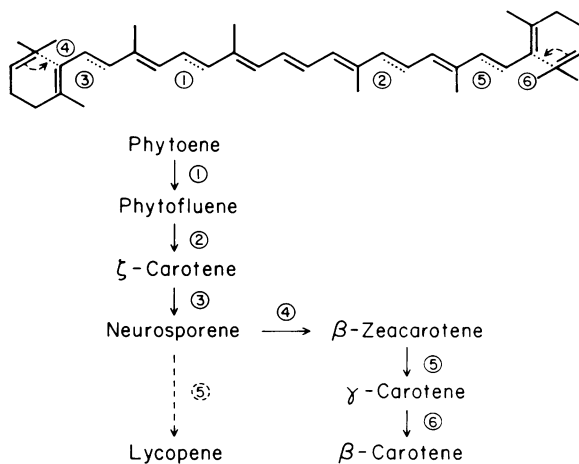

Phytoene
$\downarrow$ ①
Phytofluene
$\downarrow$ ②
ζ-Carotene
$\downarrow$ ③   ④
Neurosporene $\longrightarrow$ β-Zeacarotene
| $\downarrow$ ⑤
| ⑤ γ-Carotene
| $\downarrow$ ⑥
Lycopene β-Carotene

FIG. 2. The conversion of phytoene into β-carotene and lycopene.

Mutants of Type I seem to have normal enzyme complexes for the steps after phytoene. However, albino 5 runs through this set with less material.

In Type II we note that the supply of phytoene is certainly normal, but the subsequent steps are throttled. This is likely due to a failure of the dehydrogenation enzymes.

In Type III the principal defect lies in the cyclization steps. One of the mutants (albino 12) has suffered in addition a defect resulting in an overall diminution of carotene content, similar to that of albino 5.

It is not clear at this point whether the changes of carotene contents seen in R1 and albino 12 are due to regulating effects indirectly produced by the failure of cyclization or whether they are due to separate mutations at different loci. Experiments to clarify this point, involving heterokaryons and sexual crosses, are in progress.

The Receptor Pigment. We pointed out in the Results section that retinal can be ruled out with a high degree of confidence as being part of the receptor pigment. We now wish to discuss whether any other of the carotenes and especially whether β-carotene may be involved. It is obvious of course that the bulk of the β-carotene is not involved since a great reduction in the β-carotene content accom-

plished either physiologically by the addition of DPA or genetically by mutation does not affect the sensitivity to light. However this does not settle the question at issue. On the contrary the very fact that albino 10, which is fully photosensitive, does not show any receptor pigment with an OD through the spph as great as 0.003 proves that the receptor pigment is present at a much lower concentration than that of β-carotene in wild type.

Let us see whether we can estimate a lower limit for the receptor pigment and then decide whether or not the minimal β-carotene contents found in fully sensitive strains lie above or below this limit. We will estimate this lower limit on the basis of the finding that the phototropic response occurs down to a quantum flux of about 10^6 quanta/cm² sec (14). This tropic response occurs over a period of about 10^3 seconds giving a total stimulus of about 10^9 quanta/cm², and with this stimulus the organism must evaluate the light flux to be asymmetric with respect to azimuth. We know that this evaluation involves the lens properties of the growing zone and that the convergence of light effected by the cylindrical lens favors the distal side over the proximal side by a factor of about 1.3 (4). How many absorbing pigment molecules per growing zone do we then need to establish this difference between distal and proximal side above the noise level of the quantum fluctuations?

To estimate the number of receptor pigment molecules needed we first note that molecular extinction coefficients (ϵ) can be converted to capture cross sections (q) by means of the relation

$$q = 3.8 \times 10^{-21} \epsilon \text{ cm}^2.$$

If we estimate that the receptor pigment has an extinction coefficient similar to that of rhodopsin (4×10^4) we obtain for the capture cross section

$$q = 1.5 \times 10^{-16} \text{ cm}^2.$$

With a total incident stimulus of 10^9 quanta/cm² we obtain 1.5×10^{-7} absorptions per receptor pigment. To obtain a reliable difference in the number of absorptions between the proximal and distal side we need about 3×10^9 pigment molecules per growing zone, yielding 200 absorptions on the proximal side and 250 on the distal side.

How does this number compare with the minimal amounts of β-carotene found? Here we first have to consider that β-carotene has a very high extinction coefficient (1.4×10^5) and that therefore 10^9 molecules per growing zone are already sufficient. In table I we report a value of 10^{-7} g β-carotene/g dry weight spph. One spph weighs about 10^{-4} g. Thus we have about 10^{-11} g/spph $= 2 \times 10^{-14}$ mole/spph $= 1.2 \times 10^{10}$ molecules β-carotene/spph. If all of these are concentrated in the growing zone we still have 10 times more than the lower limit estimated on the basis of the physiological threshold. Thus our argument falls short of ruling out definitely a special form of β-carotene as part of the receptor pigment. If we could show that the receptor pigment is spread uniformly through the spph, our

argument would definitely exclude β-carotene. We note, further, that our estimate of the lower limit of the number of pigment molecules in the growing zone implies an OD due to this pigment of about 10^{-4}, about a factor 30 below our present instrumental limit.

Acknowledgments

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