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Biosynthesis of Phytoene from Isopentenyl Pyrophosphate by a *Neurospora* Enzyme System

SANDRA L. SPURGEON,[1,2] RICHARD V. TURNER, AND ROY W. HARDING[3]

Radiation Biology Laboratory, Smithsonian Institution, Rockville, Maryland 20852

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An enzyme system catalyzing the conversion of isopentenyl pyrophosphate to phytoene has been isolated from *Neurospora crassa* mycelia. This enzyme system shows an absolute requirement for Mg^{2+}, but no other cofactors. Cultures of *N. crassa* exhibit a low level of phytoene synthesizing activity when grown in the dark. A 2-min *in vivo* blue light irradiation results in a ninefold increase in activity after 24 h. This increase is dependent on the duration of the light treatment and is inhibited by cycloheximide. A similar blue light-induced elevation of phytoene synthesizing activity was demonstrated in an *albino-1* mutant. This enzyme activity was not found in either dark-grown or irradiated cultures of an *albino-2* or an *albino-3* mutant.

Cell-free systems capable of synthesizing phytoene have been obtained from both higher plants and microorganisms (1). For example, in higher plants, incorporation of radioactivity from isopentenyl pyrophosphate into phytoene was shown using extracts from tomato plastids (2) and spinach leaves (3). In the fungus *Phycomyces blakesleeanus*, a cell-free system which can synthesize phytoene from mevalonic acid has been demonstrated (4). Cell-free synthesis of phytoene has also been shown in bacteria (5, 6). Recently the enzyme system obtained from tomato plastids has been partially purified, and a number of its properties have been examined (7, 8).

In *Neurospora crassa*, incorporation of radioactivity from mevalonic acid into triterpenes and phytoene by whole homogenates of an albino mutant has been reported, but the amount of label incorporated into phytoene was low (9). In the present investigation we report, for the first time, the isolation of an enzyme system from *N. crassa* which catalyzes the formation of phytoene from isopentenyl pyrophosphate. In addition, we have examined the photoregulation of this phytoene synthesizing activity in a wild-type strain and in several albino mutants.

N. crassa is one of a number of microorganisms in which carotenoid biosynthesis is induced by light (10, 11). In *N. crassa* it has been shown that phytoene accumulates in the dark (12, 13), whereas biosynthesis of the carotenoid pigments is dependent on exposure to blue light (14, 15). These observations led to the hypothesis that the enzymes which catalyze the biosynthesis of phytoene in *N. crassa* are not photoregulated. However, it was later shown in an albino mutant that phytoene levels were increased by an *in vivo* light treatment (16). In the present work, it is shown that although the enzyme activity which catalyzes the conversion of isopentenyl pyrophosphate to phytoene is present in dark-grown cells, the activity is much higher after exposure to blue light.

MATERIALS AND METHODS

Materials. The *albino-1* (RES-6) mt A strain was obtained from Dr. R. E. Subden. All other strains were obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, California 95521, U.S.A. These included the Em 5297 wild-

[1] Present address: Lipid Metabolism Laboratory, Veterans Administration Hospital, Madison, Wisconsin 53706.

[2] Supported by a postdoctoral fellowship from the Office of Academic Studies, Smithsonian Institution.

[3] To whom requests for reprints should be addressed.

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type (FGSC 352), *albino-2* (FGSC 904), and *albino-3* (FGSC 2082). Other materials were obtained as follows:[4] $NADP^+$, FAD, and dithiothreitol from Sigma Chemical Co.; Tween 80 from J. T. Baker Chemical Co.; [1-^{14}C]isopentenyl pyrophosphate from Amersham Corp.; carrot oil from Nutritional Research Associates, Inc. Alumina, from E. M. Laboratories, Inc., was heated at 115°C overnight prior to deactivation with 1% (v/w) water. Hexane was passed through silica gel, grade 12 (W. R. Grace and Co.) before use.

Culture conditions and irradiation procedures. The strains were grown in 20-ml aliquots of Vogel's medium N (17) supplemented with 2% (w/v) sucrose and 0.8% (v/v) Tween 80 in standing cultures in the dark for 6 days at 18°C. All subsequent operations involving mycelial pads, lyophilized powders, and enzyme extracts were carried out under red safelight (60-W, natural dark ruby, incandescent lamps) unless otherwise indicated. Excess medium was removed from the mycelial pads by suction on a Büchner funnel, and each pad was transferred to a 9-cm-diameter petri dish. Vogel's medium N supplemented with 2% (w/v) sucrose, 0.8% (v/v) Tween 80, and 0.5% (w/v) sorbose was added (8 ml per pad). The pads were then incubated for 2 h in the dark at 6°C unless otherwise indicated. The mycelial pads were irradiated for the indicated times with blue light using a bank of 16 daylight fluorescent lamps and a celluloid filter which transmits between 400 and 510 nm with a maximum at 450 nm. The irradiance used was 48 $\mu W \cdot cm^{-2}$. Following irradiation, the pads were incubated at 6°C unless otherwise indicated.

Preparation of enzyme systems. At the end of the incubations, the pads were washed with ice-cold deionized water, pressed between paper towels, frozen with dry ice, lyophilized to dryness, and ground to a fine powder with a Wiley Mill. The lyophilized powder was stored at −80°C. The powder was extracted with 0.1 M potassium phosphate buffer (pH 7.5, 10 ml/g of powder) containing 1 mM dithiothreitol. The extract was centrifuged at 100,000*g* for 1 h, the lipid layer at the top of the supernatant was discarded, and the rest of the supernatant was saved, including a turbid layer at the bottom of the supernatant. The 100,000*g* pellet was extracted, and a second 100,000*g* supernatant was collected as already described. The two 100,000*g* supernatants were combined and stored at −80°C under nitrogen.

Incubation system. The incubation mixture (in a total volume of 0.7 ml) contained potassium phosphate buffer (pH 7.5), 35 μmol; Tween 80, 1.4 mg; $NADP^+$, 1.3 μmol; FAD, 0.7 nmol; dithiothreitol, 14.0 μmol; $MgCl_2$, 10.5 μmol; [1-^{14}C]isopentenyl pyrophosphate, 7.7 nmol (1×10^6 dpm), and enzyme protein. At least two protein concentrations were used to assay each extract in order to show a linear dependence of phytoene biosynthesis on the amount of added enzyme. Incubations were carried out for 45 min at 25°C under nitrogen in the dark. Protein concentrations were determined by the method of Lowry *et al.* (18) using bovine serum albumin as a standard.

Assay for [^{14}C]phytoene. Incubations were stopped by the addition of four volumes of ethanol, and the mixture was extracted three times with hexane. The hexane extract was washed with deionized water and dried over sodium sulfate, and phytoene (15-*cis* isomer) prepared from carrot oil (19) was added. The extract was chromatographed on a 0.9 × 24-cm column of 1% (v/w) water-deactivated alumina using increasing concentrations of peroxide-free diethyl ether in hexane. Phytoene was detected by determining the ultraviolet absorption spectrum of each fraction. Each fraction was assayed for radioactivity in a liquid scintillation spectrometer.

RESULTS

Enzymatic conversion of isopentenyl pyrophosphate to phytoene. The biosynthesis of phytoene from isopentenyl pyrophosphate by *Neurospora* extracts has been demonstrated. The amount of phytoene produced was determined by extraction of incubation mixtures with hexane and chromatography of the hexane extracts on 1% (v/w) water-deactivated alumina. This chromatography separated the ^{14}C-labeled phytoene from a faster moving peak of label which was shown by thin-layer chromatography and gas–liquid chromatography to be squalene. The phytoene peak was identified by the coincidence of radioactivity with the light absorbance due to authentic phytoene (15-*cis* isomer) added to the hexane extracts. Thus the phytoene synthesized by the *Neurospora* extracts is the 15-*cis* isomer as expected (20). To demonstrate the purity of the [^{14}C]phytoene obtained, aliquots recovered from the columm were chromatographed on silica gel thin-layer plates. A coincidence of radioactivity and authentic phytoene was observed. In addition, an aliquot of [^{14}C]phytoene obtained by alumina chromatography was hydrogenated and chromatographed on alumina. Over 95% of the radioactivity originally present in the [^{14}C]phytoene was recovered in the hydrogenated product. The [^{14}C]perhydrophytoene was then subjected to gas–liq-

[4] Reference to brand or firm name does not constitute endorsement by the Smithsonian Institution over others of a similar nature not mentioned.

uid chromatography. Ninety-four percent of the label applied was eluted in a single peak which was coincident with the mass of authentic perhydrophytoene.

Cofactor requirements. Phytoene synthesizing activity shows an absolute requirement only for Mg^{2+} (Table I). Omission of $NADP^+$ from the incubation mixture results in nearly a complete loss of squalene synthesis with only a slight increase in the amount of phytoene synthesized. Presumably $NADP^+$ is converted to NADPH by the crude extracts, since inclusion of NADPH in the incubation mixture (1.0 μmol per incubate) in place of $NADP^+$ stimulates squalene biosynthesis. Squalene biosynthesis would be expected to require NADPH in order to convert presqualene pyrophosphate to squalene (21).

Requirement for Mg^{2+}. Phytoene biosynthesis was found to be optimal with Mg^{2+} concentrations above 10 mM (Fig. 1). Mn^{2+} can replace Mg^{2+} only at very low concentrations and is inhibitory at concentrations above 1 mM. A similar inhibitory effect of Mn^{2+} is observed in the presence of Mg^{2+},

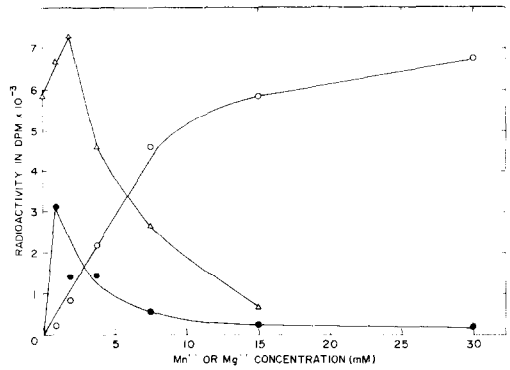

FIG. 1. Incorporation of radioactivity into phytoene as a function of Mg^{2+} or Mn^{2+} concentration. All components of the incubation mixtures were as described under Materials and Methods except for Mg^{2+} and Mn^{2+} concentrations, which were varied. The protein concentration was 0.3 mg/incubate, and all incubations were for 45 min at 25°C. The protein extracts were obtained from cultures which were grown in the dark for 6 days at 18°C, equilibrated for 2 h at 6°C, irradiated for 2 min with blue light, and incubated for 24 h at 6°C. Symbols used: variable Mn^{2+} concentration with no Mg^{2+} present, ●; variable Mg^{2+} concentration with no Mn^{2+} present, ○; variable Mn^{2+} concentration with the Mg^{2+} concentration held at 15 mM, △.

TABLE I

COFACTOR REQUIREMENTS FOR PHYTOENE BIOSYNTHESIS *IN VITRO*

Incubation system	Incorporation of label into phytoene (dpm)
Complete[a]	6000
−$NADP^+$	6700
−Tween 80	5900
−FAD	5800
−Dithiothreitol[b]	5700
−$MgCl_2$	0

[a] The complete incubation system contained 35 μmol potassium phosphate buffer (pH 7.5), 1.4 mg Tween 80, 1.3 μmol $NADP^+$, 0.7 nmol FAD, 14.0 μmol dithiothreitol, 10.5 μmol $MgCl_2$ and 7.7 nmol [1-^{14}C]isopentenyl pyrophosphate (1 × 10^6 dpm). The protein concentration in all cases was 0.3 mg per incubate. All incubations were for 45 min at 25°C. The protein extract was obtained from cultures which had been grown in the dark for 6 days at 18°C, equilibrated 2 h at 6°C, irradiated 2 min with blue light, and incubated 24 h at 6°C.

[b] The incubation mixture still contains 0.4 μmol of dithiothreitol, present originally in the buffer and protein solutions added.

although the levels of phytoene synthesized are higher than with Mn^{2+} alone.

Effect of an in vivo light treatment on the level of phytoene synthesizing activity. The level of phytoene synthesizing activity in *Neurospora* extracts increases about ninefold 24 h after an *in vivo* light treatment (Fig. 2). Most of this increase is seen in the first 12 h after irradiation, and the increase in phytoene synthesizing activity is linear with time up to about 8 h. Mixing experiments with extracts from light-treated and dark-grown cultures were carried out. No evidence was found for the presence of an inhibitor in extracts from dark-grown cultures or an activator in extracts from light-treated cultures.

Inhibition by cycloheximide. Addition of cycloheximide to mycelial pads was shown to inhibit the light-induced increase in enzyme activity, and the extent of inhibition is dependent on the time of addition after the light treatment (Fig. 3). The increase in phytoene synthesizing activity was completely blocked by cycloheximide when added up to 2 h after irradiation. Addition

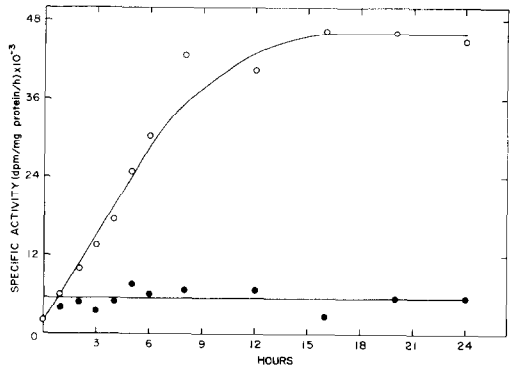

FIG. 2. Effect of an *in vivo* light treatment on the level of phytoene synthesizing activity in wild-type *N. crassa*. All cultures were grown in the dark for 6 days at 18°C and equilibrated for 2 h at 6°C. At zero time, one-half of the cultures were exposed to 2 min of blue light and then incubated in the dark at 6°C (○), while the other half remained at 6°C (●). Light- and dark-treated mycelial pads were harvested at the times indicated, and extracts were prepared and assayed for phytoene synthesizing activity. The protein concentrations used were in the range 0.2 to 1.0 mg per incubate.

of cycloheximide at longer times after the light treatment showed a gradually decreasing effectiveness, and after 8 h the light-induced increase in phytoene synthesizing activity was no longer sensitive to cycloheximide inhibition.

The possibility that the inhibitory effect of cycloheximide is due to direct inhibition of enzyme activity can be ruled out since addition of cycloheximide to incubation mixtures does not inhibit the incorporation of [1-^{14}C]isopentenyl pyrophosphate into phytoene.

Effect of temperature on the light-induced increase in enzyme activity. The increase in enzyme activity following the light treatment is temperature sensitive. In Fig. 4 data obtained using mycelial pads incubated at 25°C following irradiation are shown. For comparison the data obtained for incubation at 6°C are also included. At 25°C the increase in phytoene synthesizing activity reaches a maximum within 1 h, whereas at 6°C the increase occurs over a 12-h period. In addition, the final level of activity reached is very much reduced at 25°C showing only a threefold increase compared to the ninefold increase observed at 6°C.

Albino mutants. Incorporation of [1-^{14}C]-isopentenyl pyrophosphate into phytoene could not be detected using extracts obtained from either dark-grown or light-treated cultures of the *albino-2* and *albino-3* mutants. By using a constant irradiance, the effect of irradiation time on the increase in phytoene synthesizing activity in an *albino-1* mutant and wild-type was examined (Fig. 5). It was found that both strains show a similar dependence on irradiation time for the photoinduced increase in enzyme activity.

DISCUSSION

In the present paper, we report the isolation of an enzyme system from *N. crassa* which catalyzes the conversion of isopentenyl pyrophosphate to phytoene. This enzyme system shows an absolute requirement for a divalent metal ion, but not for other cofactors.

Regulation of carotene biosynthesis in *N. crassa* has been the subject of numerous reports in the literature. Early studies showed the presence of phytoene in mycelia grown in the dark, but only trace amounts of

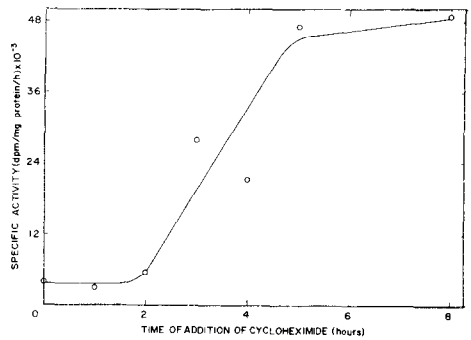

FIG. 3. Inhibition of photoinduced phytoene synthesizing activity as a function of the number of hours after irradiation before addition of cycloheximide. Cultures were grown in the dark for 6 days at 18°C and equilibrated for 2 h at 6°C. At zero time, the mycelial pads were given 2 min of blue light and placed at 6°C. At each time indicated in the figure, 20 pads were transferred to medium containing cycloheximide (12.3 μM) and kept at 6°C. In all cases, 24 h after the light treatment the pads were harvested, and extracts were prepared and assayed for phytoene synthesizing activity. The protein concentrations used were in the range of 0.3 to 0.7 mg per incubate.

other carotenoids (12, 13, 22). A short light treatment was found to greatly stimulate production of carotenoid pigments. On the basis of this *in vivo* evidence, it was thought that enzymes required for phytoene biosynthesis in *N. crassa* were not photoregulated. Development of a cell-free assay for the phytoene synthesizing system from *N. crassa* has made careful analysis of this question possible. The data presented here show that while low levels of phytoene synthesizing activity are present in dark-grown mycelial pads, a 2-min blue light treatment results in a ninefold elevation in this enzyme activity after 15 to 24 h. *Mycobacterium sp.* is another organism in which the effect of an *in vivo* light treatment on phytoene biosynthesis has been studied using cell-free extracts (6, 23). In that organism it was shown that the enzyme activity which catalyzes the conversion of geranylgeranyl pyrophosphate to prephytoene pyrophosphate is totally absent in dark-grown cultures and is photoinduced. These results account for the earlier observation that little or no phytoene is produced by dark-grown *Mycobacterium*

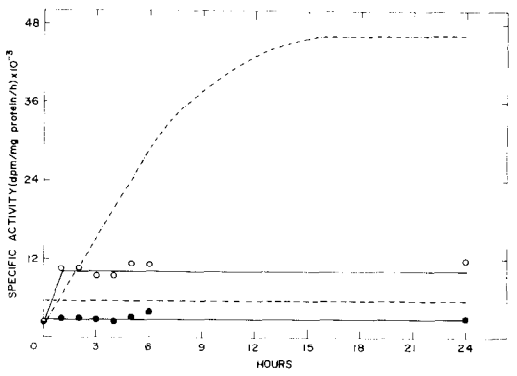

FIG. 4. Effect of temperature on the light-induced increase in phytoene synthesizing activity. Cultures were grown in the dark for 6 days at 18°C and equilibrated at 25°C for 2 h. At zero time, one-half of the cultures were irradiated for 2 min with blue light and then incubated in the dark at 25°C (○), while the other half remained at 25°C (●). Light- and dark-treated mycelial pads were harvested at the times indicated, and extracts were prepared and assayed for phytoene synthesizing activity. The protein concentrations used were in the range 0.2 to 0.6 mg per incubate. Data obtained for pads incubated at 6°C (Fig. 2) are shown for comparison as follows: 0–24 h dark, 6°C (- - -); 2 min light, 0–24 h dark, 6°C (-·-·-).

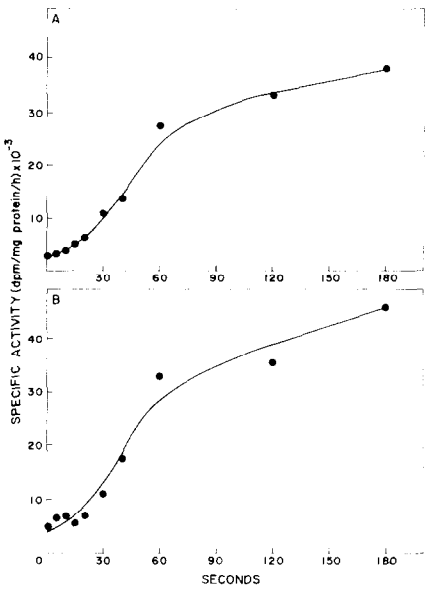

FIG. 5. Effect of irradiation time on photoinduction of phytoene synthesizing activity. Cultures were grown in the dark for 6 days at 18°C, equilibrated for 2 h at 6°C, and irradiated with blue light for the times indicated. The mycelial pads were then incubated in the dark at 6°C for 24 h, and extracts were prepared and assayed for phytoene synthesizing activity. The protein concentrations used were in the range 0.2 to 0.6 mg per incubate. A: wild-type; B: *albino-1*.

sp. cultures (24). *N. crassa* differs from *Mycobacterium sp.* in that it can synthesize phytoene in the dark. However, in both organisms, phytoene synthesizing activity is increased by an *in vivo* light treatment. It should be noted that *N. crassa* produces predominantly the 15-*cis* isomer of phytoene (20), whereas *Mycobacterium sp.* predominantly synthesizes the all-*trans* isomer (6).

Experiments carried out using cycloheximide indicate that protein synthesis is required for photoinduction of phytoene synthesizing activity in *N. crassa*. Addition of cycloheximide up to 2 h after irradiation completely blocks the increase in activity. The inhibitory effect is reduced if addition of the cycloheximide is delayed. Similar results were observed when the inhibition of carotenoid biosynthesis by cycloheximide was investigated (25).

In the experiments discussed so far, the light treatments and subsequent dark incubations were all carried out at 6°C. A pre-

vious study has shown that following irradiation carotene biosynthesis in *N. crassa* was optimal at this temperature (25). To further investigate this temperature effect, the light-induced elevation of phytoene synthesizing activity at 25°C was determined. It was found that at 25°C the photoinduced increase in this activity is much less than at 6°C. There are several hypotheses which could account for this temperature effect. Perhaps a photoproduct required for induction of protein synthesis is less stable at the higher temperature, or possibly one or more of the photoinduced enzymes are temperature labile. Although 6°C is below the optimum temperature for protein synthesis, a significant amount does occur at low temperatures in *Neurospora* (26), and in fact several enzymes are induced in this organism by lowering the temperature of the cultures to 6°C (27). As shown in the present study, lowering the temperature to 6°C is not sufficient to induce phytoene synthesizing activity. Light is also required.

All of the studies discussed so far have been with the wild-type strain. A preliminary study of several albino mutants was also undertaken in order to gain additional information about the photoregulation of phytoene biosynthesis in *Neurospora*. As shown in Fig. 5, photoinduction of phytoene synthesizing activity can also be observed in an *albino-1* mutant. Several similarities between this mutant and the wild-type can be observed. In the dark, the wild-type (12, 13) and the mutant (16) accumulate phytoene, and as shown in the present study, both strains have a low level of phytoene synthesizing activity in the dark. In addition, the photoinduced increase in activity is nearly identical in these two strains. Since the *albino-1* mutant produces little or no carotenoid pigment (28), it would seem unlikely that a carotenoid is the photoreceptor for the blue light-induced increase in phytoene synthesizing activity. *Albino-2* and *albino-3* mutants do not produce phytoene *in vivo* (28), and as expected no phytoene synthesizing activity was found in the *albino-2* and *albino-3* strains tested.

The enzyme fraction used in these experiments consists of two phases which are only partially separated by the ultracentrifuge. In addition to the clear supernatant, there is a turbid layer which sediments but does not pellet. Further studies have shown that this turbid fraction is necessary for synthesis of phytoene and squalene. Extracts which did not contain this fraction synthesized primarily acid labile compounds. This turbid fraction may consist of membrane fragments and associated enzymes. Recently in a preliminary report, evidence was presented that in *Neurospora* conversion of geranylgeranyl pyrophosphate to phytoene requires a membrane-bound enzyme activity (29). Squalene synthetase, the enzyme required for the conversion of farnesyl pyrophosphate to squalene, is probably also membrane bound in *Neurospora*, since this is the case for numerous other organisms (30, 31). Our results support the idea that one or more of the enzymes in both the phytoene and squalene biosynthetic pathways of *Neurospora* are membrane bound.

It is not known at present how many enzymes are involved in the conversion of isopentenyl pyrophosphate to phytoene in *Neurospora*. It will be necessary to isolate and investigate each of these enzymes in order to determine which ones are regulated by light.

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