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The Effect of Temperature on Photo-induced Carotenoid Biosynthesis in *Neurospora crassa*

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ABSTRACT

The temperature dependence of carotenoid synthesis in *Neurospora crassa* was investigated. The primary light reaction is independent of temperature, but the amount of carotenoid pigment which subsequently accumulates in the dark is strongly dependent on the temperature during the dark incubation. Carotenoid synthesis shows a sensitivity to both high and low temperatures, and of the temperatures tested, 6 C is optimal. Exposure to temperatures above 6 C for various times immediately following irradiation brings about a temperature-dependent reduction in the amount of carotenoid pigment that is synthesized in a total dark incubation time of 24 hours. This sensitivity to incubations at temperatures above 6 C is reduced by either continuous irradiation during the entire time at the higher temperature or by a short irradiation at the end of this period, and the relative effectiveness of these two types of light treatments is presented. Carotenoid production is also sensitive to amino acid analogues and inhibitors of protein synthesis during a critical period after irradiation.

It is proposed that the light reaction leads to the production of a compound which can be degraded in a temperature-dependent competitive reaction. This compound (or a product derived from it) can also induce the *de novo* synthesis of an enzyme (or enzymes) required for carotenoid production. An alternative hypothesis, that a repressor is directly inactivated in the light reaction, can be ruled out by the results presented.

In *Neurospora crassa* (5, 23–26), *Fusarium aquaeductuum* (3, 11, 14, 16, 21), and an unclassified species of Mycobacterium known as *Mycobacterium sp.* (2, 7, 18–20), there is little or no pigment production unless the cultures are first exposed to light. The visible action spectra for initiation of carotenoid production by light in these organisms are similar as far as has been determined, and in each case a typical blue light response is observed (2, 7, 12, 19, 24).

It has previously been proposed that there are at least three phases of carotenoid production in *N. crassa* (6, 15): (*a*) a light reaction; (*b*) a period of protein synthesis; and (*c*) an accumulation of the carotenoid pigments. The proposal that protein synthesis is required in order for carotenoid accumulation to occur is based on studies with cycloheximide (6, 15) which has been shown to be an inhibitor of protein synthesis in *N. crassa* (9).

In *N. crassa*, carotenoid synthesis has been reported to occur at 4 C (5, 6), and studies of the pathway of biosynthesis of the carotenoid pigments in this organism were previously carried out using this temperature. In the present study, the temperature dependence of carotenoid production in *N. crassa* is investigated in detail, and a model for the photoinduction of carotenoid synthesis is proposed.

MATERIALS AND METHODS

Culture Conditions and Irradiation Procedure. Wild type (EM 5297a) *Neurospora crassa* was grown in 20 ml of Vogel's minimal medium (22) in standing cultures, for 6 days in the dark at 18 C. The medium was supplemented with 0.8% Tween 80 (polyoxyethylene sorbitan mono-oleate) to prevent conidiation. Under a red safelight (60-w, natural dark ruby, incandescent lamp), the excess medium was removed from the pads by suction on a Büchner funnel, and the pads were placed into 15-cm diameter Petri dishes, three pads per dish.

Medium (8 ml/pad) was then added to the mycelial pads, and they were incubated in the dark for 2 hr at the temperature which would be used during the subsequent light treatment. The medium added to the pads was Vogel's minimal medium supplemented with 0.8% Tween 80 and 0.5% sorbose. The Tween 80 and sorbose are both used to inhibit conidiation around the edges of mycelial pads that are incubated at 25 and 37 C for more than a few hours. However, the temperature effects which will be presented can still be observed when Tween 80 and sorbose are omitted from the medium added to the pads.

At the end of the 2-hr incubation in the dark, the mycelial pads were irradiated for the indicated times with blue light using a bank of 16 daylight fluorescent lamps and a filter which transmits between 400 and 510 nm with a maximum at 450 nm. The irradiance used was 54 $\mu w/cm^2$.

Time Course and Variation of Irradiation Time Procedures. In time course investigations, the mycelial pads were prepared and irradiated for 2 min at a particular temperature and then incubated in the dark at the same temperature. The accumulation of carotenoid pigment was measured as a function of time.

In experiments in which the irradiation time was varied, a total incubation time of 24 hr was used. This 24-hr incubation was divided into two portions—an irradiation period which was varied in length, followed by a dark period. The temperature during the entire 24-hr incubation was held constant.

Dependence of Carotenoid Synthesis on the Temperature during Irradiation. In these studies mycelial pads were irradiated for 2 min at different temperatures. All the pads were then rapidly cooled to 6 C and incubated in the dark at 6 C for 24 hr. In order to rapidly lower the temperature of the mycelial pads to 6 C following an irradiation, the pads were submerged in distilled water already chilled to 6 C, filtered with suction on a Büchner funnel, placed in chilled Petri dishes, and fresh medium (8 ml/pad) was added. The medium was Vogel's minimal supplemented with Tween 80 and sorbose as already described. The same results were obtained in experiments in which the pads were rapidly cooled to 6 C by submerging them

in Vogel's minimal medium supplemented either with Tween 80 or Tween 80 plus sorbose instead of using distilled water as described above.

Dependence of Carotenoid Synthesis on the Temperature after Irradiation. The pads were irradiated for 2 min at a particular temperature and kept at the same temperature in the dark for various lengths of time before being rapidly cooled to 6 C. The pads were then incubated further in the dark at 6 C. In all cases, the total time in the dark after the 2-min irradiation (time at higher temperature plus time at 6 C) was 24 hr.

These experiments were extended using the following treatment procedures: (*a*) as described in the previous paragraph, mycelial pads were irradiated for 2 min at a particular temperature and then incubated for various lengths of time at that temperature before they were rapidly cooled to 6 C (controls); (*b*) mycelial pads were irradiated at a particular temperature for 2 min, incubated at the same temperature for various times, irradiated for 2 min a second time, and then cooled to 6 C; (*c*) mycelial pads were irradiated continuously for various times at a particular temperature and rapidly cooled to 6 C. The total incubation time (time at the higher temperature plus time at 6 C) was 24 hr in all three treatment procedures.

Addition of Cycloheximide at Various Times after Irradiation. Mycelial pads were prepared and irradiated for 2 min at 6 C, and the pads were incubated in the dark at 6 C. At different times, the pads were refiltered, and Vogel's minimal medium (8 ml/pad), supplemented with 0.8% Tween 80, 0.5% sorbose, and cycloheximide (at a concentration of 12.3 μM), was added. Controls were carried out for each addition time using the same medium without cycloheximide.

Blasticidin and Amino Acid Analogue Studies. Mycelial pads were obtained from cultures grown 4 days at 25 C in the dark, in Vogel's minimal medium supplemented with 0.8% Tween 80. Excess medium was removed from the pads by pressing them between paper towels. The pads were placed back into Petri dishes, and Vogel's minimal medium (2 ml/pad) supplemented with 0.8% Tween 80 was added. The mycelium was incubated for 2 hr in the dark at 25 C, and then irradiated at 25 C for 5 min at an intensity of 200 ft-c with a 15w, cool white fluorescent lamp. The mycelial pads were pressed between paper towels either immediately following the irradiation or at later times, and Vogel's minimal medium (2 ml/pad) containing 0.8% Tween 80 plus the appropriate compounds was added. The mycelium was incubated in the dark at 6 C for a total of 24 hr following the 5-min irradiation treatment.

Extraction and Assay of Carotenoid Pigments. The carotenoids were extracted from the mycelium by pressing the pads between paper towels and extracting twice with 3-ml portions of methanol and then extracting three times with 3-ml portions of acetone. For each treatment, six pads (three per Petri dish) were used, and the extraction was carried out in triplicate using two pads per extraction. The methanol and acetone extracts for a particular extraction were combined, dried by addition of Na_2SO_4, allowed to stand overnight at 6 C, and then filtered with Whatman No. 1 filter paper.

The absorption spectrum in the range 380 to 580 nm of each extract was determined with a Cary 14 recording spectrophotometer. Extracts of controls which were not exposed to light showed very little absorption and no typical carotenoid maxima. Extracts of light-treated cultures had a major maximum at 473 nm and a minor maximum at 500 nm. The absorbance at 473 nm (using a cuvette with a 2-cm path length) in an extract of two mycelial pads was used as a measure of the total amount of carotenoid pigment synthesized.

RESULTS

Dependence of the Rate of Accumulation of Carotenoid Pigment on Temperature. The effect of short irradiation times on the amount of pigment which will subsequently be synthesized in the dark was determined. A 2-min irradiation treatment is more than sufficient to produce the maximum response for irradiations at 6, 12, 18, 25, and 37 C when a subsequent 24-hr dark incubation at 6 C is used.

The time course of biosynthesis of carotenoid pigment in the dark at a particular temperature following a 2-min irradiation at the same temperature is presented in Figure 1. Although there is a long lag at 6 C before carotenoids can be detected, eventually more pigment accumulates at this temperature than at any of the others tested. No carotenoid pigment is detected after a 24 hr incubation in the dark at 37 C or 1 C.

Effect of Long Irradiation Times on Carotenoid Synthesis. A 2-min light treatment provides a saturating irradiation dose as already mentioned; however, the response can be increased further if long irradiation times are used (Fig. 2). For each temperature, the amount of pigment produced is increased as the irradiation portion of the 24-hr incubation is varied from 0 to 24 hr. However, the amount of pigment synthesized at 18, 25, and 37 C is always less than that produced at 6 and 12 C.

Dependence of Carotenoid Synthesis on the Temperature during and following Irradiation. The photoinduction of carotenoid synthesis is independent of the temperature during a short irradiation treatment (Table I).

Carotenoid synthesis is sensitive to temperatures above 6 C after a 2-min irradiation. As shown in Table II, the higher the temperature during the dark incubation, the lower the amount of pigment which accumulates.

The next series of experiments were carried out to determine how rapidly the photoinduction of carotenoid synthesis is inactivated by incubation at temperatures above 6 C immediately after the light treatment. Mycelial pads were irradiated at temperatures above 6 C and kept at the higher temperatures in the dark for various lengths of time before the temperature was reduced to 6 C (Fig. 3). As the time at 37 C is increased, there is a very rapid effect on the amount of carotenoid pigment that the mycelial pads can synthesize during the total 24 hr incubation. At 25 C there is about a 15 min lag before the incubation at

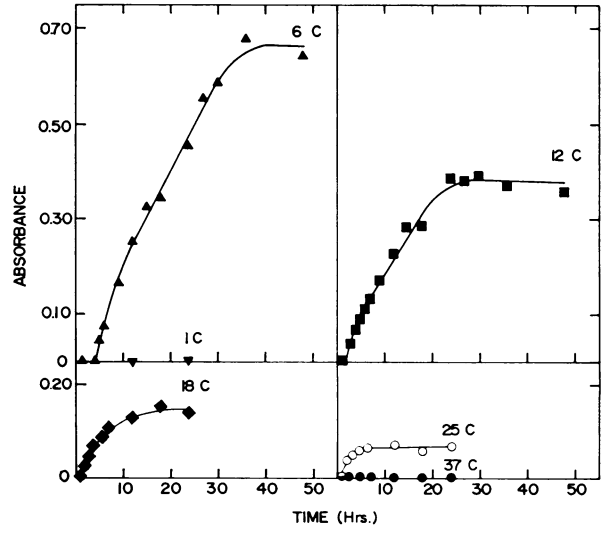

FIG. 1. Time course accumulation of carotenoid pigment (absorbance at 473 nm) at different temperatures in the dark following a 2-min irradiation. The standard deviations of the absorbance values varied from 0.00 to 0.07.

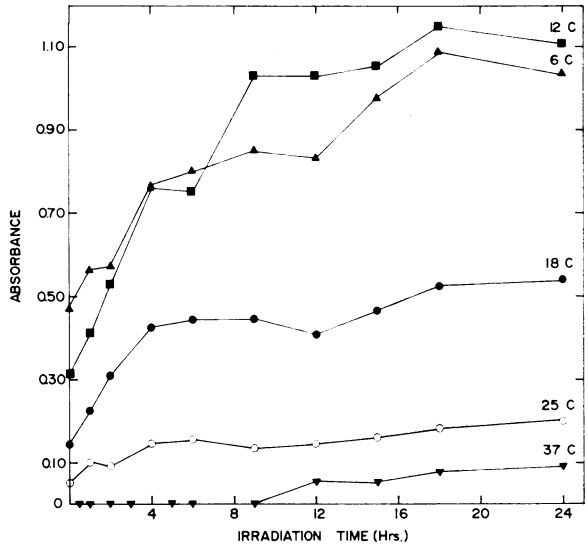

FIG. 2. Effect of varying the irradiation portion of a 24-hr incubation from 0 to 24 hr on the amount of carotenoid pigment (absorbance at 473 nm) which is synthesized. The temperature used throughout the incubation is indicated above each curve. The absorbance values plotted directly on the ordinate were measured in extracts of pads irradiated only 2 min and then incubated 24 hr in the dark. The standard deviations of the absorbance values varied from 0.00 to 0.11.

Table I. *Dependence of Carotenoid Synthesis on the Temperature during Irradiation*

Mycelial pads were irradiated at various temperatures, cooled rapidly to 6 C, and incubated in the dark for 24 hr at 6 C. The standard deviations for the absorbance values are presented.

Temperature during Irradiation	Absorbance
C	*473 nm*
6	0.43 ± 0.04
12	0.47 ± 0.06
18	0.45 ± 0.05
25	0.48 ± 0.06
37	0.43 ± 0.05

Table II. *Dependence of Carotenoid Synthesis on the Temperature after Irradiation*

Mycelial pads were irradiated for 2 min at 6 C and then incubated for 24 hr in the dark at different temperatures. The standard deviations for the absorbance values are presented.

Temperature during Dark Incubation	Absorbance
C	*473 nm*
37	0
25	0.08 ± 0.01
18	0.17 ± 0.01
12	0.34 ± 0.02
6	0.49 ± 0.02

the higher temperature begins to have an adverse effect. The lag at 18 C is about 30 min to 1 hr and at 12 C at least 1 hr. The slopes of the curves decrease as the temperature used is varied from 37 C to 12 C.

The adverse effect of incubations at temperatures above 6 C on carotenoid synthesis should be reduced by increasing the irradiation time. This prediction is based on the results presented in Figure 2, and confirmed by results of experiments presented in Figure 4. The units along the abscissa represent the length of time the mycelial pads were left at the higher

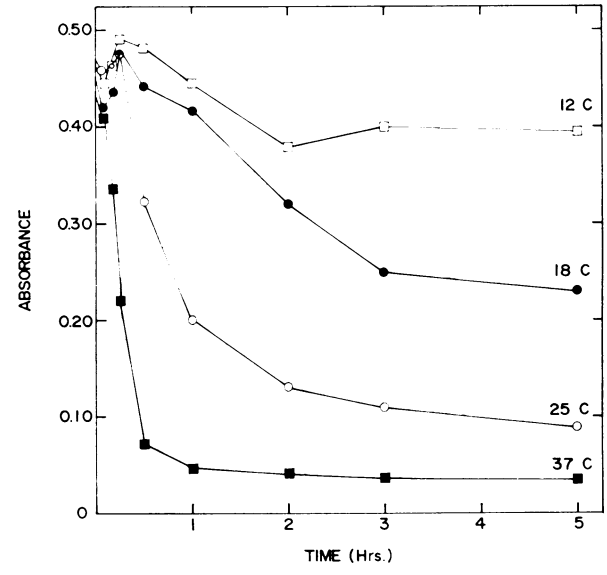

FIG. 3. Effect of temperatures above 6 C following a 2-min irradiation on the amount of pigment synthesized in a 24-hr dark period. The level of pigment produced (absorbance at 473 nm) is plotted *versus* the length of time the pads were at a temperature above 6 C. The time at the higher temperature plus the time at 6 C was 24 hr in all cases. The standard deviations of the absorbance values varied from 0.00 to 0.08.

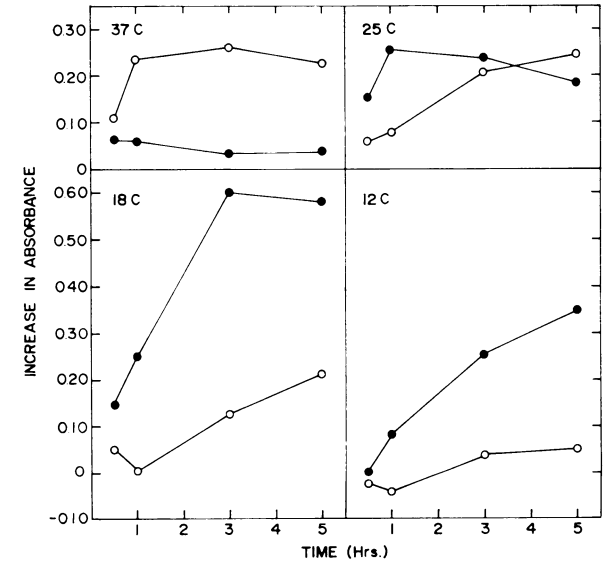

FIG. 4. Reduction by irradiation of the adverse effect of incubations at temperatures above 6 C on carotenoid synthesis. The increase in carotenoid synthesis (absorbance at 473 nm), over that of the controls, induced by either a second 2-min irradiation given at the end of an incubation at a higher temperature (○) or by continuous irradiation during the entire time at the higher temperature (●) is plotted *versus* the length of time at the higher temperature. The higher temperature used (37, 25, 18, or 12) is indicated above each series of curves. The time at the higher temperature plus the time at 6 C was 24 hr in all cases. The standard deviations of the absorbance values used to calculate the increases in absorbance varied from 0.00 to 0.12.

temperature (37, 25, 18, or 12 C) before being cooled to 6 C. The units along the ordinate show the increase in absorbance at 473 nm in carotenoid extracts of the pads induced by one of two types of light treatments. In each case the increase in absorbance of an extract is calculated by subtracting the value for a corresponding control which only had a 2-min irradiation at the beginning of the incubation at the higher temperature.

The second 2-min irradiation (Fig. 4, open symbols), given at the end of the incubation at 37, 25, or 18 C, shows an increase in effectiveness as this incubation time is increased. A limiting increase is reached for 37 and 25 C. A second 2-min irradiation, given at the end of incubations at 12 C, is essentially ineffective. Continuous light (closed symbols) applied during the entire incubation time at 37 C is less effective than the 2-min light treatment applied at the end of these periods at 37 C. However, continuous light is more effective at 25, 18, and 12 C.

A time course of accumulation of carotenoid pigment at 6 C after a second 2-min irradiation, given at the end of an incubation at 37 C, is shown in Figure 5. Carotenoid pigment begins to accumulate after a lag of at least 5 hr at 6 C following the second irradiation treatment. Such a lag is also observed when the pads are given an initial 2-min irradiation and then incubated in the dark at 6 C (Fig. 1).

Inhibition of Carotenoid Synthesis by Antibiotics and Amino Acid Analogues. The proposal that protein synthesis is required following irradiation in order for carotenoid production to take place (6, 15) will be an important part of any model that is presented to take into account the sensitivity of carotenoid synthesis to temperatures above 6 C after the light treatment. For this reason, earlier experiments with cycloheximide were repeated and extended under the same experimental conditions used to obtain the data shown in Figure 1. In addition, blasticidin-S·HCl, which is also an inhibitor of protein synthesis in *Neurospora crassa* (10), and three amino acid analogues were tested to determine whether any of these compounds inhibit carotenoid synthesis.

As shown in an earlier paper (6), cycloheximide was found in the present study to completely block carotenoid production in *Neurospora crassa* only if it is added immediately after

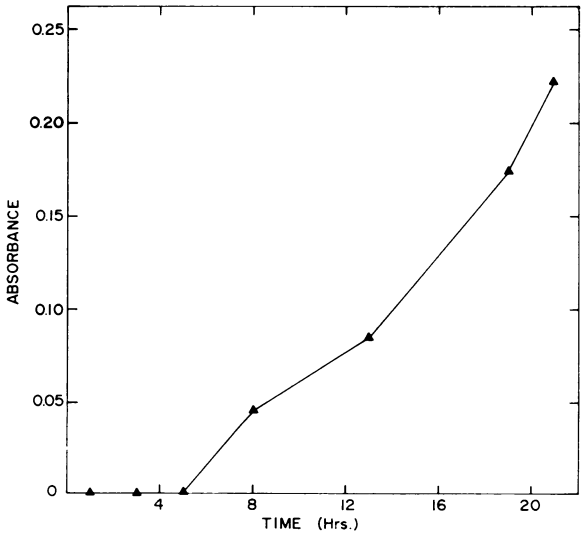

FIG. 5. Time course accumulation of carotenoid pigment (absorbance at 473 nm). The treatment sequence was: 2 min of irradiation at 37 C, 3 hr of incubation in the dark at 37 C, 2 min of irradiation at 37 C, incubation for 0 to 21 hr in the dark at 6 C. The standard deviations of the absorbance values varied from 0.00 to 0.04.

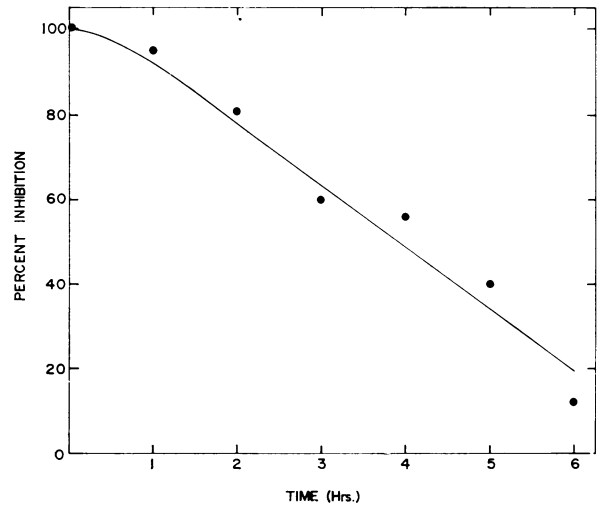

FIG. 6. The percentage of inhibition of carotenoid synthesis *versus* the number of hours after a 2-min irradiation at 6 C before cycloheximide (12.3 μM) was added. The total incubation time in the dark at 6 C after irradiation was 24 hr in all cases. The standard deviations of the absorbance values used to calculate the percent inhibitions varied from 0.00 to 0.07.

irradiation. The inhibitory effect is reduced if addition of cycloheximide is delayed (Fig. 6). The percentage of inhibition of carotenoid synthesis is plotted in Figure 6 *versus* the number of hours after irradiation at which cycloheximide is added. If addition of the inhibitor is delayed for 6 hr, there is very little inhibition of carotenoid synthesis. On the other hand, addition of the inhibitor immediately after irradiation causes a complete inhibition. The increase in carotenoid synthesis (Fig. 5) after a second 2-min irradiation is also completely blocked by the addition of cycloheximide immediately after the second light treatment.

Blasticidin-S·HCl is also a good inhibitor of carotenoid synthesis in *Neurospora*, although it is less effective than cycloheximide. For example, at a concentration of 12.3 μM, cycloheximide added immediately after irradiation inhibits carotenoid synthesis by 100% and blasticidin inhibits synthesis by 77%. At a concentration of 1.23 μM, cycloheximide inhibits 63% and blasticidin inhibits 42%.

Several amino acid analogues inhibit carotenoid synthesis if used at higher concentrations. For example, at a concentration of 12.3 mM, DL-*p*-fluorophenylalanine added immediately after irradiation inhibits carotenoid production by 82%, L-ethionine by 55%, and 4-methyl-DL-tryptophan by 56%. Blasticidin and the three amino acid analogues all show a marked reduction in inhibition if the addition is delayed 3 hr after the light treatment, and there is little or no inhibition if the addition is delayed until 6 hr after irradiation.

Inhibition of carotenoid synthesis by the amino acid analogues can be reduced by the simultaneous addition of L-amino acids (Table III). The reduction is specific in that L-phenylalanine reduces the effect of its analogue, DL-*p*-fluorophenylalanine, L-methionine reduces the inhibition of L-ethionine, and L-tryptophan reduces the effect of 4-methyl-DL-tryptophan. The reduction of the inhibition by 4-methyl-DL-tryptophan by L-tryptophan is complete at the lowest concentration of L-tryptophan used, while the same concentration of the other amino acids causes only a partial reduction of inhibition by each corresponding analogue. Addition of each of the amino acids alone has no effect on the level of carotenoid pigment synthesized.

Table III. *Reduction by L-Amino Acids of Inhibition of Photo-induced Carotenoid Biosynthesis by Amino Acid Analogues*

The inhibitor (12.3 mM) plus the amino acid indicated were added together to the pads just after the light treatment, and the mycelium was incubated for 24 hr at 6 C. Two different concentrations of amino acids were used (treatment A = 1.5 mM and treatment B = 12.3 mM), or no amino acid was added, as indicated (None). The standard deviations for the absorbance values used to calculate the percentage of inhibitions varied from 0.00 to 0.05.

Amino Acid	Inhibition of Carotenoid Synthesis				
	DL-Fluoro-phenylalanine		L-Ethionine		4-Methyl-DL-tryptophan
	A	B	A	B	A
	%				
None	86	86	52	48	53
L-Phenylalanine	38	16	58	47	45
L-Methionine	84	81	30	0	57
L-Tryptophan	78	80	53	46	0

DISCUSSION

Rau (11) has investigated the effect of temperature on carotenoid synthesis in *Fusarium aquaeductuum*. Carotenoid synthesis in the dark following an irradiation at 0 C was found to be strongly temperature-dependent. The higher the temperature used, the more carotenoid pigment was found to accumulate. In addition, the lag in carotenoid production that was observed was decreased as higher temperatures were used. In these studies Rau tested quite a few temperatures from 5 to 30 C.

Zalokar (24) has reported that the production of carotenoid pigment by *N. crassa* is also sensitive to lower temperatures. Zalokar used only temperatures of 25 and 0 C, and he found that the synthesis of the carotenoids drops to zero at 0 C. In the present investigation, results are presented (Fig. 1) which confirm Zalokar's conclusion that carotenoid production is zero at very low temperatures (1 C). However, it is shown that carotenoid synthesis in *N. crassa* also shows a sensitivity to higher temperatures. Of the temperatures tested, 6 C was found to be optimal. This is in sharp contrast to the results mentioned above which were reported by Rau for *Fusarium* (11).

To account for the data obtained in the present investigation, it is proposed that in the dark a compound accumulates which serves as a substrate in a light reaction. The light reaction is independent of temperature, as indicated by the results presented in Table I. It is proposed that the level of the substrate is rapidly depleted by the light reaction, since it is observed that for short irradiation times, a 2-min light treatment provides a saturating irradiation dose. The proposed dark-accumulated substrate may be a photoreceptor which undergoes a direct photoconversion to a photoproduct. An analogous example would be the case in which protochlorophyll(ide) is converted by irradiation into chlorophyll(ide) *a* (17). Another possibility is that a photoreceptor may photosensitize the oxidation of the proposed dark-accumulated substrate of the light reaction. This type of reaction has been studied extensively *in vitro* using various photosensitizing compounds (4).

The results obtained using antibiotics and amino acid analogues can be used to make the proposal that the product of the light reaction (or a compound derived from it) photoinduces the *de novo* synthesis of an enzyme (or enzymes) required for carotenoid production but absent in dark-grown cultures. Both cycloheximide and blasticidin-S·HCl are inhibitors of total protein synthesis in *N. crassa* (9, 10), and one of the analogues, L-ethionine, has been shown to be directly incorporated into total protein in this organism (8). Both antibiotics and all three amino acid analogues inhibit carotenoid synthesis if these compounds are added during a critical interval of time following irradiation.

From the data shown in Table II, it is concluded that carotenoid synthesis is sensitive to temperatures above 6 C during the dark incubation after the light treatment. This is also demonstrated by the results presented in Figure 3. It is proposed that the temperature sensitivity shown there is due to the degradation of the product of the light reaction (or a compound derived from it) in a temperature-dependent reaction. As a result of this competitive reaction, less enzyme synthesis is induced, and consequently, less carotenoid production occurs.

Additional irradiation reduces the adverse effect of the incubation at temperatures above 6 C on carotenoid synthesis (Figs. 4 and 5). It is proposed that the substrate for the light reaction can be resynthesized after it is depleted by an initial saturating irradiation dose. As a result, an additional irradiation treatment given at a later time or continuous irradiation over a long period of time will regenerate the photoproduct which is involved in the proposed competitive reaction. For example, a second irradiation presented at the end of a dark incubation at 37, 25, or 18 C (Fig. 4, open symbols) causes an increase in carotenoid synthesis over that of the controls. Furthermore, the second irradiation shows an increase in effectiveness as the incubation time at each of these temperatures is increased.

At 12 C the second irradiation is ineffective, indicating that little or no substrate for the light reaction is resynthesized at this temperature in the dark. On the other hand, continuous irradiation during the entire incubation at 12 C increases the level of carotenoid synthesis which is induced. This indicates that the substrate for the light reaction is resynthesized faster in continuous light than in the dark.

One explanation for the observation that continuous irradiation at 37 C is not very effective can be stated as follows. At this temperature, the proposed competitive reaction might occur almost as rapidly as the resynthesis of the substrate for the light reaction. In continuous light at 37 C, the labile photoproduct will never reach a very high level. It is more efficient to allow the substrate for the light reaction to reaccumulate in the dark at 37 C, give a second 2-min irradiation, and then immediately reduce the temperature of the mycelium to 6 C.

Based on inhibitor studies with *Mycobacterium sp.*, Rilling (19) has also proposed that a product of the light reaction induces the synthesis of an enzyme required for carotenoid accumulation. An alternative mechanism of photoinduction of carotenoid synthesis in nonphotosynthetic organisms such as *Mycobacterium sp.* and *Neurospora crassa* which must be considered is that the light reaction involves the direct inactivation of a repressor compound (1, 7, 13). Using the results from inhibitor studies alone, it is impossible to distinguish between these two possibilities. However, the data presented in Figure 4 are inconsistent with the idea that direct inactivation of a repressor by a light reaction induces carotenoid synthesis in *N. crassa*. For example, any repressor which reaccumulates during an incubation in the dark at a particular temperature should be subsequently inactivated by a second 2-min irradiation. This second light treatment should not show an increase in effectiveness as the dark incubation time at the higher temperature is increased. In addition, continuous irradia-

tion during an incubation should always be as effective as a short irradiation given at the beginning and end of such an incubation. As already pointed out, this is not the case at 37 C.

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