

Photoreception in *Neurospora crassa*: Correlation of reduced light sensitivity with flavin deficiency

(riboflavin auxotrophs/blue light responses/circadian rhythmicity/carotenoids/fluorometric assay)

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ABSTRACT The effect of flavin deficiency on blue light responses in *Neurospora crassa* was studied through the use of two riboflavin mutants (*rib-1* and *rib-2*). The photoresponses assayed were the suppression of circadian conidiation, the phase shifting of the circadian conidiation rhythm, and the induction of carotenoid synthesis. Flavin deficiency was induced in the *rib-1* mutant by restrictive growth temperatures and in the *rib-2* mutant by low levels of supplemental riboflavin. At 26°C, a semirestrictive growth temperature, the *rib-1* mutant is about 1/80th as sensitive to light for the photosuppression of circadian conidiation. Flavin deficiency in the *rib-1* and *rib-2* strains was effective in reducing the photosensitivity for phase shifting and carotenogenesis to about 1/16th and 1/4th of normal, respectively. Experiments with permissive temperatures, riboflavin supplementation, and revertants at the *rib* locus all indicated that the effects on light sensitivity were due solely to the presence of the *rib* mutations. These results provide evidence that one or more flavin photoreceptors are involved in the blue light responses of *Neurospora*.

Physiological responses to blue light are known in a large number of organisms. Examples include phototropism in coleoptiles of monocots (1) and sporangiophores of fungi (2), induction of carotenoid synthesis in fungi (3), and entrainment of circadian pupal emergence in fruit flies (4). The photoreceptor pigments involved remain unidentified but most of the available evidence supports the hypothesis that a flavin is acting as the photoreceptor for many of these blue light responses. This evidence (reviewed in refs. 5 and 6) includes (i) the similarity between the absorption spectrum of riboflavin and the action spectra of both the blue light responses and light-induced absorbance changes involving a *b*-type cytochrome and (ii) the effects on blue light responses of inhibitors such as phenylacetic acid and potassium iodide which interact with flavins.

Spectrophotometric studies with whole cells and cell fractions suggest that a primary photoreception event for the fungus *Neurospora crassa* is a flavin-mediated reduction of a *b*-type cytochrome (7, 8). Experiments supporting cytochrome *b* involvement have been done with a cytoplasmic mutant, *poky*, which exhibits a low cytochrome *b* content, a decrease in blue light-induced absorbance changes, and a decreased sensitivity to light for the suppression of circadian conidiation (9, 10).

One approach to elucidation of the nature of a photoreceptor is to use a system in which the photoreceptor levels can be perturbed. If the photoreceptor is indeed a flavin, then a decrease in photoreceptor levels should occur in the case of a generalized flavin deficiency and might result in a decrease of light sensitivity. To detect this effect, one needs a system having a blue light response that can be monitored quantitatively and in which a flavin deficiency can be specifically induced. Riboflavin-re-

quiring mutants have been isolated in *Neurospora* (11, 12) and were used here to examine the effects of flavin deficiency on light sensitivity. Flavin deficiency in the mutants was induced by growth at an appropriate temperature (*rib-1*) or by growth on limiting concentrations of riboflavin (*rib-2*). The effect on blue light photoreception was then assayed. The three blue light responses studied were: (i) suppression of circadian conidiation by constant illumination, (ii) phase shifting of the circadian conidiation rhythm by brief exposures to light, and (iii) induction of carotenoid synthesis. The results reported here are consistent with a flavin photoreceptor for at least some of the blue light responses in *Neurospora crassa*.

MATERIALS AND METHODS

Fungal Strains. The following strains were obtained from the Fungal Genetics Stock Center (Humboldt State University, Arcata, CA): *bd* (41-4, FGSC no. 1859); *csp-1* (UCLA 37, FGSC no. 2554); *rib-1* (51602t, FGSC no. 1955); and *rib-2* (Yso539r, FGSC no. 1873). The general properties of the *rib* mutants are described elsewhere (11, 12). Double mutants of genotype *bd rib-1* and *bd rib-2* were constructed according to standard procedures (13). The strains used for the isolation of *rib* revertants contained the *csp-1* mutation as a contamination check. *rib* revertants were selected for by incubation of far-UV-irradiated conidia under nonpermissive growth conditions involving temperature or medium composition. UV light was chosen as a mutagen because it has been shown to be effective in the reversion of the *rib* mutations (14).

Materials. Riboflavin and Tween-80 were from ICN Pharmaceuticals. The riboflavin was checked for purity by thin-layer chromatography on cellulose plates (Eastman) with two different solvent systems (*n*-butanol/acetic acid/water and aqueous 5% sodium phosphate) (15). *Megasphaera elsdenii* flavodoxin (16) was a generous gift from V. Massey (University of Michigan). Apoflavodoxin was prepared according to the dialysis technique of Mayhew (17). FMN was prepared by purifying FAD on DEAE-cellulose (18) and then hydrolyzing the FAD to FMN with *Naja naja* venom (19). All other biochemicals were from Sigma.

Photoreponse Assays. (i) *Photosuppression of circadian conidiation.* Growth tubes were inoculated, kept for 1 day in the light and then for 2 days under dim red light (Kodak no. 1A filter), and then transferred into constant white light. A glucose/arginine medium (20) was used in all cases and the experiments were carried out in 16 × 450 mm Pyrex growth tubes with 8 ml of medium per tube. Suppression of rhythmicity by the constant white light was measured at temperatures ranging from 20°C to 28°C and at light intensities ranging from 50 to 1000 lx. Cool white fluorescent lighting (Sylvania, F40CW) was used in all experiments, and the intensities were measured with a Li-Cor LI-185 photometer coupled to a LI-210S photometric sensor (for lx) or a LI-200S radiometric sensor (for W/m²). All ex-

periments were carried out in a Lab-Line constant temperature room ($\pm 0.25^\circ\text{C}$) with the temperatures reported being those of the medium in the growth tubes as measured by a copper/constantan thermocouple attached to a recorder system (21).

(ii) *Photoinduced phase shifting of circadian conidiation.* Growth tubes were inoculated and placed in constant darkness; after 24 hr they were transferred to 6°C for 12 hr (dark) in order to synchronize the conidiation rhythm. Following this cold pulse, the growth tubes were shifted to the experimental temperature and, after about 100 hr of growth, were exposed to a pulse of fluorescent light (cool white; 800 lx; 0.5 W/m^2) for various time intervals. Light pulses were given in all cases at Circadian Time 2200, which is defined as the center of a conidial band (21). The phase shift resulting from a light pulse was obtained by subtracting the actual time of occurrence of the first conidial band formed after the light pulse from the calculated position (by linear regression analysis) of that band as if no light pulse had been given.

In constant darkness, the *bd rib* mutants will not show a clear conidiation rhythm, particularly under conditions of flavin deficiency. In order to stimulate conidiation, aeration of the growth tubes (*bd* and *bd rib* strains) was used (22). Sterile filtered (Gelman $0.2\text{-}\mu\text{m}$ in-line filter) and humidified (100% relative humidity) air was pumped through the growth tubes at a flow rate of approximately 20 ml/min. The growth tube medium used for the phase-shifting experiments had the same composition as that used in the photosuppression assays except that arginine was omitted and, in certain cases, riboflavin was added. The use of this medium and aeration sets up conditions such that all strains will clearly show circadian conidiation. Light sensitivity is apparently not altered by aeration (unpublished data). Exposure of riboflavin-supplemented medium to light was minimized in all cases.

(iii) *Photoinduced carotenoid synthesis.* Mycelial pads were grown in 250-ml Erlenmeyer flasks containing 25 ml of sucrose/Tween-80 medium (23) and, in certain experiments, riboflavin. Because conidial carotenoid formation is constitutive, this medium, which inhibits the formation of conidia, was used so that only mycelial carotenoid photoinduction was assayed. Inoculated cultures were placed in dim red light for 4–6 days, depending on the medium, temperature, and strains involved.

Mycelial pads were harvested, rinsed in fresh culture medium (without supplemental riboflavin), moistened with fresh medium, and placed, with the top/bottom pad orientation maintained, in 15×150 mm Petri plates. After 1 hr the pads, with the Petri dish lids off, were exposed to light (cool white fluorescent; 1000 lx; 0.63 W/m^2) for the various time intervals. The pads were then moistened with chilled (6°C) Tween-80 medium containing 0.5% *l*-sorbose (autoclaved separately) and riboflavin at an appropriate level. The pads were then incubated at 6°C (23) in darkness for 48 hr—i.e., an interval sufficient to allow maximal carotenoid synthesis and minimize any secondary effects of flavin deficiency on carotenogenesis.

After the 6°C incubation, the pads were lyophilized, weighed, ground to a fine powder, and extracted with methanol (6 ml) and then acetone (9 ml). The methanol and acetone extracts were combined, and filtered through Whatman no. 1 filter paper. Peak absorbance of the extracts as determined on a Cary 14 recording spectrophotometer was 473 nm. The absorbance of the extracts at that wavelength as determined with a Gilford 250 spectrophotometer was used as the measure of total photoinduced carotenoid synthesis. Values are expressed in absorbance units per 100 mg of mycelial dry weight.

Flavin Analysis. The fluorometric assay of flavin nucleotides makes use of the fluorescent quenching caused by the binding of FMN to *Megasphaera elsdenii* apoflavodoxin (19, 24). This

technique allowed analysis of the flavin levels in all strains, including those cases in which supplemental riboflavin was used in the growth medium. Flavins were extracted from mycelial tissue by homogenization and subsequent hot-water extractions according to established techniques (15, 25).

Mycelial pads were grown in darkness on 2% sucrose and Vogel's salts liquid medium (containing riboflavin supplementation for *rib-2*) and prepared for assay according to the following steps. First, the mycelia were harvested by filtration, rinsed, and minced; then, 3 ml of water at 80°C was added and the temperature was held at 80°C for 5 min. The sample was homogenized for 2 min at 50,000 rpm in a Sorvall microhomogenizer, and then three successive extractions, each with 3 ml of 80°C water, were made and the extracts were combined. The samples were cooled and centrifuged at $20,000 \times g$ for 10 min, and the supernatant fraction was used for the subsequent assays. All steps in the preparation and handling of the samples were done under dim red light.

The apoflavodoxin assay of FAD and FMN was performed as described (19, 24). A SLM System 4000 spectrofluorometer with excitation at 445 nm and emission at 525 nm was used for the assays. Each value shown in Table 1 is the mean of assays on three separate samples.

RESULTS

Photosuppression of Circadian Conidiation. The *bd* strain has its circadian conidiation fully suppressed—i.e., conidiation is continuous—by constant illumination at about 7 lx with the threshold for a detectable effect at about 1 lx (20). In contrast, when grown at a moderately restrictive temperature of 26°C (35% decrease in dry weight as compared to optimal) and kept in constant illumination with intensities as high as 550 lx (0.35 W/m^2), the *bd rib-1* strain exhibits circadian conidiation (Fig. 1) to near the end of the growth tube (6–7 days). At intensities higher than 550 lx, continuous conidiation was observed. The *bd rib-1* strain is therefore about 1/80th as sensitive to light as the *bd* strain. The period length (20.5 hr) observed in constant light (550 lx) was slightly shorter than that (21.4 hr) in constant darkness.

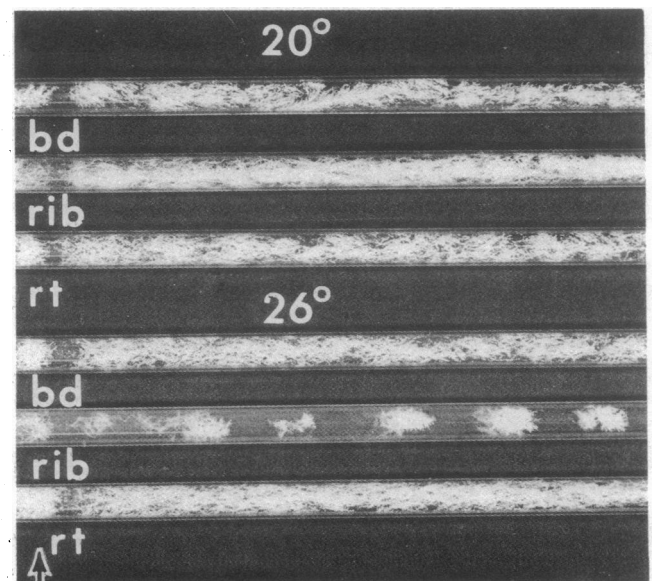


FIG. 1. Effect of constant light (550 lx) on circadian conidiation at 20°C and 26°C for the *bd* and *bd rib-1* strains. Arrow, point of transfer to constant light; *bd*, *bd* strain; *rib*, *bd rib-1* strain; *rt*, *bd rib-1^{rt}* strain, revertant of *rib-1*.

The effect on light sensitivity was clearly correlated with the temperature sensitivity of the *rib-1* mutation. At lower, less-restrictive temperatures (20–24°C), circadian conidiation was suppressed by light (Fig. 2); at 26°C it was not. A further demonstration of the role of the *rib-1* mutation in decreasing light sensitivity was that reversion of *rib-1* to wild type resulted in normal light sensitivity (Fig. 1). The effect on light sensitivity was found to cosegregate with the *rib-1* mutation (50 *bd rib-1* isolates tested).

The circadian conidiation of *bd rib-1* observed in constant light at 26°C was under growth conditions borderline between a sufficient flavin deficiency to cause the effect and a consequent severe interference with growth and conidiation that would occur at only a slightly higher temperature. The decrease in light sensitivity was therefore seen only under certain limited culture conditions. The borderline conditions are also indicated in that approximately 20% of the growth tubes showed only mycelial growth (i.e., no conidiation) at 26°C. The auxotrophic strain *bd rib-2* could not be assayed for photosuppression because, due to photosensitization and photodegradation processes, growth will not occur in a riboflavin-supplemented medium exposed to constant light.

Photoinduced Phase Shifting. The data for this response indicate a smaller effect, in comparison to photosuppression, of flavin deficiency on sensitivity to light. Fig. 3 shows that, with both the temperature-sensitive (*bd rib-1*) and auxotrophic (*bd rib-2*) strains under restrictive conditions, the dose required to reach saturation for the response was about 16-fold greater than that required for the *bd* strain. If one compares the response at the lowest dose for which reliable data could be obtained (i.e., 15 sec, 800 lx), then the *bd rib* strains were only one-third to one-half as sensitive. With increasing dose, the response of both mutant strains became more normal, so that there was little difference in response with respect to the controls by 4 min of exposure.

The reduction in phase shifting was clearly due to the presence of the *rib* mutations; normal light sensitivity could be restored in a number of ways. For the *bd rib-1* strain, riboflavin supplementation (2.5 $\mu\text{g}/\text{ml}$ of medium), reversion of the riboflavin requirement, or growth at 20°C (data not shown) resulted in near-normal light sensitivity. For the *bd rib-2* strain, higher levels of riboflavin supplementation (2.5 $\mu\text{g}/\text{ml}$ vs. 0.1 $\mu\text{g}/\text{ml}$) and reversion of the riboflavin requirement resulted in normal light sensitivity.

Photoinduced Carotenoid Synthesis. As with the phase shifting response, a relatively small effect of flavin deficiency on

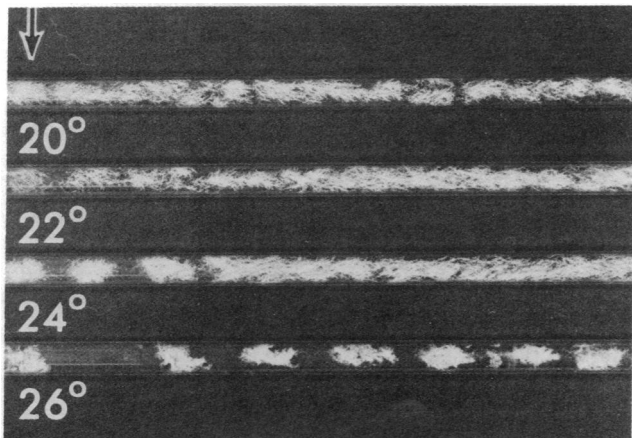


FIG. 2. Effect of temperature (20–26°C) on circadian conidiation in constant light (550 lx) for the *bd rib-1* strain. Arrow, point of transfer to constant light.

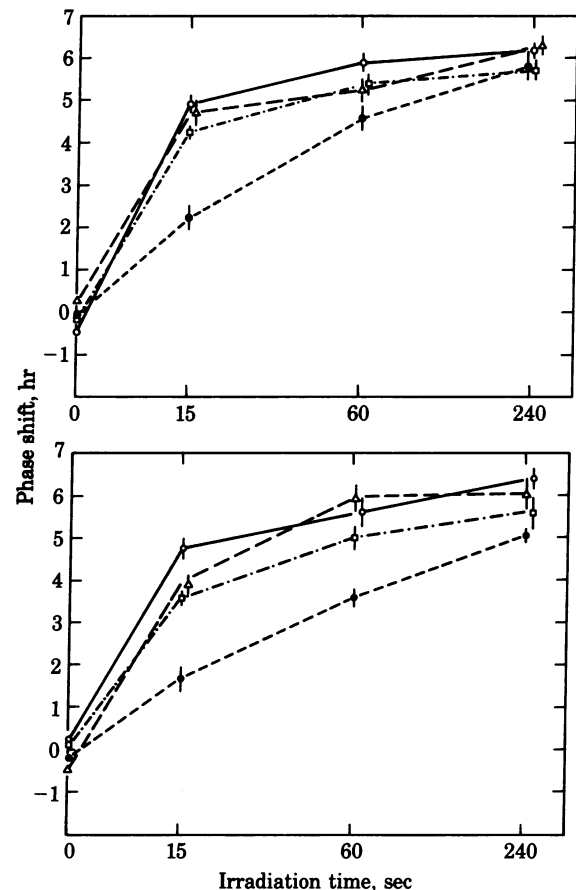


FIG. 3. Dose-response curves for phase shifting. Each point is the mean \pm SEM of six replicates. Negative shift is delay. (Upper) *bd* and *bd rib-1* strains. Experiments were done at 26°C with a light intensity of 800 lx. \bullet , *bd rib-1* strain on unsupplemented medium; \circ , *bd rib-1* strain with riboflavin supplementation at 2.5 $\mu\text{g}/\text{ml}$; \square , *bd rib-1*^{rt} strain, revertant of *rib-1*; Δ , *bd* strain. (Lower) *bd* and *bd rib-2* strains. Experiments were done at 25°C with a light intensity of 800 lx. \bullet , *bd rib-2* strain with riboflavin supplementation at 0.1 $\mu\text{g}/\text{ml}$; \circ , *bd rib-2* strain with riboflavin supplementation at 2.5 $\mu\text{g}/\text{ml}$; \square , *bd rib-2*^{rt} strain, revertant of *rib-2*; Δ , *bd* strain.

light sensitivity was observed. The response level of the *rib* strains under restrictive conditions was reduced by about one-third (*bd rib-2*) or one-half (*bd rib-1*) that of normal when a subsaturating pulse of light (30 sec, 1000 lx) was used (Fig. 4). Comparison of saturating doses shows that the mutant strains are about one-fourth as sensitive as the controls. The restrictive condition for *bd rib-1* was growth at 26°C (35% reduction in dry weight compared to optimal), and for *bd rib-2* it was growth on riboflavin at 0.1 $\mu\text{g}/\text{ml}$ (41% reduction in dry weight compared to optimal). The response both in *bd* and the controls was rapidly saturated, and little increase in induced carotenoid synthesis was seen in the interval from 2 to 8 min of exposure. With an 8-min exposure, the *bd rib-1* strain at 26°C (Fig. 4 Upper) reached the levels seen with 2 min of exposure in either other strains or *bd rib-1* under permissive conditions. The response level of *bd rib-2* was less affected throughout the entire range of doses used; however, significant differences in response levels between the restrictive and permissive conditions were seen at the two lowest doses (Fig. 4 Lower).

Consistent with the *rib-1* or *rib-2* mutations acting to alter the photoinduced carotenoid levels were the findings that reversion of the mutations or growth of the mutants with sufficient riboflavin in the medium (2.5 $\mu\text{g}/\text{ml}$) restored approximately

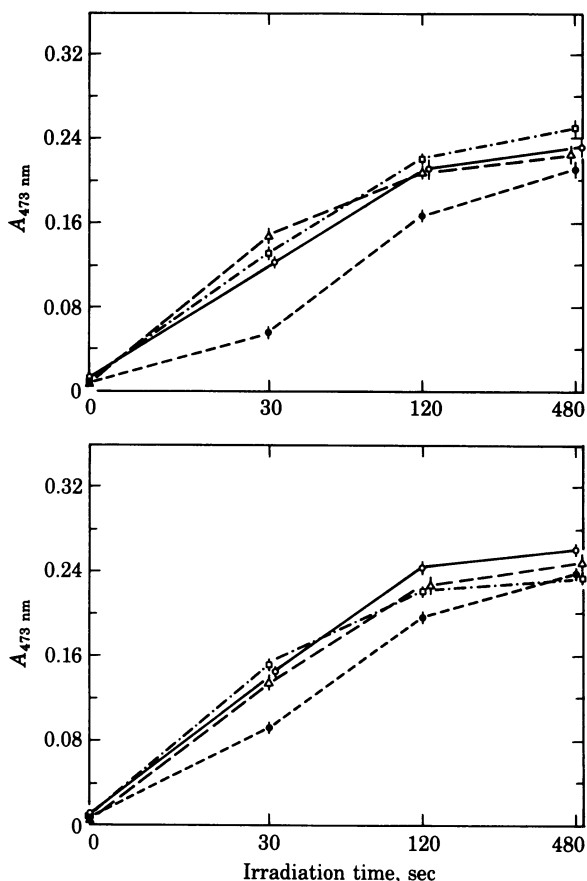


FIG. 4. Dose-response curves for the photoinduction of carotenoid synthesis. Experiments were done with light intensity of 1000 lx. Each point is the mean \pm SEM of 10 replicates. (Upper) *bd* and *bd rib-1* strains at 26°C. ●, *bd rib-1* strain on unsupplemented medium; ○, *bd rib-1* strain with riboflavin supplementation at 2.5 μ g/ml; □, *bd rib-1^{re}* strain, revertant of *rib-1*; △, *bd* strain. (Lower) *bd* and *bd rib-2* strains at 25°C. ●, *bd rib-2* strain with riboflavin supplementation at 0.1 μ g/ml; ○, *bd rib-2* strain with riboflavin supplementation at 2.5 μ g/ml; □, *bd rib-2^{re}* strain, revertant of *rib-2*; △, *bd* strain.

normal responses (Fig. 4). For the *bd rib-1* strain, growth at a lower permissive temperature (20°C) also allowed normal levels of photoinduced carotenoid synthesis to occur (data not shown). Although the effect of the *rib* mutations on light sensitivity was of a relatively small magnitude, it clearly correlated with the flavin deficiency.

Fluorometric Assay of FMN and FAD. As a measure of the degree of flavin deficiency induced in the *rib* mutants, the levels of flavin nucleotides (which are possible flavin photoreceptors) were determined (Table 1). The FAD and FMN levels were substantially lower in both *rib* strains under restrictive conditions. The reduction was particularly evident in the *bd rib-1* strain: about 60% decrease for both FAD and FMN was seen.

Table 1. Flavin nucleotide levels for the *bd* and *bd rib* strains

Strain	<i>t</i> , °C	Flavin level*	
		FMN	FAD
<i>bd</i> [†]	26	1.64 \pm 0.06	2.60 \pm 0.20
<i>bd rib-1</i> [†]	26	0.65 \pm 0.19	0.88 \pm 0.16
<i>bd</i> [†]	25	1.75 \pm 0.12	2.38 \pm 0.25
<i>bd rib-2</i> [‡]	25	0.83 \pm 0.09	1.50 \pm 0.28

* μ g of flavin per g of mycelia (wet weight), mean \pm SEM.

[†] Grown on medium without supplemental riboflavin.

[‡] Grown on medium with supplemental riboflavin, at 0.1 μ g/ml.

The *bd rib-2* strain was slightly less affected; decreases of 52% (FMN) and 37% (FAD) were observed. The ratio of FAD to FMN in the *bd* extracts was approximately 3:2, and this proportion was not drastically altered by the induction of flavin deficiency in the *bd rib* strains.

DISCUSSION

Despite the limited degree of induced flavin deficiency, the use here of *rib* mutants of *Neurospora* has provided evidence that a flavin is involved in blue light photoreception. The simplest interpretation of the observed correlation between flavin deficiency and reduced light sensitivity is that the photoreceptor pigment concentration has been decreased sufficiently to have caused a measurable change in photoreception efficiency. This is particularly apparent for the photosuppression response which shows the greatest alteration in light sensitivity (about 1/80th as sensitive). The effect on photosuppression parallels a phenomenon seen in the *poky* strain of *Neurospora* (9, 10). The *poky* strain has extramitochondrial cytochrome levels that are 16% of normal, and these decreased levels correlate with a decrease in light sensitivity for the photosuppression response. Taken together, the results obtained with the *rib* and *poky* mutants are consistent with a flavin-cytochrome *b* complex acting as the photoreceptor for the photosuppression of circadian conidiation.

Why should the photosuppression response be affected to such a magnitude but not the other responses monitored? For the phase shifting response, the effect of flavin deficiency was to decrease the light sensitivity to 1/3 to 1/2 of normal (sub-saturating dose) or to 1/16 of normal (saturating dose) (Fig. 3). For carotenogenesis, similar deficiencies decreased the sensitivity to 1/2 to 2/3 of normal (sub-saturating dose) or to 1/4 of normal (saturating dose) (Fig. 4). These results indicate that the responses may be mediated by different blue light photoreceptors. Other preliminary evidence from action spectra and methylene blue red-light studies also suggests that the photoreceptors for carotenogenesis and photosuppression may be different (ref. 10; unpublished data).

The levels of flavin nucleotides for the strains given here (Table 1) confirm a flavin deficiency but provide only a rough indication of what the effect on a presumptive flavin photoreceptor might be because different flavoproteins have different binding affinities for their flavin components. In *Neurospora*, available data indicate that some flavoprotein activities are retained to a greater extent than others in flavin-deficient mycelia (26). For example, the activity of NADH dehydrogenase under flavin starvation is reduced less than that of nitrate reductase (50% vs. 90% reduction). Thus, if the photoreceptor responsible for photosuppression had a low affinity for flavin, it would be affected severely by starvation whereas smaller effects would be observed if the photoreceptor(s) for the other photoresponses had a high affinity for flavin.

In addition, the phase shifting and carotenogenesis photoresponses appear to be qualitatively different from photosuppression in which constant light suppresses the expression of an ongoing physiological activity. The other responses have evolved for high sensitivity in that a brief pulse of light results in a large response. Because of the possible presence of different photoreceptors or different types of signal transduction pathways, the flavin levels may not have been decreased sufficiently to reveal a large effect for all of the responses studied.

The interpretation taken here is that the decreases in light sensitivity are significant enough to indicate a flavin photoreceptor for photosuppression. The smaller effects of flavin deficiency on photoinduced phase shifting and carotenogenesis, although consistent with a flavin photoreceptor, do not exclude

the involvement of another type of photoreceptor pigment [e.g., a carotenoid (27)] for these responses. The smaller reduction in response levels could be caused by secondary effects resulting from flavin deficiency. Flavin deficiency can produce multiple effects in *Neurospora* such as an increase (about 2-fold) in the levels of peroxidase and cytochrome *c* oxidase (26). The effect of such metabolic alterations on light sensitivity cannot be estimated at present, although studies with other types of auxotrophic strains might provide a way to delineate general effects on light sensitivity of nutrient limitation. Possible effects mediated by mitochondrial flavoprotein dehydrogenases, which have been implicated in the photoinhibition of respiration (28), also should be considered.

J.P. is in the Genetics Ph.D Program at the University of Illinois.

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