STUDIES OF THE BLUE LIGHT

RECEPTOR IN <u>PHYCOMYCES</u>

Tractatus by

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a sine qua non in the ritual

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From years of study and contemplation
an old man brews a work of clarity,
a gay and involuted dissertation
discoursing on sweet wisdom playfully.

An eager student bent on storming heights
has delved in archives and in libraries,
but adds the touch of genius when he writes
a first book full of deepest subtleties.

A boy, with bowl and straw, sits and blows,
filling with his breath the bubbles from the bowl.
Each he praises like a hymn and each one glows;
into the filmy beads he blows his soul.

Old man, student, boy, all these three,
from the maya of the universe create illusions.
None is better or worse.
But in each of them the Light of Eternity sees its reflection
and burns more joyfully.

<div align="center">Joseph Knecht</div>

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1977). Finally, the fluorescence spectroscopy described in chapter VI was done

in collaboration with Robert Fugate and Pill-Soon Song of Texas Tech University

in Lubbock, Texas; the electron paramagnetic resonance spectroscopy, also described

in chapter VI, was done in the laboratory of Sunney Chan in Caltech's Division of

Chemistry and Chemical Engineering.

Finally, this dissertation is dedicated to my mother and father, who made

it all possible, and to my Florence, who makes it all worthwhile.

Précis

A large number of organisms possess a variety of responses to blue and long-wavelength ultraviolet light. Riboflavin and β-carotene have long been considered the major candidates for the photoreceptor mediating these so-called blue-light physiological responses. The fungus Phycomyces possesses several responses to blue light, among which are induction of β-carotene synthesis in the mycelium and sporangiophore growth and tropism. In this thesis, it is conclusively demonstrated that a carotenoid is not the photoreceptor for the responses of Phycomyces sporangiophores to blue light. The contention that the photoreceptor is riboflavin is further strengthened by action spectral evidence consistent with the direct excitation of the photochemically active flavin triplet state. Light-induced cytochrome optical absorbance changes are investigated in in vivo preparations of Phycomyces; an action spectrum determination shows such absorbance changes to be mediated by a flavin photoreceptor. However, no evidence linking these absorbance changes to possible steps in the sensory transduction pathway was found. Indeed, the findings that the flavin-mediated cytochrome absorbance changes occur with a low quantum yield in Phycomyces and that they also occur in cells from a human cervical carcinoma speak against their possible relevance to the physiological blue-light receptor. Electron paramagnetic resonance spectroscopy and fluorescence lifetime spectroscopy are also investigated as possible probes of the flavin photoreceptor. However, the widespread occurrence of riboflavin in cellular roles other than photoreception makes it difficult to separate out that particular flavin which functions as the physiological blue-light receptor. It represents a case of a photoreceptor which is at once ubiquitous and elusive. Finally, blue-light-induced synthesis of β-carotene is investigated in the wild-type and in several sensory mutants of Phycomyces.

Any man dat iss unable to build a filter out of toot'picks, if he has to, would maybe better buy his results along with his fine equipment.

Max Gottlieb

ENUMERATION OF CHAPTERS

Chapter One

BLUE LIGHT AND LIFE

As far as is known, light from the sun (and, to some extent, other stars) is the ultimate source of all biological energy. All plants, many protists and several classes of bacteria use sunlight to store chemical energy, not only for themselves but also for all other organisms on earth. Animals obtain their energy either secondhand by eating plants or thirdhand by eating other animals. Fungi and most bacteria obtain much of their energy by catabolizing organic molecules supplied by living organisms and/or present in dead organisms.

Living organisms utilize light in two ways: as a source of energy (in simple biosynthetic steps and in energy storage) and as a means of obtaining information about their environment. The latter ranges from the initiation of simple physiological functions in bacteria to the enormously complex vision of vertebrate animals. The several molecules which life has settled upon to play the role of light receptor perform their functions very efficiently. Biological photoreceptors have relatively large extinction coefficients (10^4-10^5 liter mole^{-1}cm^{-1}) and broad absorption bands to maximize the collection of light. Furthermore, the quantum yields for the photochemical reactions which they undergo are large, ranging from about 0.1 to 1.

The disciplines of biochemistry and photochemistry each deal with many hundreds or thousands of molecules. The intersection of these two disciplines delimits the subject of photobiology, which deals with the handful of molecules life has settled upon to play the role of photoreceptor for light-regulated physiological responses (Presti and Delbrück, 1978): photoreactivating enzyme mediates the light-induced repair of pyrimidine dimers formed by the action of short-wavelength ultraviolet light on DNA; chlorophylls and bacteriochlorophylls function to gather light energy and initiate photochemistry leading to the generation of

ATP and NAD(P)H in plant and protist chloroplasts and in photosynthetic bacteria; carotenoids (long chain, often cyclized, polyenes) and bilins (linear tetrapyrroles) function as photosynthetic antenna pigments by absorbing light energy and transferring it with high efficiency to chlorophyll; protochlorophyll, a biosynthetic precursor of chlorophyll, in some algae and many plants absorbs light and becomes reduced to form chlorophyll, concomitantly bringing about a variety of developmental changes which result in a fully functional chloroplast; retinal plays the role of chromophore in the animal visual pigments (rhodopsins) and in bacteriorhodopsin, the photoreceptor for the generation of ATP (Stoeckenius, 1976; Oesterhelt, 1976) and the control of a tactic response (Hildebrand and Dencher, 1975) in Halobacteria; phytochrome, a linear tetrapyrrole-protein found in all plants and in some protists, is involved in the regulation of a large number of morphogenetic phenomena such as seed germination, seedling development, and opening and closing of flowers and leaves; stentorin, a polyaromatic pigment (Møller, 1962), functions as the receptor for the photophobic response in the motile protist Stentor coeruleus (Wood, 1976); and provitamin D is converted to vitamin D via the absorption of ultraviolet light, a photochemical reaction which takes place in the skin of animals.

Few molecules other than the ones mentioned above have been implicated as biological photoreceptors. However, there is one notable omission from the above list, namely the molecule or molecules which mediate the responses of a variety of organisms to blue and long-wavelength ultraviolet light. The universality of the so-called blue-light responses is remarkable, with examples existing among every type of organism: bacteria, protists, fungi, plants, and animals.

Blue-light Responses

Probably the oldest known example of a blue-light response is the growth

toward light (phototropism) of various fungal and plant structures; examples include

the aerial fruiting bodies (sporangiophores) of the fungi Phycomyces (Curry and

Gruen, 1959; Delbrück and Shropshire, 1960) and Pilobolus (Page and Curry, 1966),

the coleoptiles of Avena (oat) (Shropshire and Withrow, 1958; Thimann and Curry,

1961) and Zea mays (corn), and the growing tips of the alga Vaucheria geminata

(Kataoka, 1975). Motile protists such as Euglena (Diehn, 1969; Checcucci et al.,

1976) and Nitzschia (Nultsch, 1971) exhibit directed motion toward blue light

(phototaxis). The synthesis of carotenoid pigments is known to be stimulated

by blue light in several organisms, including the bacterium Mycobacterium (Rilling,

1964; Batra and Rilling, 1964; Howes and Batra, 1970), and the fungi Fusarium

(Rau, 1967), Neurospora (Zalokar, 1955; De Fabo et al., 1976), and Phycomyces

(Bergman et al., 1973). Additional examples of blue-light responses include enhance-

ment of respiration in the alga Chlorella (Kowallik, 1967; Pickett and French,

1967); entrainment of the circadian rhythms of conidia formation in the fungus

Neurospora (Sargent and Briggs, 1967) and of pupae emergence in the fruit fly

Drosophila (Frank and Zimmerman, 1969; Klemm and Ninnemann, 1976); chloroplast

rearrangement in the moss Funaria (Zurzycki, 1972); retardation of flower opening

in the plant Oenothera lamarckiana (Saito and Yamaki, 1967); and stimulation

of sexual development in the fungus Nectria (Curtis, 1972), of cell division in

the fern Adiantum (Wada and Furuya, 1974), of branching and thallus formation

in the alga Scytosiphon lamentaria (Dring and Lüning, 1975a,b), of conidia formation

in the fungus Trichoderma (Gressel and Hartmann, 1968), and of sporangiophore

initiation in the fungus Phycomyces (Bergman, 1972). WHEW!

Photophysiological responses of some phytochrome-containing organisms are regulated by a combination of long-wavelength ultraviolet, blue, red, and far-red light (Satter and Galston, 1976). Phytochrome-mediated responses are generally regulated via the absorption of red (∿660 nm) and far-red (∿730 nm) light by the pigment. The effect of this light is to interconvert phytochrome between two forms: a red light absorbing form and a far-red light absorbing form. However, phytochrome also has absorption peaks in the blue and long-wavelength ultraviolet regions of the spectrum and light in these regions transforms phytochrome from one form to the other, although less efficiently than far-red and red light (Butler et al., 1964). Thus it is not surprising that some physiological effects of blue light can be explained by its absorption by phytochrome. However, a number of other interactions between blue and red light have been found to involve another blue-light receptor in addition to phytochrome. Examples of photophysiological responses which are regulated by both phytochrome and an unknown blue-light receptor distinct from phytochrome are anthocyanin pigment synthesis in Sorghum seedlings (Drumm and Mohr, 1978), chloroplast movement in the alga Mougeotia (Haupt, 1971), and phototropism in corn coleoptiles (Chon and Briggs, 1966).

Sporangiophores of the fungus Phycomyces are exquisitely sensitive to light, the action of which is to induce a transient increase in the velocity of growth of the sporangiophore. When illumination is asymmetric, the lens properties of the sporangiophore focus the light such that a net growth increase occurs on the side away from the light source; this results in growth toward the light, i.e., phototropism. A similar mechanism is probably operative in sporangiophores of the related fungus Pilobolus. Phototropism enables the fruiting bodies of these fungi to grow out from the depths of crevices in order to disperse their spores somewhere

other than the present home of the organism. It is thus a tremendously valuable
ability for these organisms to have.

Phototropism of coleptiles (e.g., oat and corn) apparently occurs by a
different mechanism even though the photoreceptor may be the same as the fungal
one. Here the first action of light is to induce a transient decrease of the velocity
of growth. However, in contrast to the fungus Phycomyces, the coleoptile is rather
opaque so that asymmetric illumination still results in growth toward the light
source (since growth would be faster on the side away from the light). Here again,
phototropism is a tremendously useful ability for the coleoptile to have, for it
must have light in order to regulate its development (via phytochrome and proto-
chlorophyll) and eventually to obtain energy via photosynthesis. Similarly, photo-
tropism or phototaxis in any organism possessing photosynthesis would be a valuable
aid in enabling the organism to maximize its collection of energy-yielding light.

Nature of the Blue-light Receptor

For many of the organisms possessing responses to blue light, effectiveness
of different wavelengths of light in eliciting the various physiological responses
has been measured. Such a set of measurements constitutes an action spectrum
for a particular response of an organism to light. Although the exact wavelengths
for the various maxima in the action spectra vary somewhat from organism to
organism (not surprising, since the various spectra were determined by different
procedures by different experimenters over a period of several decades) the shape
is always the same (figure I-1). These action spectra are all characterized by
an action band between 400 and 500 nm (blue light). This band usually exhibits
a maximum at approximately 450 nm with subpeaks or shoulders at about 420 nm

Figure I-1

Examples of action spectra for some physiological responses of several organisms to blue light: (A) phototropism in the fungus Phycomyces (Delbrück and Shropshire, 1960), (B) phototropism in the fungus Pilobolus (Page and Curry, 1966), (C) phototropism in coleoptiles of the plant Avena (Thimann and Curry, 1961), (D) phototaxis in the motile protist Euglena (Diehn, 1969), (E) stimulation of carotenoid synthesis in the fungus Neurospora (DeFabo, Harding and Shropshire, 1976), (F) stimulation of carotenoid synthesis in the fungus Fusarium (Rau, 1967), (G) stimulation of carotenoid synthesis in the bacterium Mycobacterium (Rilling, 1964), (H) enhancement of respiration in the alga Chlorella (Pickett and French, 1967), (I) chloroplast rearrangement in the moss Funaria (Zurzycki, 1972), and (J) entrainment of the circadian rhythm of pupae emergence in the fruit fly Drosophila (Klemm and Ninnemann, 1976).

A

B

C

D

E

F

G

H

I

J

350   400   450   500

WAVELENGTH (nm)

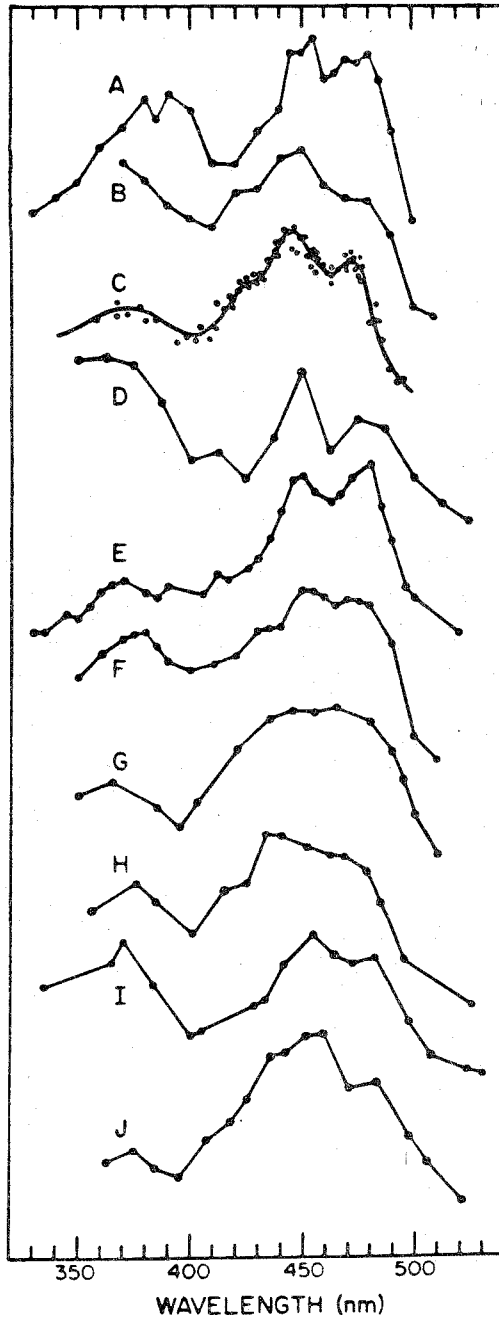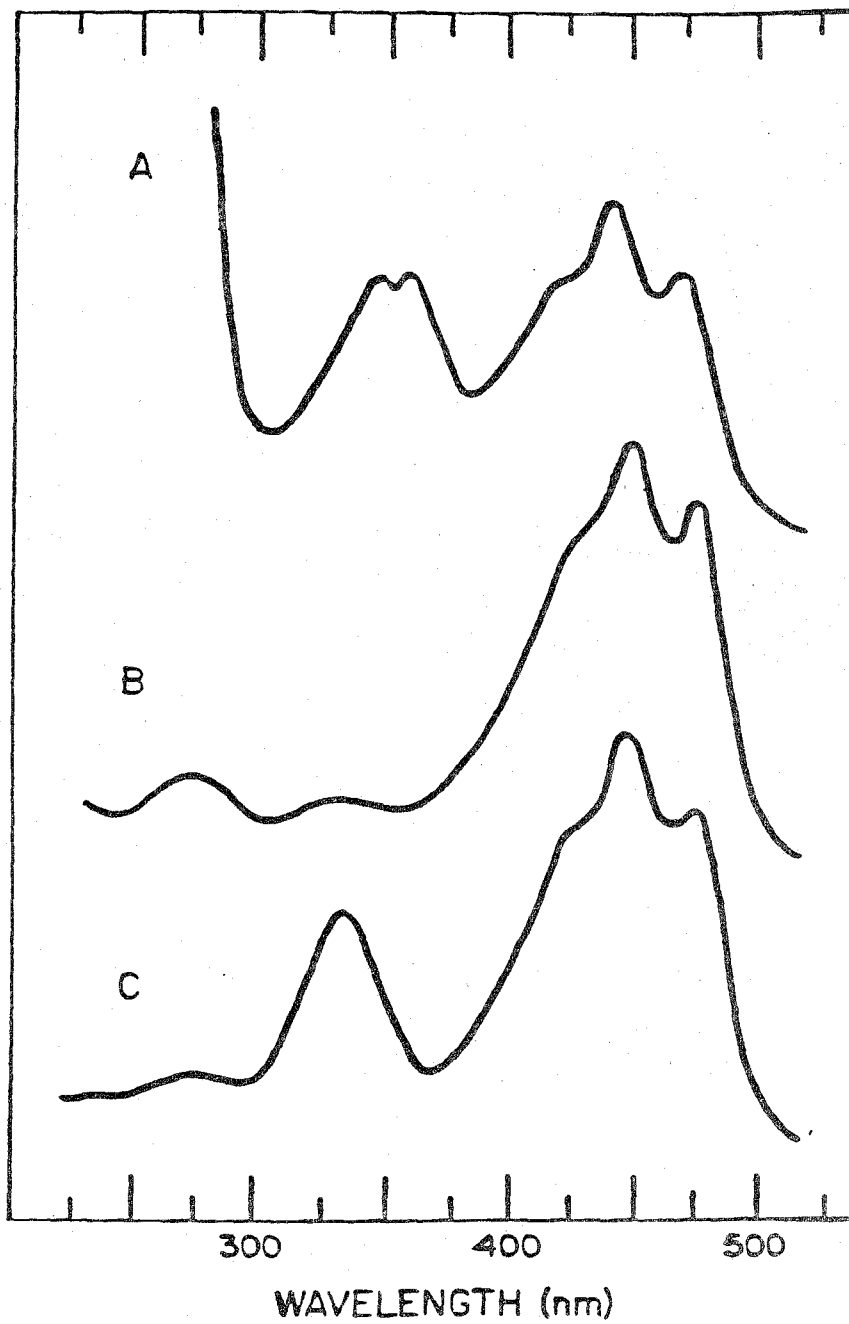## Figure I-2

Absorption spectra of (A) riboflavin in ethanol at 77°K (Sun et al., 1972), (B) β-carotene in hexane at room temperature (Vetter et al., 1971) and (C) 15,15'-cis-β-carotene in hexane at room temperature (Vetter et al., 1971). The vertical scales are arbitrary. The 450 nm peak of riboflavin is actually 10 times smaller than that of the carotenes.

WAVELENGTH (nm)

and 480 nm. In addition, most of the spectra possess an action band in the long-wavelength ultraviolet with a maximum at approximately 370 nm. Moreover, they all show no effectiveness for light having wavelength longer than about 500 nm. The resemblance of these action spectra to the absorption spectra (figure I-2) of riboflavin and of some carotenoids, especially β-carotene, has provided the basis for the belief that these molecules are the most likely candidates for the blue-light photoreceptor(s) (figure I-3). Both molecules have broad absorption bands in the blue region of the spectrum (400-500 nm) and reasonably large extinction coefficients at 450 nm: 1.25×10^4 liter mole^{-1}cm^{-1} for riboflavin and 1.3×10^5 liter mole^{-1}cm^{-1} for β-carotene.

Carotenoids were first proposed more than four decades ago as receptors for phototropism in Avena, Phycomyces, and Pilobolus (Castle, 1935; Wald and DuBuy, 1936; Bünning, 1937a,b,c). The carotenoid photoreceptor hypothesis remained unchallenged until almost fifteen years later when A. W. Galston (Galston and Baker, 1949; Galston, 1949, 1950) suggested that riboflavin might be playing the role of the photoreceptor for phototropism. This proposal was based both on the similarity of the riboflavin absorption spectrum to the action spectra for phototropism and on the ability of riboflavin to sensitize the photooxidation of the plant growth hormone indoleacetic acid (auxin). It was thought at that time that photodestruction of indoleacetic acid might be the primary photochemical step leading to tropism in coleoptiles. Later, however, it was discovered that indoleacetic acid is not destroyed during phototropism, but rather laterally transported.

Carotenoids are widely distributed among living organisms (Goodwin, 1976). They occur throughout the plant and protist kingdoms, in some bacteria,

Figure I-3

The longtime candidates for the elusive blue-light receptor: (A) riboflavin,

(B) β-carotene and (C) 15,15'-cis-β-carotene.
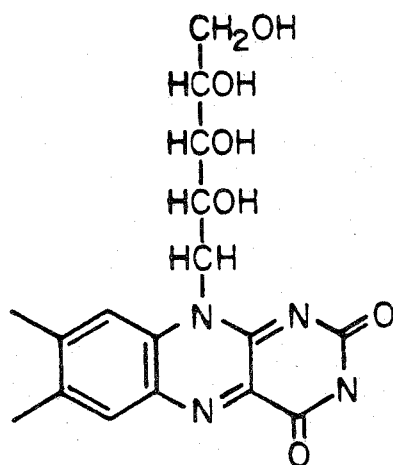
A
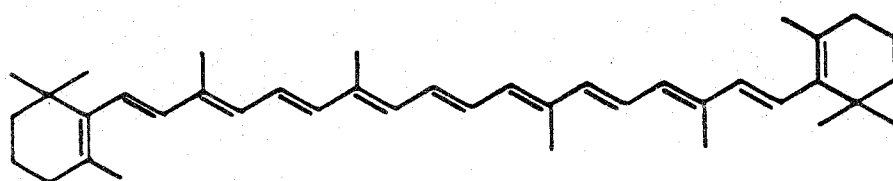
B
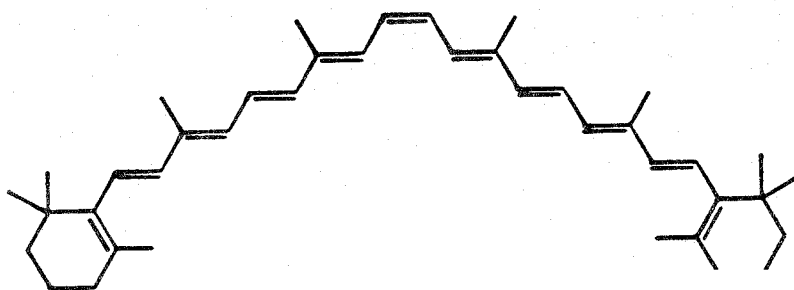
C

in many fungi, and in insects, birds, and other animals. In photosynthetic organisms carotenoids serve as antenna pigments, absorbing light and transferring the energy with high efficiency to chlorophyll. Even more importantly, they serve a protective function by efficiently quenching potentially dangerous triplet chlorophyll and singlet oxygen molecules (Krinsky, 1968, 1971; Burnett, 1976). In some fungi carotenoids serve as the biosynthetic precursors for trisporic acid derivatives, molecules which function in the sexual interaction. Finally, carotenoids are responsible for many of the brilliant yellow, orange, and red colors in flowers, fruits and vegetables, and the plumage of birds. It is carotenoids that make grapefruits, egg yolks, and buttercups yellow, oranges and carrots orange, tomatoes and lobsters red, and flamingos pink. Except for animals, all organisms that contain carotenoids can make their own. Animals have lost the ability to biosynthesize carotenoids and are therefore dependent upon what they eat for a supply. In addition to being used for pigmentation in animals, some of the ingested carotenoids are converted to vitamin A, a molecule which functions (in the form of retinal = vitamin A aldehyde) as the chromophore for the animal visual pigments as well as performing several other necessary, but as yet not clearly defined, biological functions.

Riboflavin (also known as vitamin B_2) is present in all living organisms (as far as is known). It is synthesized by most bacteria, protists and fungi, and by all plants. Animals cannot make their own riboflavin and so for them it is an essential element of their diet. (Check the ingredients in your vitamin-enriched breakfast cereal or loaf of bread, riboflavin will be there.) Because of the great versatility of riboflavin in one- and two-electron transfer reactions, it appears, in the guise of riboflavin 5'-phosphate (flavin mononucleotide, FMN) or flavin

adenine dinucleotide (FAD), in a variety of enzymes involved in many key metabolic reactions (figure I-4). For example, at least six different flavoproteins are involved in electron transfer reactions which lead into the mitochondrial respiratory chain. Other flavoproteins are involved in amino acid metabolism and the biosynthesis and degradation of nucleotides. Riboflavin itself is a molecule of ancient origin. It is found in the most primitive of extant organisms. Indeed, flavodoxins, a low molecular weight class of electron-transport flavoproteins found in a variety of microorganisms, are believed to represent some of the oldest extant proteins (Rossmann et al., 1974).

Thus both riboflavin and β-carotene are molecules which enjoy wide biological prevalence. Carotenoids, in particular β-carotene, have long been candidates for the role of photoreceptor for the various blue light-controlled processes. The main points supporting this candidacy were the presence of carotenoids in the blue light-sensitive organisms, the general similarity of the β-carotene absorption spectrum and the various physiological action spectra, and the fact that carotenoids are closely related to retinal, the molecule already well known as the photoreceptor for vision in animals. The notion that riboflavin, rather than a carotenoid, might be playing the role of blue-light receptor has had its ups and downs since Galston first raised the point three decades ago. However, over the past ten years the consensus has come to be that riboflavin is probably functioning as the receptor for blue light-mediated physiological responses in most of the organisms that have been studied.

One strong argument providing support for a flavin rather than a carotenoid photoreceptor is the presence of a band in the long-wavelength ultraviolet (maximum at about 370 nm) in many of the action spectra for blue-light responses.

Figure I-4

The molecular structures of flavins: riboflavin (aka vitamin B_2), lumiflavin, riboflavin 5'-phosphate (aka flavin mononucleotide, aka FMN) and riboflavin adenine diphosphate (aka flavin adenine dinucleotide, aka FAD). In vivo, riboflavin is converted to FMN by the enzyme riboflavin kinase. FMN is converted to FAD by the enzyme FMN adenylyl transferase. Flavoproteins contain either FMN or FAD as the flavin prosthetic group. The flavin may be either non-covalently or covalently bound. Covalent bonds usually occur via the 8α methyl to a histidine or cysteine residue of the protein.

H_2N

OH OH

CH_2

O

$OPO^{\ominus}$

O

$OPO^{\ominus}$

O

$5'$ CH_2

$(CHOH)_3$

$1'$ CH_2

10

8α
CH_3 8 9

7α
CH_3 7 6 N 5 4 4a O 3 N H 2 O N 1

FLAVIN ADENINE DINUCLEOTIDE

FLAVIN MONO NUCLEOTIDE

LUMIFLAVIN

RIBOFLAVIN (VITAMIN B_2)

FMN

FAD
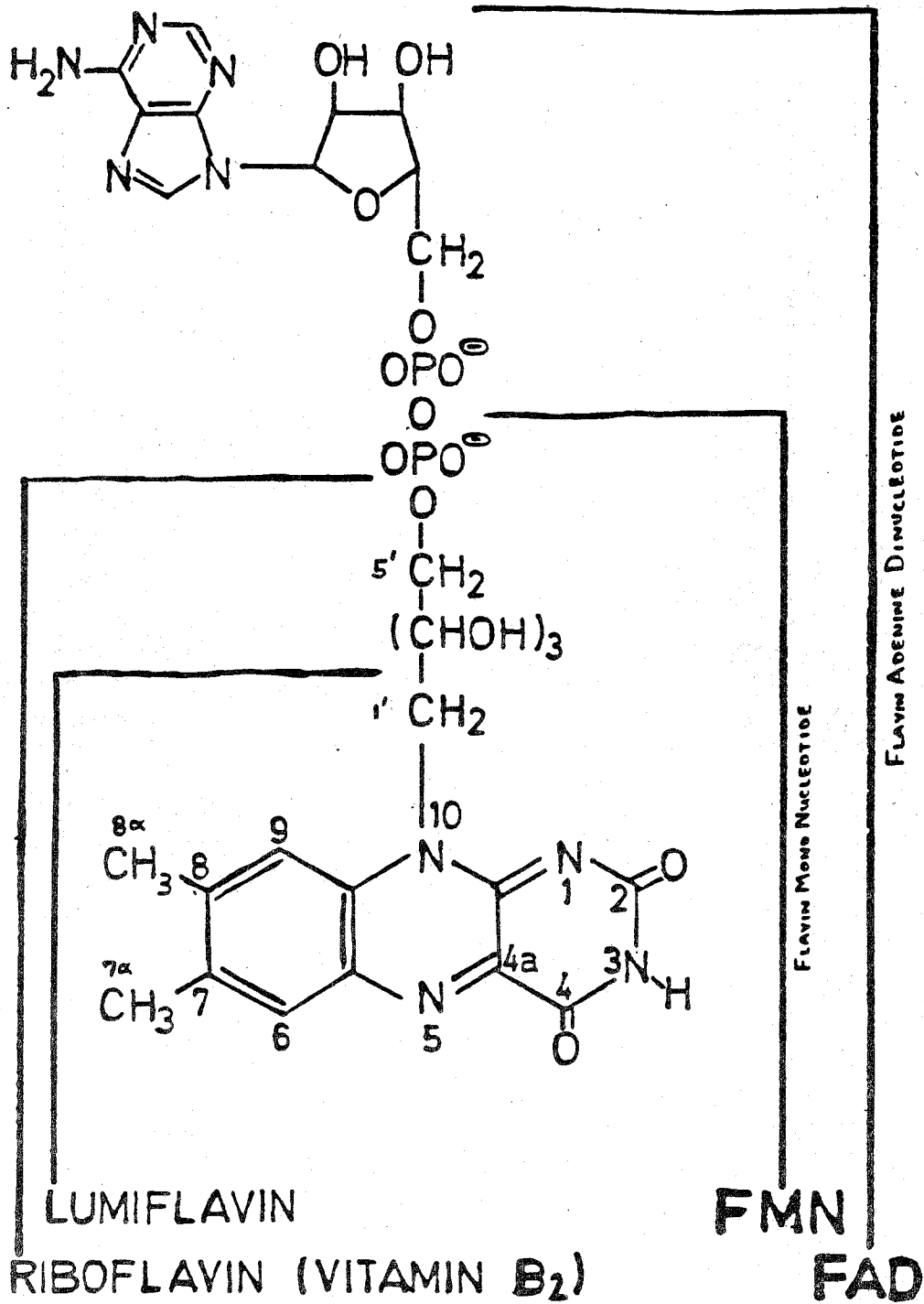
Whereas riboflavin has a significant absorption in precisely this region, carotenoids, in general, do not. This argument, however, has been challenged by the demonstration (Hager, 1970) that certain carotenoids will acquire an absorption in the long-wavelength ultraviolet if water is added to solutions of the carotenoid in alcohol. The appearance of this ultraviolet absorption was attributed to the formation of pigment aggregates. It was further conjectured that carotenoids which normally do not exhibit ultraviolet absorptions might do so under membrane-bound conditions. Song and Moore (1974) reexamined these arguments and concluded that the appearance of an ultraviolet absorption in some carotenoid-alcohol-water solutions could indeed be due to stacking interactions between carotenoid molecules. However, β-carotene showed no tendency to form stacked dimers or aggregates under their experimental conditions.

The 15,15'-cis-isomer of β-carotene exhibits an absorption in the long-wavelength ultraviolet at about 340 nm (figure I-2). Thus the carotenoid photoreceptor argument could be kept alive by postulating that it was a cis-isomer which was absorbing long-wavelength ultraviolet light. However, a careful carotenoid analysis of the fungus Phycomyces indicated the absence of cis-isomers of β-carotene (Presti et al., 1977; Chapter III). Thus, at least in this case, the cis-β-carotene photoreceptor argument cannot be applied.

In several systems the shorter wavelength ultraviolet region has also been investigated for physiological effectiveness. The action spectra for phototropism in Phycomyces (Curry and Gruen, 1959; Delbrück and Shropshire, 1960), for stimulation of carotenoid synthesis in Mycobacterium (Howes and Batra, 1970) and for chloroplast rearrangement in Funaria (Zurzycki, 1972), show a sharp band of considerable magnitude at about 280 nm. Riboflavin possesses such an absorption

maximum, whereas β-carotene does not. This action spectrum band may thus

be taken as additional evidence in support of a flavin photoreceptor in these cases.

(It should be kept in mind, however, that the amino acids tryptophan and tyrosine

absorb 280 nm light; thus energy transfer from the protein moiety of the photo-

receptor to the chromophore might contribute to action spectra bands in the 280 nm

wavelength region.)

The upshot is that β-carotene can probably be ruled out as the blue-light

receptor in those cases where the action spectra show significant effectiveness

of ultraviolet light, especially long-wavelength ultraviolet light (∼370 nm).

A strong argument in favor of a non-carotenoid (and therefore, most

likely a flavin) photoreceptor being responsible for the blue-light responses of

a number of organisms derives from growth of these organisms under conditions

where their carotenoid content is reduced. Specifically, it has been reported

that carotenoid deficiencies in Phycomyces (Meissner and Delbrück, 1968),

Neurospora (Sargent and Briggs, 1967), Euglena (Checcucci et al., 1976), Pilobolus

(Page and Curry, 1966), Avena (Bara and Galston, 1968), and Drosophila (Zimmer-

man and Goldsmith, 1971) had little or no effect on the physiological responses

of these organisms to blue light. These studies (using mutants in the cases of

Phycomyces, Neurospora, and Euglena,inhibitors of carotenoid biosynthesis in

the cases of Pilobolus and Avena, and carotenoid-free medium in the case of

Drosophila) were, however, subject to the limitation that there definitely was

or at least could have been more than enough residual β-carotene present to serve

efficiently as photoreceptor. Although these studies strongly suggest that β-caro-

tene (or other related carotenoids) cannot be functioning as the blue-light receptor

in these organisms, conclusive proof was lacking until mutants of Phycomyces

containing no detectable β-carotene were shown to possess phototropic responses to blue light identical to those of the wild type (Presti et al., 1977; Chapter III). These results definitely rule out the possibility that a carotenoid is functioning as the photoreceptor for phototropism in Phycomyces and strengthen the contention that a carotenoid is not the photoreceptor in other blue light-sensitive organisms as well.

Riboflavin is an exceedingly well-suited molecule for participation in physiological photochemistry. Free riboflavin, for example, may react photochemically by dehydrogenating a variety of external hydrogen donors or by an intramolecular dehydrogenation of its own ribityl group (Hemmerich, 1976). Many of the photochemical reactions of riboflavin occur through the relatively long-lived triplet state (figure I-5). In free riboflavin, at least, this triplet state is readily populated via intersystem crossing from the excited singlet configuration (Moore et al., 1977; Grodowski et al., 1977).

Potassium iodide has been found to quench (depopulate) the flavin excited singlet state (Weber, 1950) and the lowest triplet state (Song and Moore, 1968). The inhibition of the phototactic responses of Euglena by potassium iodide has therefore been cited as evidence in support of a flavin photoreceptor (Diehn and Kint, 1970; Mikolajczyk and Diehn, 1975). Potassium iodide, but not potassium chloride, has also been found to inhibit phototropism to blue light in corn seedlings (Schmidt et al., 1977b). Moreover, phenylacetic acid, a molecule known to covalently bind to irradiated flavins (Hemmerich et al., 1967), inhibits phototropism in corn seedlings (Schmidt et al., 1977b).

Further evidence supporting a flavin photoreceptor for phototropism in the fungus Phycomyces has come from the determination of an action spectrum

Figure I-5

The electronic structure of riboflavin is shown schematically along with the experimental values in nanometers for the energies of several major singlet-singlet transitions (Sun et al., 1972) and an unassigned triplet-triplet transition (Schreiner et al., 1975). (The electronic transitions of FMN and FAD are virtually identical to those of riboflavin.) All major transitions have been classified as $\pi \rightarrow \pi^*$ (Sun et al., 1972). The energy separation between S_0 (singlet ground state) and T_1 (the lowest triplet state) is approximately 600 nm based on phosphorescence emission spectra (Sun et al., 1972). The quantum yield for intersystem crossing from excited singlet states to T_1 is approximately 0.7 (Moore et al., 1977). Fluorescence accounts for the fate of most other absorbed energy. The fluorescence lifetime of riboflavin in ethanol is 5.6 ns (Fugate and Song, 1976). (The fluorescence lifetimes of FMN and FAD in pH 7 phosphate buffer are 4.6 and 2.3 ns, respectively [Spencer and Weber, 1969]). The quantum yield for phosphorescence from T_1 is approximately 0.005. The phosphorescence lifetime of the triplet state is about 170 ms at 77°K (Sun et al., 1972) and about 90 µs at room temperature (Vaish and Tollin, 1971).
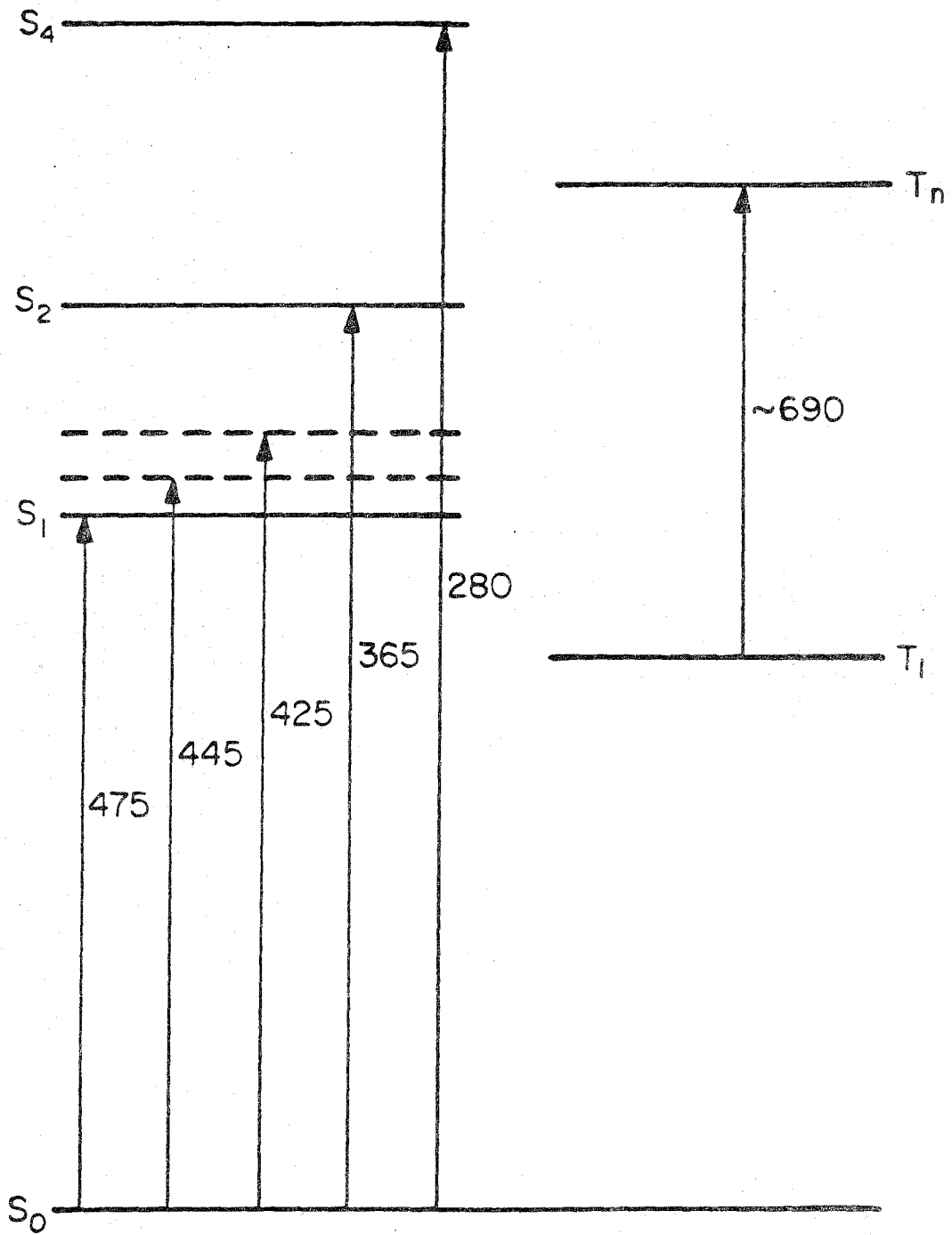
in the region between 575 and 630 nm (yellow-orange-red light) using a tunable laser (Delbrück et al., 1976; Chapter IV). An action band was detected having a maximum near 595 nm and a magnitude approximately 10^{-9} times the size of the major multiple band in the blue region of the spectrum. These parameters conform with a direct optical excitation of the lowest triplet state of riboflavin (figure I-5) (the corresponding triplet state of β-carotene is in the infrared [Land et al., 1971]) and suggest that the blue-light receptor in Phycomyces is a flavin and that the photochemistry leading eventually to the physiological response can occur from the lowest triplet state.

An additional piece of evidence supporting a flavin photoreceptor for the growth responses of the Phycomyces sporangiophore to light comes from studies by Lipson (1975). Modeling dose-response data in terms of inactivation and re-generation of the photoreceptor, he has derived an extinction coefficient of approximately 1.5×10^4 liter mole^{-1}cm^{-1} for the receptor at its blue-light absorption maximum. This is very close to the flavin value of 1.25×10^4 liter mole^{-1}cm^{-1}.

The site of the receptor for phototaxis of Euglena is believed to be contained within the paraflagellar body (Checcucci,1976), a quasi-crystalline structure (Kivic and Vesk, 1972) located within the flagellar membrane near the base of the flagellum. Fluorescence microscopy of living cells has suggested the presence of flavins in the paraflagellar body (Benedetti and Checcucci,1975). Isolation of paraflagellar bodies as a pure fraction would make unequivocal determination of their flavin content possible; however, this task has not yet been accomplished. Nonetheless, in vivo microspectrofluorometry of the Euglena paraflagellar body has further strengthened the contention that flavins are indeed a component of this structure (Benedetti and Lenci, 1977).

The role of a photoreceptor is to absorb light energy and pass this signal on by some mechanism along a transduction chain. Thus the excited state created by the absorption of light by the photoreceptor molecule must be long-lived enough to do useful photochemistry. Specifically, such an excited state must be able to do more than rapidly dissipate its energy as heat. It has been argued (Song et al., 1972; Song and Moore, 1974) that very short lifetimes of excited singlet states and inefficient intersystem crossing to the metastable triplet state make carotenoids ill-suited for the role of a photobiological receptor. Although this argument is cogent, it does not completely rule out the possibility that carotenoids might be functioning as the receptor for some responses to blue light. Specifically, carotenoids could function as photoreceptors in the same way that they do in photosynthesis, that is, absorbing light and efficiently transferring the energy to another more photochemically active molecule (Song et al., 1976).

In summary, it is probably true that riboflavin, and not β-carotene, is playing the role of blue-light receptor in most, if not all, cases of blue-light photophysiology.

Coda on Blue Light and Life

The fact that riboflavin plays so many roles in living organisms makes it extremely difficult to separate out that particular flavin which is functioning as the physiological blue-light receptor (contrary to the situation for other physiological photoreceptors). Although the weight of evidence supports the hypothesis that the blue-light receptor is a flavin in many cases of blue light-regulated physiology, elucidation of the mechanism by which the photoreceptor flavin acts and an assay specific for the photoreceptor are still forthcoming. The detailed nature

of the blue-light receptor is as obscure now as it ever has been. It is not even known for certain where in the cell the photoreceptor is located, although the plasma membrane is probably the most likely location in many cases (e.g., the presumed flavin photoreceptor for phototropism in Phycomyces has been shown to be oriented with respect to the axis of the cell [Jesaitis, 1974], suggesting a plasma membrane-bound molecule). Indeed, one might say that riboflavin seems to be at once a ubiquitous and an elusive photoreceptor.

At this time, one can do no more than speculate as to how many yet undiscovered physiological responses to light might exist among the creatures of the microbial world. Because of its widespread occurrence, riboflavin might play a photoreceptor role in prokaryotes whose origin is so primeval that they do not possess chlorophyll or heme.

Among the many organisms that exhibit physiological responses to blue light there may in fact exist several different blue-light receptors with differing mechanisms of action. Retinal was apparently discovered and put to use by life as a photoreceptor at several independent points in evolution, the results being retinal-based vision in animals and retinal-based energy and sensory phototrans- duction in the Halobacteria. Given the ubiquity of riboflavin among living organisms, it would be far less surprising here than it was for retinal that life would put to use the photochemical properties of this molecule at several independent points in evolution.

Chapter Two

PHYCOMYCES: MATERIALS AND METHODS

Phycomyces blakesleeanus (from φῦκος = seaweed, μύκης = fungus, and mycologist Albert Francis Blakeslee) belongs to the family Mucoraceae, the order Mucorales, the class Zygomycetes and the kingdom fungi. Among its close relatives are the common bread molds Mucor and Rhizopus; its more distant ones include Penicillium roqueforti, Penicillium camemberti and mushrooms. Phycomyces grows as filamentous hyphae, called mycelium, on the surface of dung or other sufficiently nutritious matter. It lacks internal septa and thus is a single giant cell containing many identical nuclei. As part of its life cycle, Phycomyces produces aerial stalks called sporangiophores (diameter ∼0.1 mm) which grow upwards from the mycelium to a height of several centimeters. At the tip of a sporangiophore is a spherical structure called a sporangium (diameter ∼0.5 mm) which contains approximately 10^5 spores, the seeds of the next asexual generation. Strains of Phycomyces belong to one of two sexes or mating types called (-) and (+). Different mating types are not distinguishable by any morphological feature; however, when mycelia of opposite mating types come near each other, a mating process takes place wherein hyphae intertwine, fuse and develop into a thorny structure called a zygospore. The zygospore, after a period of dormancy of several months, germinates (without forming a mycelium) to produce a single sporangiophore whose sporangium contains several thousand spores representing the meiotic products of two nuclei from the parent strains.

Both mycelium and sporangiophores of Phycomyces respond to light in several ways. In mycelium, light stimulates the biosynthesis of the yellow-orange pigment β-carotene. Although wild-type Phycomyces produces significant amounts of β-carotene in the absence of light, growth under continuous illumination for several days will increase its β-carotene production more than 10-fold (Bergman

et al., 1973). Light also regulates the initiation of sporangiophores from the mycelium (Bergman, 1972) and affects the subsequent development of these structures (Russo, 1977; Galland and Russo, unpublished, 1978). Sporangiophores are exquisitely sensitive to light, the action of which is to induce a transient increase in the velocity of growth. This photoresponse is called the light-growth response of the sporangiophore or photomecism (from μηκος = length). One of the most remarkable features of the Phycomyces light-growth response is the wide range of intensities over which it operates. A photographic film is doing well if it covers a range of 10^3 between threshold and overexposure. The human eye, with its rod and cone light-receptor systems, is sensitive over a range of about 10^{10}-10^{11} in intensity. The Phycomyces sporangiophore responds to light over a similar range of 10^{10}-10^{11} in intensity (figure III-2), a remarkable feat for a single fungal cell. When illumination is asymmetric, the lens properties of the sporangiophore focus the light such that a net growth increase occurs on the side distal to the light source; consequently, there is a tropism (τροπος = turn) toward the light. Phototropism is a consequence of photomecism coupled with the fact that the growing zone of the sporangiophore rotates ($\sim 10^\circ$/minute) so that a sensitive dark-adapted area is continuously coming into the region where light is focused (Dennison and Bozof, 1973; Dennison and Foster, 1977; Medina and Cerdá-Olmedo, 1977a). Thus, unlike simple photomecism in which the growth rate increases only transiently and then returns to a basal rate after a few minutes, the phototropic response does not adapt to unilateral illumination but persists until an equilibrium is attained with the tendency of the sporangiophore to grow up (geotropism). This equilibrium usually results in the sporangiophore bending toward a unilateral light source with an angle of about 75° from the vertical.

Figure II-1

Tentative scheme for the sensory pathway in <u>Phycomyces</u>, adapted from Bergman

<u>et al</u>. (1973) and Ootaki <u>et al</u>. (1977). Light gives rise to responses in both mycelia

and sporangiophores. The action of light on the mycelium is to stimulate the

initiation of sporangiophores and the synthesis of β-carotene; on sporangiophores,

light gives rise to tropic and growth responses. Sporangiophores also grow away

from nearby barriers (the so-called avoidance response) and away from the surface

of the earth (the geotropic - more precisely negative geotropic - response).

Mutations affecting the sensory pathway for phototropism have been found to

fall into seven complementation groups (Ootaki <u>et al</u>., 1974; Eslava <u>et al</u>., 1976),

presumably defining seven genes. Mutations in the genes <u>madA-C</u> appear to affect

the input side of the sensory pathway. Mutations in the genes <u>madD-G</u> affect

the output side of the sensory pathway since sporangiophores of these mutants

show altered responses to all tropic stimuli.

PHOTO
RECEPTOR

BLIND

STIFF

TROPIC AND
GROWTH
RESPONSES

AVOIDANCE

GEOTROPISM

LIGHT

madA,madB
phototropism----(−)
sporangiophore
photoinitiation----(−)
avoidance-------(+)

madC
phototropism----(−)
sporangiophore
photoinitiation----(+)
avoidance-------(+)

madD,madE,madF,madG
phototropism----(−)
sporangiophore
photoinitiation----(+)
avoidance-------(−)

MYCELIAL RESPONSES
sporangiophore photoinitiation
photostimulation of carotenoid synthesis

LIGHT   DARK   LIGHT

The responses of the <u>Phycomyces</u> sporangiophore to light are eminently quantifiable, thereby making highly precise measurements possible and permitting the detection of relatively small effects with low noise levels.

The responses of <u>Phycomyces</u> to light are examples of so-called blue-light responses, the photoreceptor for which is believed to be riboflavin (this thesis). In light-induced stimulation of β-carotene synthesis (Chapter VII), it is conceivable that β-carotene may also be involved in the photoreception process.

The details of the sensory transducer pathway which couples light via a photoreceptor to the physiological responses of sporangiophore growth, sporangio-phore initiation and stimulation of carotenoid biosynthesis remain unelucidated. All that can be said for certain is that the thus far discovered mutations affecting the sensory pathway for phototropism have been found to fall into seven comple-mentation groups (Ootaki <u>et al</u>., 1974; Eslava <u>et al</u>., 1976), presumably defining seven genes (figure II-1). Cyclic AMP may play a role in the regulation of <u>Phyco-myces</u>' responses to light, as moderate doses of light have been found to quickly and transiently decrease the level of cyclic AMP in sporangiophores (Cohen, 1974) and to enhance the activity of a cyclic AMP phosphodiesterase extracted from sporangiophores (Cohen and Atkinson, 1978). At the output end of the sensory pathway for sporangiophore growth responses to light, regulation of the cell-wall-synthesizing enzyme chitin synthetase is a plausible mode of controlling growth. Irradiation of a mycelial homogenate with large doses of blue light has been found to increase the <u>in vitro</u> chitin synthetase activity by about 30% (Jan. 1974). The physiological significance of this result, if any, is unknown.

If a flavin does form the chromophore of the blue-light receptor in an organism, it might be possible to replace it by a flavin analogue by growing the

organism on medium containing the analogue. If the analogue had absorption
peaks different from those of riboflavin and if it did indeed become incorporated
into the photoreceptor and was functional, then one should observe changes in
the action spectrum for the physiological response of the organism to blue light.
Such a shift in action spectrum would provide strong evidence that the receptor
was a flavin and might yield further information (depending on the structure of
the analogue) about the nature of the chromophore-protein interactions in the
photoreceptor.

M. Delbrück and colleagues have experimented with growing Phycomyces
on a variety of riboflavin analogues. (The analogues generally differ from ribo-
flavin by substitutions of one atom for another [e.g., carbon for nitrogen or sulfur
for oxygen] or replacing one or both of the methyl groups with other groups or
atoms [e.g., chlorine, amino, dimethyl amino].) The result has been that most
of the analogues do not get utilized by the organism because of specificities in
the flavin uptake system or in the kinase enzyme which phosphorylates the flavin
to FMN. At least one analogue, 5-deazariboflavin (an analogue in which one of
the riboflavin heterocyclic nitrogens is replaced with carbon), does appear to
be taken up by the organism. However, presumably because its chemical properties
differ dramatically from riboflavin, growth on 5-deazariboflavin results in death
of the organism unless a mutation occurs which renders the flavin uptake system
nonfunctional so that the lethal analogue cannot enter the cell (Delbrück and
Ootaki, 1978). Because of its lethal effect on the organism, an analogue such
as 5-deazariboflavin is not very useful for probing the flavin photoreceptor.

Another riboflavin analogue, roseoflavin (an analogue in which the methyl
group in position 8 is replaced by dimethyl amino) (Otani et al., 1974; Otani, 1976),

has given indications that either it or possibly its monomethyl amino photoproduct
(Matsui and Kasai, 1976) can substitute into the blue-light receptor, be functional
and not have a ruinous effect on the general metabolism of the organism (Jayaram,
Hamilton and Delbrück, unpublished, 1978). The absorption spectrum of roseo-
flavin has its maximum at about 500 nm, a 50 nm red shift relative to riboflavin.
Therefore, one might expect that if roseoflavin substitutes for even a small per-
centage of the photoreceptor flavin, it could bring about a considerable change
in the blue light maximum of the Phycomyces action spectrum for phototropism.

Strains

The strains used in work to be described in this thesis are listed in table II-1.
NRRL1555(-), also known as DEL, is the standard wild type strain for physiological
studies. All other strains listed in table II-1 were obtained from this wild type
via one or more mutageneses using N-methyl-N'-nitro-N-nitrosoguanidine.

The symbol car indicates a mutation affecting the biosynthesis of β-
carotene (figure III-1). carB mutants are blocked in the four dehydrogenation
steps between phytoene and lycopene, carR mutants are blocked in the two cycli-
zation steps between lycopene and β-carotene (Aragón et al., 1976; de la Guardia
et al., 1971). carA mutants are affected in an unknown regulatory step in β-caro-
tene synthesis. The symbol mad denotes mutation in the sensory pathway for
phototropism (figure II-1).

S131, S132 and S134 were isolated by Medina and Cerdá-Olmedo (1977b)
using nitrosoguanidine mutagenesis. After our spectroscopic studies (Chapter V)
had been completed, it was determined that the strain S131 which we used does
not appear to be madA but rather mad something else. Moreover, the strain S134

Table II-1

Strains of <u>Phycomyces</u> used in this thesis

Strain	Genotype	Reference	Chapter
NRRL1555 (DEL)	wild-type (-)		VI, VII
C2	carA5(-)	1	IV, V, VI
C5	carB10(-)	1	VII
C21	madA7(-)	2, 3	VII
C47	madA35(-)	2, 3	VI, VII
C68	madD59(-)	2, 3	VII
C114	madB106(-)	2, 3	VI, VII
C148	madC119carA5(-)	2, 3	V
C173	carB32carR21(-)	1, 4	III
C174	carB33carR21(-)	1, 4	III
S131	madA7carA115(-)	5	V
S132	madB104carA116(-)	5	V
S134	madE102carA118(-)	5	V

References:

(1) Meissner and Delbrück, 1968

(2) Bergman <u>et al</u>., 1973

(3) Ootaki <u>et al</u>., 1974

(4) Ootaki <u>et al</u>., 1973

(5) Medina and Cerdá-Olmedo, 1977b

appears to be madB rather than madE. In fact, there is a yet to be explained phenomenon which appears to change the C110 madE mutation into a madB mutation when a carA mutation is introduced by nitrosoguanidine mutagenesis (E. D. Lipson, personal communication, 1978). This phenomenon cannot, however, be a general interaction between carA and madE since the combination carAmadE has been obtained from recombination in crosses with no loss of the madE phenotype (A. Eslava and T. Ootaki, personal communication, 1978).

Culture Conditions

Several types of media were used for the work reported in this thesis:

Potato-dextrose agar (PDA), consisting of 4% Difco potato dextrose agar and 0.5 µg/ml thiamine.

PDAY, consisting of PDA plus 0.1% yeast extract.

Glucose-asparagine-yeast (GAY) liquid (Heisenberg and Cerdá-Olmedo, 1968), consisting of 30 g D-glucose, 2 g L-asparagine, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 0.25 mg thiamine, 1 g yeast extract and 1 liter H_2O.

A glucose-asparagine-trace element minimal medium (Sutter, 1975) (SIV), consisting of 20 g D-glucose, 2 g L-asparagine, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 g KH_2PO_4, 28 mg $CaCl_2$, 2 mg thiamine, 1.5 mg $Fe(NO_3)_3 \cdot 9H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.3 mg $MnSO_4 \cdot H_2O$, 0.05 mg $NaMoO_4 \cdot 2H_2O$, 0.05 mg $CuSO_4 \cdot 5H_2O$, 2 mg citric acid, 15 g (1.5%) agar and 1 liter H_2O.

SIVY, a complete medium consisting of SIV enriched with 0.1% yeast extract.

SIVYCA, a complete acidified (promotes colonial growth) medium consisting of SIVY further enriched with 0.1% Bacto-Casitone (pancreatic digest

of the milk protein casein) and acidified with HCl to pH 3.2 at 25°C.

Phycomyces cultures were inoculated from spores which had been heat-activated for approximately 20 minutes at 48°C, a procedure which promotes germination (for more details see the Phycomyces review by Bergman et al., 1969).

Light Intensity Measurements

Light intensity measurements were made using a PIN 10DP silicon photodiode manufactured by United Detector Technology (UDT) of Santa Monica, California. Such a photodiode produces a current which is proportional to the intensity of the incident light. The responsitivity of the diode (i.e., photocurrent produced as a function of incident light intensity) varies with the wavelength of the incident light. For blue light, the approximate responsivity of the photodiode at 450 nm was used (0.13 amps/watt). Since the PIN 10DP photodiode is maximally sensitive to infrared radiation (responsivity at 850 nm is almost 2.5 times than at 450 nm), infrared cutoff filters (Schott KG-1) were used over the photodiode when appropriate.

Fractionation of Plasma Membrane

Phycomyces mycelium for fractionation was grown in 500 ml Erlenmeyer flasks each containing 200 ml of SIVY medium and inoculated with about 10^4 heat-activated spores. These cultures were allowed to grow for 3-4 days on a gyrotory shaker (200 rpm) under continuous fluorescent room light at 21°C.

Mycelium was collected by vacuum filtration over Miracloth and washed with several liters of distilled H_2O. The subsequent cell disruption and fractionation procedures are based on those of Schmidt, Thomson and Butler (1977a). About

20 g of mycelium were suspended in about 200 ml of buffer (25 mM imidazole acetate, 10 mM $MgSO_4$, 0.02% NaN_2, pH 6.8 at $25^{\circ}C$) in 500 ml Erlenmeyer flasks. Glass beads (diameter ∽4 mm) were then added up to the surface of the suspension and the flasks were placed on a gyrotory shaker (275-300 rpm) at ice temperature for approximately 3 hours. Twice during the disruption period, the buffer containing the released cytoplasm was removed by filtration and replaced with fresh buffer. Cell disruption was monitored with a microscope and reasonably good breakage was obtained after 3 hours of shaking with glass beads. (Younger mycelium, such as the 15 hour cultures used by Schmidt et al. [1977a], would break more easily; however, one cannot be sure whether the blue-light receptor is present in such young cells.) The particulate portion of the homogenate was collected by vacuum filtration and resuspended in fresh buffer. It was then washed at least 3 times by centrifugation at 1500 g for 10 minutes until the washing supernatant was clean. The resulting pellet consists primarily of cell walls with plasma membrane attached (both short hyphal tubes and open patches) and will be referred to as the "wall-membrane fraction." It also contains some unbroken mycelium (hyphal tubes too long and/or tangled for the cytoplasm to have been washed out) and thus is con-taminated by cytoplasm and mitochondria. A plasma membrane enriched fraction is prepared by resuspending some crude cell wall-membrane material in 25 mm imidazole acetate buffer (pH 6.8 at $25^{\circ}C$) without $MgSO_4$. (The motivation behind the previous presence and present absence of $MgSO_4$ is the suggestion that Mg^{+2} preserves the connection between plasma membrane and cell wall [Nurminen et al., 1970].) This suspension is then sonicated using a probe sonicator set at about 50 W. The sample, kept cool by ice, is sonicated 10 times for 5-second periods separated by 30-second intervals. This procedure separates some of the plasma

membrane from the cell wall. The cell wall fragments were pelleted by centri-fugation at 1000 g for 5 minutes; this pellet was discarded. The supernatant was then centrifuged again at 10,000 g for 10 minutes in order to pellet mitochondria. This pellet was also discarded and the supernatant, which according to Schmidt et al. (1977a) had no cytochrome oxidase activity (and therefore no mitochondria), was centrifuged at 50,000 g for 80 minutes to pellet the plasma membrane fragments. The resulting pellet will be called the "membrane-enriched fraction." This fraction no doubt also contains substantial amounts of endoplasmic reticulum membrane.

Chapter Three

A CAROTENOID IS NOT THE PHOTORECEPTOR FOR

PHOTOTROPISM IN PHYCOMYCES

Carotenoids are widely distributed among living organisms. They were first proposed more than four decades ago as receptors for phototropism in Avena, Phycomyces and Pilobolus (Castle, 1935; Wald and DuBuy, 1936; Bünning, 1937a,b,c). These proposals were based quite reasonably on the fact that the major pigments extracted from the phototropic structures were carotenoids whose absorption spectra were very similar to the phototropic action spectra.

The major pigment in wild type Phycomyces is β-carotene. The accumulation of this pigment results in brilliant yellow-orange mycelium, sporangiophores, and young sporangia. In Phycomyces, β-carotene probably serves as a biosynthetic precursor to trisporic acid-type molecules which are believed to be mating-type specific chemical messengers in the sexual interaction (Sutter, 1975, 1976). Because of the similarity of the absorption spectrum of β-carotene (especially cis-β-carotene) (figure I-2) to the action spectra for various blue light-regulated physiological phenomena (figure I-1), β-carotene has for decades been considered as a likely candidate for the blue light photoreceptor.

It has been reported that carotenoid deficiencies in Phycomyces (Meissner and Delbrück, 1968), Neurospora (Sargent and Briggs, 1967), Euglena (Checcucci et al., 1976), Pilobolus (Page and Curry, 1966), Avena (Bara and Galston, 1968), and Drosophila (Zimmerman and Goldsmith, 1971) had little or no effect on the physiological responses of these organisms to blue light. These studies (using mutants in the cases of Phycomyces, Neurospora and Euglena, inhibitors of carotenoid biosynthesis in the cases of Pilobolus and Avena, and carotenoid-free medium in the case of Drosophila) were subject to the limitation that there definitely was or at least could have been more than enough residual β-carotene present to serve efficiently as photoreceptor. Thus, although this suggests that

Figure III-1

The biosynthesis of β-carotene from the C_{40} precursor phytoene. In Phycomyces, there is evidence that both pathways for the conversion of neurosporene to β-carotene are operative, although the one via lycopene is the quantitatively more important route (Bramley and Davies, 1976). The four dehydrogenation steps (steps 1-4) are carried out by four copies of the product of gene carB and the two cyclization steps (steps 5 and 6) are carried out by two copies of the product of gene carR (de la Guardia et al., 1971; Aragón et al., 1976).
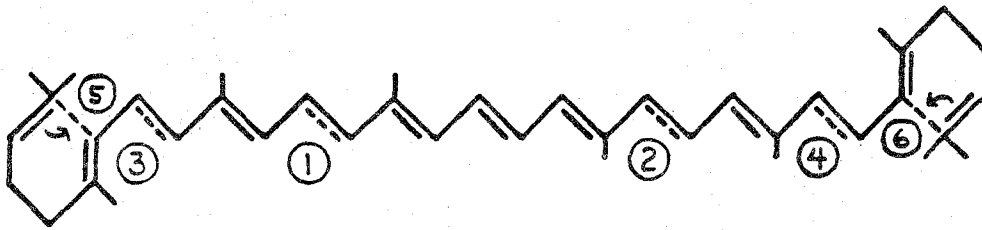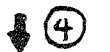
PHYTOENE
    ↓ ①
PHYTOFLUENE
    ↓ ②
ζ- CAROTENE
    ↓ ③   ⑤
NEUROSPORENE ➡ β-ZEACAROTENE
    ↓ ④   ↓ ④
LYCOPENE   ➡   γ-CAROTENE ➡ β-CAROTENE
      ⑤   ⑥

β-carotene is not the photoreceptor for the physiological responses of these organisms to blue light, no conclusive evidence ruling out β-carotene has previously been presented. De Fabo et al. (1976), in a recent study of photocarotenogenesis in Neurospora, have also pointed out the shortcomings of the published studies using albino mutants of Phycomyces and Neurospora to argue against β-carotene as the blue light receptor. Indeed, they conclude that at least for photocarotenogenesis in Neurospora, β-carotene is probably the photoreceptor.

The Phycomyces carBcarR mutants C173 and C174 are blocked in all six steps of the biosynthesis of β-carotene from phytoene (figure III-1). Table III-1 summarizes the carotenoid content of these carBcarR mutants. For both strains the principal carotenoid is phytoene. In C173 there are small quantities of phytofluene and ζ-carotene; these products of phytoene dehydrogenation are due to the leakiness of the carB mutation in this strain. The C174 strain exhibits no detectable quantities of these other pigments. In both cases, there were small amounts of various pigments which extracted with the carotenoids but which had absorption spectra not like that of β-carotene or any of its biosynthetic precursors. These several pigments (probably carotenoid derivatives) have been grouped together into the category "miscellaneous pigment." In both strains absolutely no β-carotene was detected. The carotenoid assay employed is sensitive to as little as 0.12 µg β-carotene per total sample. Dividing this number by the total sample mass places an upper limit of less than 0.01 µg/g for the β-carotene concentration in these strains. Comparing this to the value of 220 µg/g for the β-carotene concentration in wild-type sporangiophores as measured by Meissner and Delbrück (1968), the carBcarR strains have less than 4×10^{-5} of the wild-type amount of β-carotene.

Table III-1

Carotenoid content of carBcarR mutants.

Carotenoid concentrations are given in μg carotenoid per g dry weight

of sporangiophores or mycelium.

	C173		C174	
	Mycelium	Sporangiophores	Mycelium	Sporangiophores
Phytoene	407	624	257	726
Phytofluene	8.1	13	–	–
ζ-carotene	0.4	1.3	–	–
Miscellaneous pigment	1	1.6	0.9	4.9
Total sample size (grams dry weight)	14.8	18.6	20.4	12.7

Figures III-2 and III-3 indicate that phototropism in the carBcarR strains

C173 and C174 is identical to that in the wild-type NRRL1555, both with respect

to absolute threshold of the response and to the time course of bending.

In addition to total carotene analysis of the carBcarR strains, sporangio-

phores of the wild-type NRRL1555 were assayed specifically for the presence

of cis-isomers of β-carotene. These isomers of β-carotene have a major absorp-

tion peak in the long-wavelength ultraviolet region of the spectrum and thus,

in contrast to all-trans-β-carotene which does not absorb significantly in this

region (figure I-2), correspond more closely to many of the action spectra for

physiological responses to blue and long-wavelength ultraviolet light (figure I-1).

For this reason cis-β-carotene has been repeatedly proposed as a candidate for

the blue-light photoreceptor, although (to our knowledge) no assay for its presence

in blue-light sensitive organisms has ever been reported. Although the wild-type

sporangiophores were found to contain abundant quantities of trans-β-carotene,

no cis-isomers were detected.

These results indicate that β-carotene cannot be the photoreceptor

for phototropism in Phycomyces. Where there is clarity, there is no choice.

Methods

Three strains of Phycomyces were used in this work: C173 carBcarR,

C174 carBcarR and the standard wild-type NRRL1555. The double car mutants

were obtained from the wild-type NRRL1555 by two independent mutageneses

with N-methyl-N'-nitro-N-nitrosoguanidine (Ootaki et al., 1973; Meissner and

Delbrück, 1968).

Mycelium for carotene analysis was grown in 500 ml flasks each containing

Figure III-2

Phototropic threshold data for the carBcarR mutants C173 and C174 and the wild-type NRRL1555. Each point represents the average of at least 10 sporangiophores exposed for 6 hours to horizontal blue light of the indicated intensity. There are two small, but very reproducible, differences between the phototropic threshold data of the carBcarR mutants and the wild type: the phototropic angles of the two carBcarR mutants (1) were slightly greater than that of the wild type at the lowest light intensity for which data were taken, and (2) were less than that of the wild type at the highest intensity for which data were taken. These differences are probably due to a screening effect of β-carotene; shading of the photoreceptor for the tropic response by β-carotene would decrease the phototropic response to very low intensity light, but would protect the photoreceptor from excessive inactivation by high intensity light and would thereby increase the tropic response of the wild type relative to the carBcarR mutants.
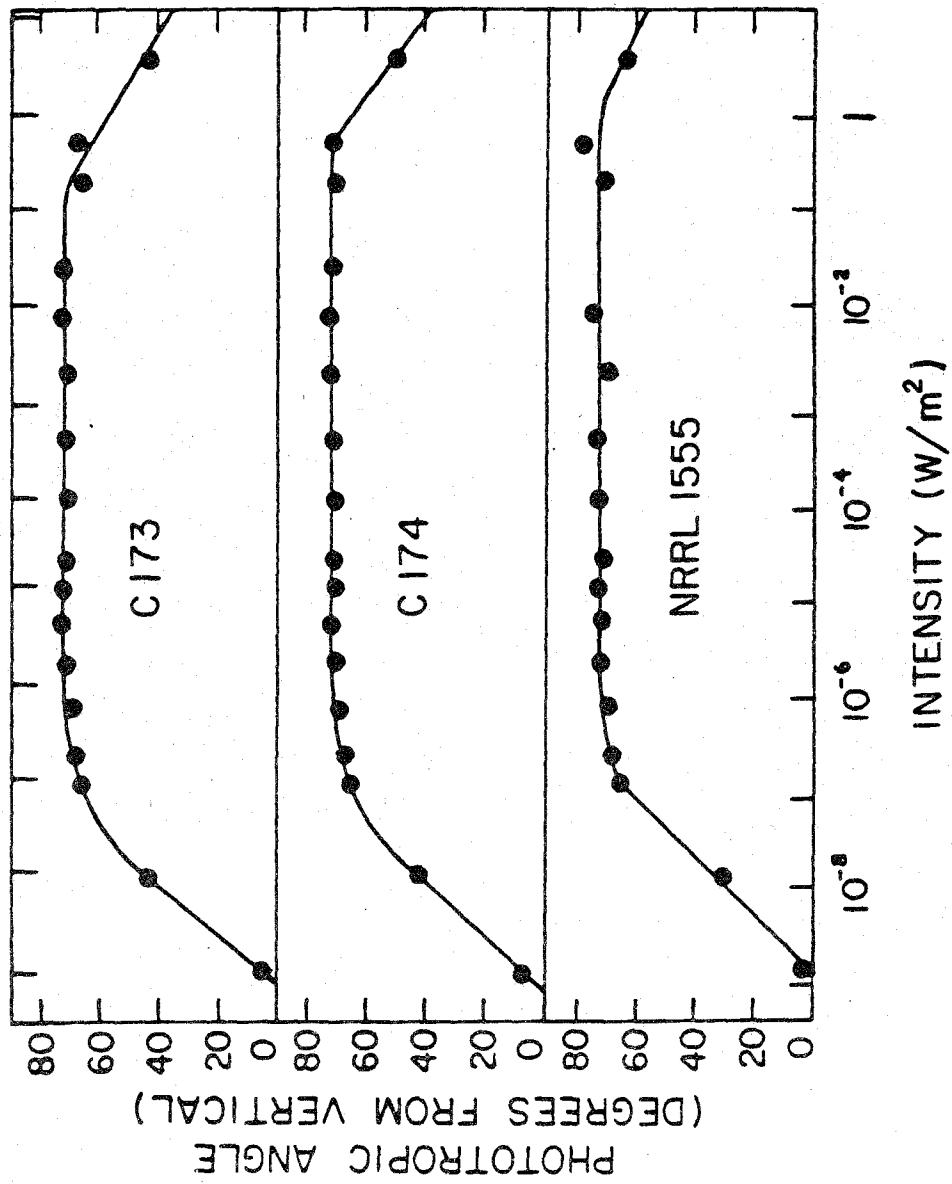
Figure III-3

Time course for the phototropic response of the carBcarR mutants C173 and C174 and the wild-type NRRL1555. Single sporangiophores, initially growing vertically, were preadapted for 30 minutes to symmetrical blue illumination of approximate intensity 10^{-4} W/m^2 on a rotating turntable. At time zero, rotation was stopped and the angle of tilt measured as a function of time. Each point represents the average of at least five different sporangiophores.
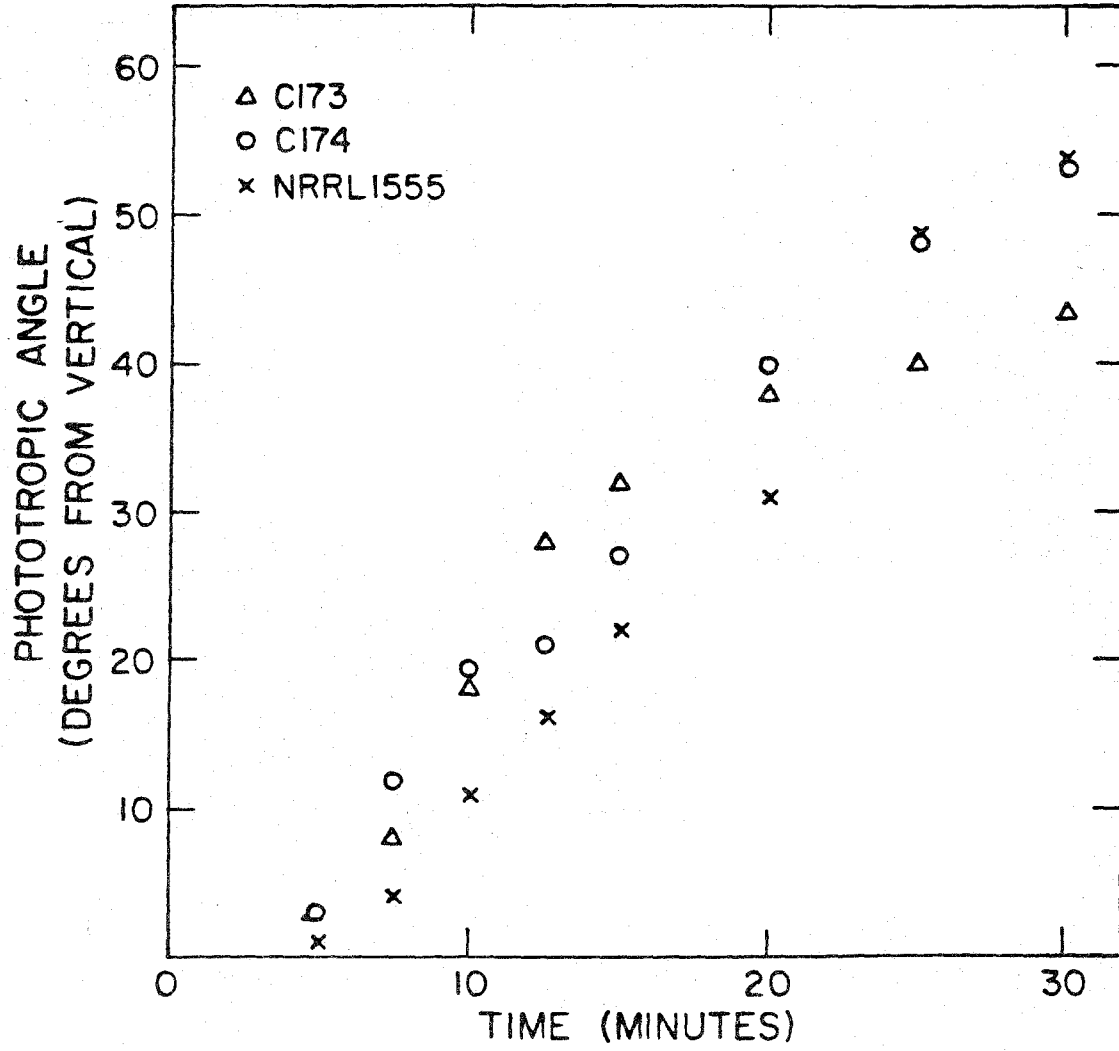
200 ml of GAY liquid medium and inoculated with about 200 heat-activated spores. The mycelium was harvested by filtration after four days of growth on a gyrotory shaker (140 rpm) under room light at 22°C.

Sporangiophores for carotene analysis were grown on PDAY agar medium (this medium gives a better yield of sporangiophores than glucose-asparagine media) in stainless steel trays (46 x 27 x 6 cm) covered with glass plates. About 500 heat-activated spores were inoculated per tray and sporangiophores were harvested after 5-6 days of growth under room light at 21°C.

Sporangiophores for phototropic response measurements were grown from spores inoculated in 3 ml vials containing about 2 ml of PDAY agar medium.

For carotenoid analysis, mycelia or sporangiophores were homogenized in a Waring blender and thoroughly extracted with acetone and methanol. The extract was transferred to diethyl ether and saponified overnight at room temperature in the dark with an equal volume of 10% potassium hydroxide in ethanol. Following saponification, the lipids were retransferred to diethyl ether and washed with water until free of alkaline. The lipid extract thus obtained was dried over anhydrous sodium sulfate, flash evaporated, and redissolved in petroleum ether (PE 30°-60°). The PE solution was stored at 2°C overnight to precipitate most of the steroids. The PE solution was then centrifuged in a clinical centrifuge and the supernatant was concentrated and chromatographed on a MgO:Hyflo supercel (1:1 w/w) column. Carotenoid fractions were eluted with increasing amounts of acetone in PE (Ootaki et al., 1973). The eluate prior to the phytofluene was rechromatographed on a Woelm neutral aluminum oxide column and phytoene eluted thereon with 2-5% diethyl ether in PE. Carotenoids were identified by their ultraviolet and visible spectra and by their adsorption behavior on the column.

Carotenoid concentrations were calculated according to the method described
by Davies (1976). Cis-isomers of β-carotene were assayed for by chromatographing
the pigment extract on a calcium hydroxide column and eluting with PE according
to the method of Polgár and Zechmeister (1942).

Phototropic thresholds of Phycomyces sporangiophores were measured
in a threshold box as described by Bergman et al. (1973). It consists of ten chambers,
each illuminated with horizontal blue light (tungsten-halogen lamp, Schott KG-1
infrared cutoff filter, Corning 5-61 blue filter) of a different intensity. The
phototropic response was measured after a 6 hour exposure to the horizontal light.

Phototropic time course measurements were made using horizontal blue
light of approximate intensity 10^{-4} W/m^2. Single sporangiophores, initially growing
vertically, were preadapted for 30 min to symmetrical blue illumination on a rotating
turntable. At time zero, rotation was stopped and the angle of tilt was measured
as a function of time.

Chapter 4

FURTHER ACTION SPECTRAL EVIDENCE

FOR A RIBOFLAVIN PHOTORECEPTOR:

DIRECT EXCITATION OF THE LOWEST TRIPLET STATE

The riboflavin molecule absorbs light with high efficiency, having an extinction coefficient at neutral pH of 1.25×10^4 liters mole^{-1} cm^{-1} at its $S_0 \rightarrow S_1$ absorption maximum of 445 nm. Riboflavin is also a photochemically active molecule. It may react by dehydrogenating a variety of external hydrogen donors or even its own ribityl side chain, as well as by ligating other groups (Hemmerich, 1976). Riboflavin photochemistry is known to take place via both the lowest triplet and first excited singlet states. If riboflavin is the photorecep- tor for the responses of Phycomyces to light, and if the photochemistry relevant to these responses occurs from the triplet state (usually populated via the first excited singlet state by intersystem crossing), then it should be possible to obtain physiological responses by direct optical excitation of the triplet state of riboflavin.

From phosphorescence emission spectra (Sun et al., 1972), the lowest triplet state of riboflavin can be estimated to lie about 600 nm above the singlet ground state. The direct optical transition from the ground singlet state S_0 to the lowest triplet state T_1 is a highly forbidden transition because it involves the flipping of an electron spin magnetic moment. The fact that the $S_0 \rightarrow T_1$ transition occurs at all is due to spin-orbit coupling which introduces singlet character to triplet states and vice versa, so that pure singlet and triplet states, in fact, do not exist. "Forbidden" $S \rightarrow T$ transitions can then be viewed as occurring between the respective pure components of the actual hybrid state. Optical $S_0 \rightarrow T_1$ transitions are nevertheless extremely weak and it can be estimated (Appendix I) that the $S_0 \rightarrow T_1$ transition in riboflavin will have an oscillator strength of only about 10^{-9} that of the $S_0 \rightarrow S_1$ transition in the blue region of the spectrum.

Because of the extreme smallness of the $S_0 \rightarrow T_1$ in riboflavin, a physio- logical effect of 590-600 nm light would not be easy to detect. Thus, it is not

surprising that previous action spectra for Phycomyces and other blue-light sensitive organisms have shown no effectiveness for light of these wavelengths.

In this chapter we shall describe the determination of an action spectrum for the growth response of the Phycomyces sporangiophore to light between 575 and 630 nm (yellow-orange-red light) using a tunable dye laser. The results are consistent with the direct optical excitation of the lowest triplet state of riboflavin.

Methods

The action spectrum measurements were done using the C2 carA strain of Phycomyces. This is an albino mutant containing very little β-carotene but having normal light growth responses of the sporangiophore. Sporangiophores were grown from spores inoculated in 3 ml vials containing about 2 ml of PDA medium.

The light growth response action spectrum measurements used the null response procedure described in detail in Delbrück and Shropshire (1960). The sporangiophore is exposed during alternate 5-minute periods to a standard (blue) light source and to a test source of variable wavelength. If the intensities of these two sources are adjusted so that they are equally effective in controlling the growth rate, the specimen will grow at a uniform rate. If they are not balanced, a periodic variation in growth rate will be seen.

The blue source was a tungsten filament 3 volt lamp with a 455 nm inter-ference filter (bandwidth 10 nm). This source was located 15 cm from the specimen and was used without optics. Its intensity was adjusted until a uniform growth response (growth response null) was attained or closely approached. The null was assessed by interpolation, with an error of about ±5% on the linear scale. A cross

section for the effectiveness of the variable wavelength laser light relative to the standard blue light was obtained by taking the ratio of the laser light photon flux to the blue light photon flux at the growth response null.

The variable wavelength source was a Coherent Radiation Model 480 tunable dye laser with rhodamine 6G dye. The dye laser was pumped by a Coherent Radiation Model 52G two watt argon ion laser. The beam profile of the dye laser was measured using a 10 μm diameter pinhole. It was approximately Gaussian in both the vertical and horizontal directions, with full widths at half maximum of 1.2 and 1.0 mm, respectively. The laser beam impinged on the middle of the growing zone of the specimen, 1 mm below the sporangium (figure IV-1). It is known that the growing zone is of fairly uniform sensitivity in the region between 0.6 and 1.5 mm below the sporangium (Delbrück and Varju, 1961). Both the blue source and the laser beam impinged at an angle of about 30° above the horizontal. This angle optimizes straight growth of the specimen. The laser beam was linearly polarized in the vertical direction.

Intensities of both light sources were measured using a UDT PIN 10DP silicon diode and double checked with a Hewlett Packard 8330A radiant fluxmeter. The intensity of the blue source ranged from 20-1000 $\mu W/m^{2}$ and that of the laser beam from 20-130 kW/m^{2}.

The specimen was placed on an x-y stage and covered with a transparent plastic house to eliminate interference from air currents. The stage was mounted on a turntable which rotated at 2 rpm. This insures symmetric illumination around its axis. The x-y stage permits accurate centering of the specimen on the axis of rotation. The turntable was mounted on a sturdy x-y-z manipulator permitting accurate positioning of the specimen relative to the laser beam. The specimen

Figure IV-1

This figure depicts a <u>Phycomyces</u> sporangiophore and the geometry of the light

sources used in our action spectrum measurements. The standard blue light (450 nm)

and the tunable dye laser light (575-630 nm) are impinging during alternate five-

minute intervals. The blue light is a broad beam. The laser light is a narrow beam,

about 0.6 mm in width, centered on the center of the growing zone, 1 mm below

the sporangium. The specimen is rotated at 2 rpm to make both illuminations

effectively symmetric around the axis of the specimen. Average growth of the

specimen, in the area of laser illumination, is about 0.1 mm during each five-minute

interval. The measured growth at the sporangium is about 0.2 mm during such

an interval. Light growth responses in our set-up consisted of periodic variations

of up to 20% of the growth per five-minute interval.

450 nm
STANDARD LIGHT

575 − 630 nm
LASER LIGHT

0.1 mm

2 rpm
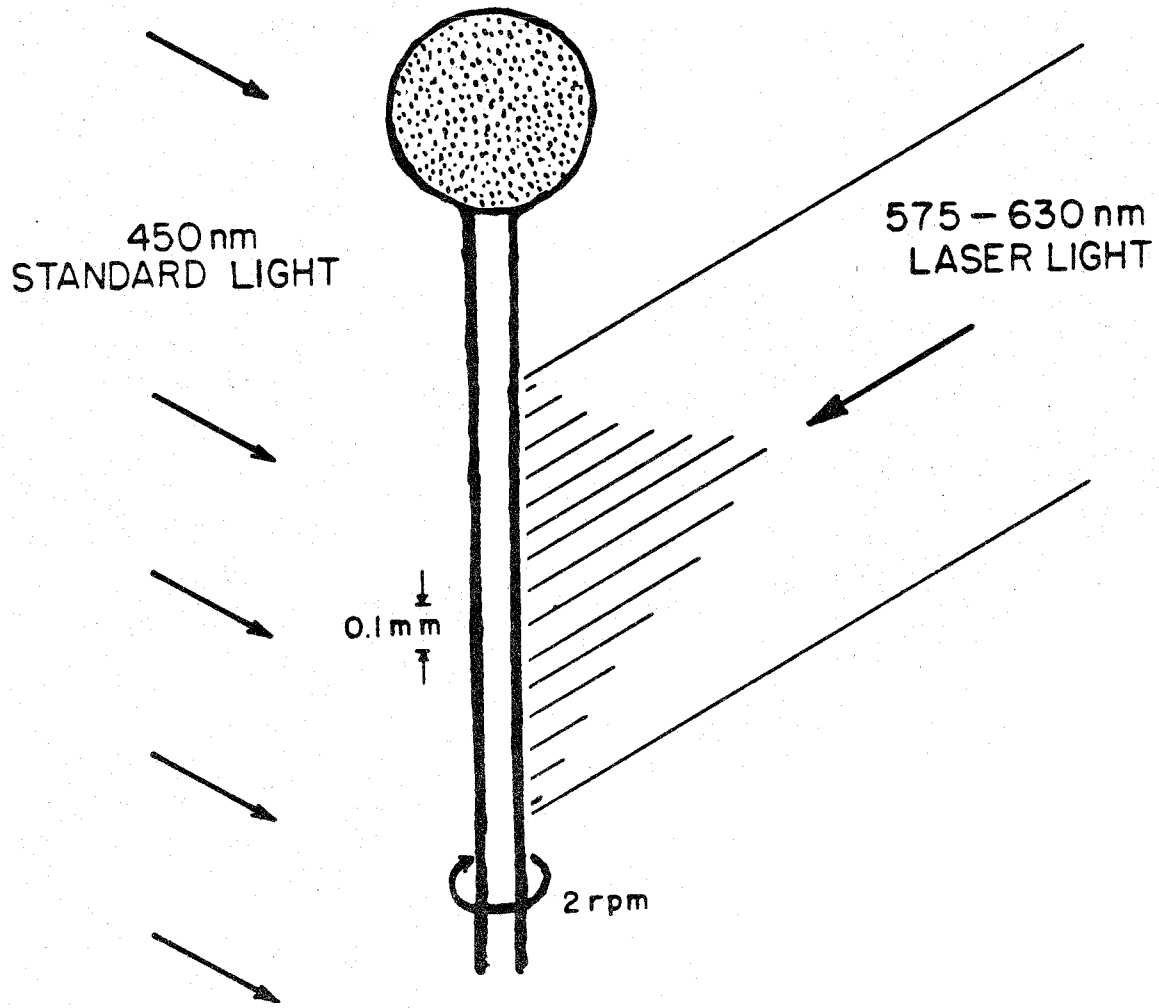
was observed against a low intensity red background light through a 40X Gaertner telescope equipped with a filar micrometer. At time zero of each five-minute interval, the upper meniscus of the sporangium was set to the midline of the micrometer using the z motion of the manipulator; at 4'50" the movable hairline of the micrometer was set to the meniscus, thus measuring the growth during the interval. At 5' (≡ 0') the specimen was moved back to the midline. During each five-minute interval, growth measured at the sporangium amounts to about 0.2 mm, and in the middle of the growing zone (at the laser beam) about 0.1 mm.

Because of the very low sensitivity of Phycomyces to the test wavelengths, precautions were taken to rigorously exclude contamination of the dye laser light with scattered blue light from the argon ion laser. Two 2 mm diaphragms were inserted in the light path, one at the dye laser exit and one several centimeters from the specimen. In addition, a Corning 3-70 sharp cut-off yellow filter was present between the two diaphragms. The beam reflected by the yellow filter fell on a silicon diode and was used to monitor the dye laser intensity.

The spectral purity of the beam emerging from the second diaphragm was checked in two ways. 1) It was viewed through a stack of three 5 mm Corning 5-61 blue filters. This stack represented an absorbance of 10.5 at 620 nm and an absorbance of 0.3 at 450 nm. The stack transmitted faintly visible 620 nm light. Blue contamination of the beam was therefore less than 10^{-10} of the test wavelength. 2) Phycomyces was used directly to test for blue contamination. A rotating sporangiophore was equilibrated with 50 μW/m^2 blue, alternating with 50 kW/m^2 laser light at 620 nm. When a Corning 5-61 blue filter (absorbance of 0.1 at 450 nm and of 3.5 at 620 nm) was inserted into the laser beam, a periodic growth response equivalent to total elimination of the laser source was observed,

showing that the 620 nm component was the actinic one in the laser beam. These controls show that the nominal test wavelength is indeed the actinic light in the laser beam.

Another control showed that 620 nm light does produce regular phototropic responses. A specimen was equilibrated as described above. Rotation was stopped, the blue source eliminated, the laser beam admitted continuously, and the specimen rotated 180° each time the phototropic response was well expressed. Figure IV-2 shows a series of responses. Latency and tropic rates correspond closely to those observed with blue light.

Since the laser beam intensities are relatively high, the question arises as to whether or not the growth effects due to the laser are linearly proportional to the light intensity. To test this, the effectiveness of the dye laser stimulation of _Phycomyces_ was tested as a function of intensity at several wavelengths. It was found that changes in laser intensities could be matched by proportional changes in the intensity of the blue light.

Several reasons conspire to make the assignment of a power per unit area ambiguous for the laser beam: 1) the laser beam is spatially nonuniform in intensity; 2) it covers only about half of the growing zone; 3) due to growth of the specimen, the beam moves about 0.1 mm back and forth on the growing zone during each five-minute interval; 4) the beam impinges at an angle of 30° from the horizontal. For these reasons the effective intensity of the laser beam, relative to that of the broad blue beam, has an intrinsic uncertainty of about a factor of two. Fitting of our equilibrium values to those obtained with broad beams must therefore allow for a possible scaling factor of this order.

Figure IV-2

Phototropic responses of a _Phycomyces_ sporangiophore to 620 nm light. The specimen was preadapted for one hour by rotating at 2 rpm in the presence of 50 $\mu W/m^2$ blue light alternating in five-minute intervals with the equivalent 620 nm laser light of 50 kW/m^2. At time zero rotation was stopped, the blue light switched off, the laser light left on continuously, and the angle of tilt measured as a function of time. Whenever the tilt became appreciable the laser light was switched to the opposite side (actually, the specimen was rotated 180°). The letters R and L between arrows indicate the intervals during which illumination was from RIGHT or LEFT. Latency of response ($\sim$6 min) and rate of tilt ($\sim 4^{\circ}$/min) are the same as those for phototropic responses to blue light at the equivalent intensity. The phototropic response could be eliminated by placing blue filters (Corning 5-61) in the laser beam. This shows that red 620 nm light, rather than blue contamination in the laser beam, is indeed the actinic light.
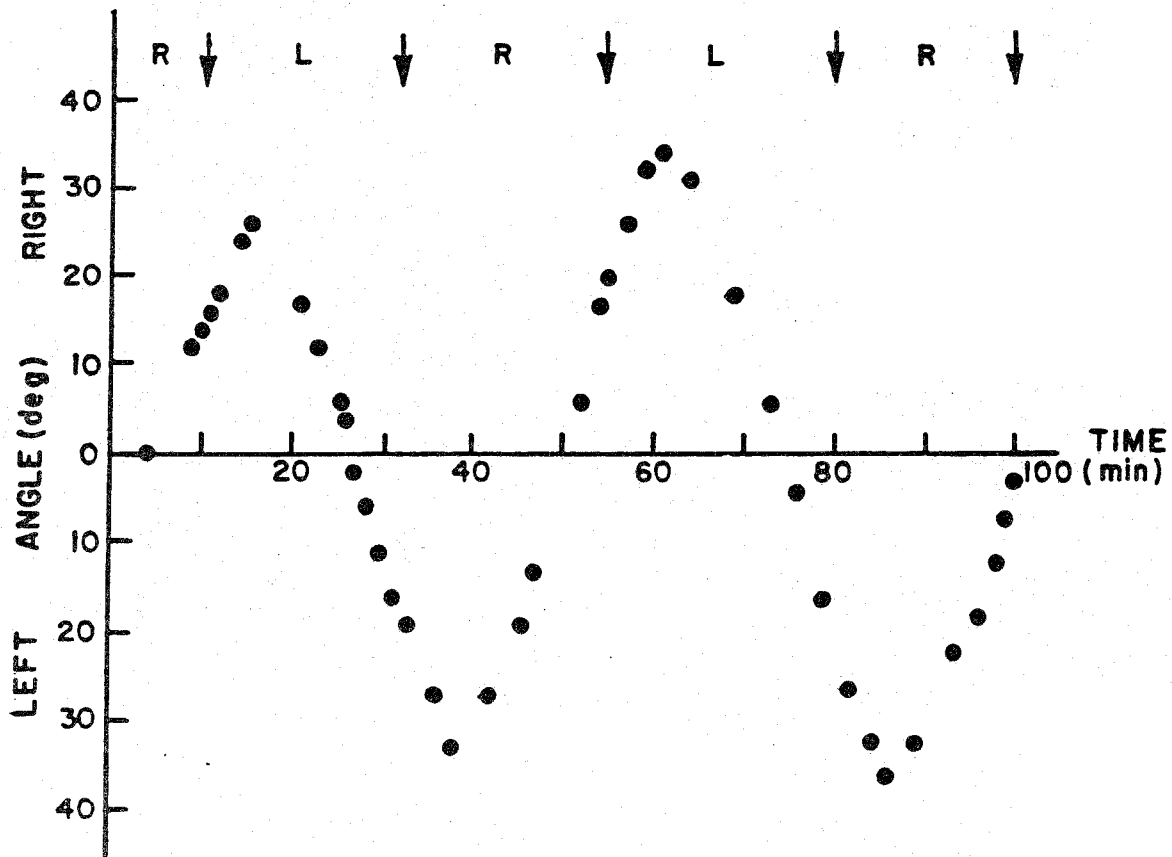
Results and Discussion

Figure IV-3 combines data from three light response action spectra for

the albino strain C2 of Phycomyces. The values from 445 to 560 nm refer to photo-

tropic response measurements which were carried out by Foster (1972). Tropic

response and growth response action spectra are known to agree if internal screening

is irrelevant (Delbrück and Shropshire, 1960), which it is for albino strains. The

values from 575-630 are our present growth response measurements. The solid

line is the graph of an equation developed by Foster (1972) to fit his two sets of

data:

$$q = \exp\left[-\frac{1}{2}\left(\frac{\nu - 22222}{877}\right)^2\right] + 0.5 \exp\left[-\frac{1}{2}\left(\frac{\nu - 20704}{447}\right)^2\right] \qquad \text{(IV-1)}$$

This equation expresses the photon action cross section as the sum of two Gaussians,

centered at 450 nm (22222 cm^{-1}) and 483 nm (20704 cm^{-1}). The peaks at 450

and 483 nm have long been adduced as evidence for riboflavin as the photoreceptor.

They correspond to $S_0 \rightarrow S_1$ absorption peaks of riboflavin; the peak at 450 nm

represents the Franck-Condon favored transition envelope and the peak at 483 nm

represents the 0 $\rightarrow$ 0 band. The fact that these absorption bands can be fit with

Gaussian curves may be considered as an illustration of the central limit theorem

of probability theory; that is to say, when there are a large number of independent

effects contributing to the broadening of an absorption band, the band will tend

toward a Gaussian shape.

It is seen that the present measurements fit equation IV-1 perfectly

from 575 to 585 nm. As explained earlier, the fit in absolute height is somewhat

arbitrary and was in fact slightly forced by scaling the experimental values upward

Figure IV-3

Light response action spectrum for <u>Phycomyces</u> sporangiophore. The photon action

cross section q is plotted as a function of frequency (wavelength). Symbols used

are: $\nabla \nabla \nabla$ tropic response equilibria using a 400 μW/m^2 440 nm blue light standard

(Foster, 1972); $\Delta \Delta \Delta$ tropic response equilibria using a 400 nW/m^2 440 nm blue

light standard (Foster, 1972); ———, - - -, - · - the graph of equation IV-1 and

its two components, the Gaussian fit to the phototropic response equilibria data

of Foster (1972); • • • growth response equilibria matched with 20-1000 μW/m^2

455 nm blue light.
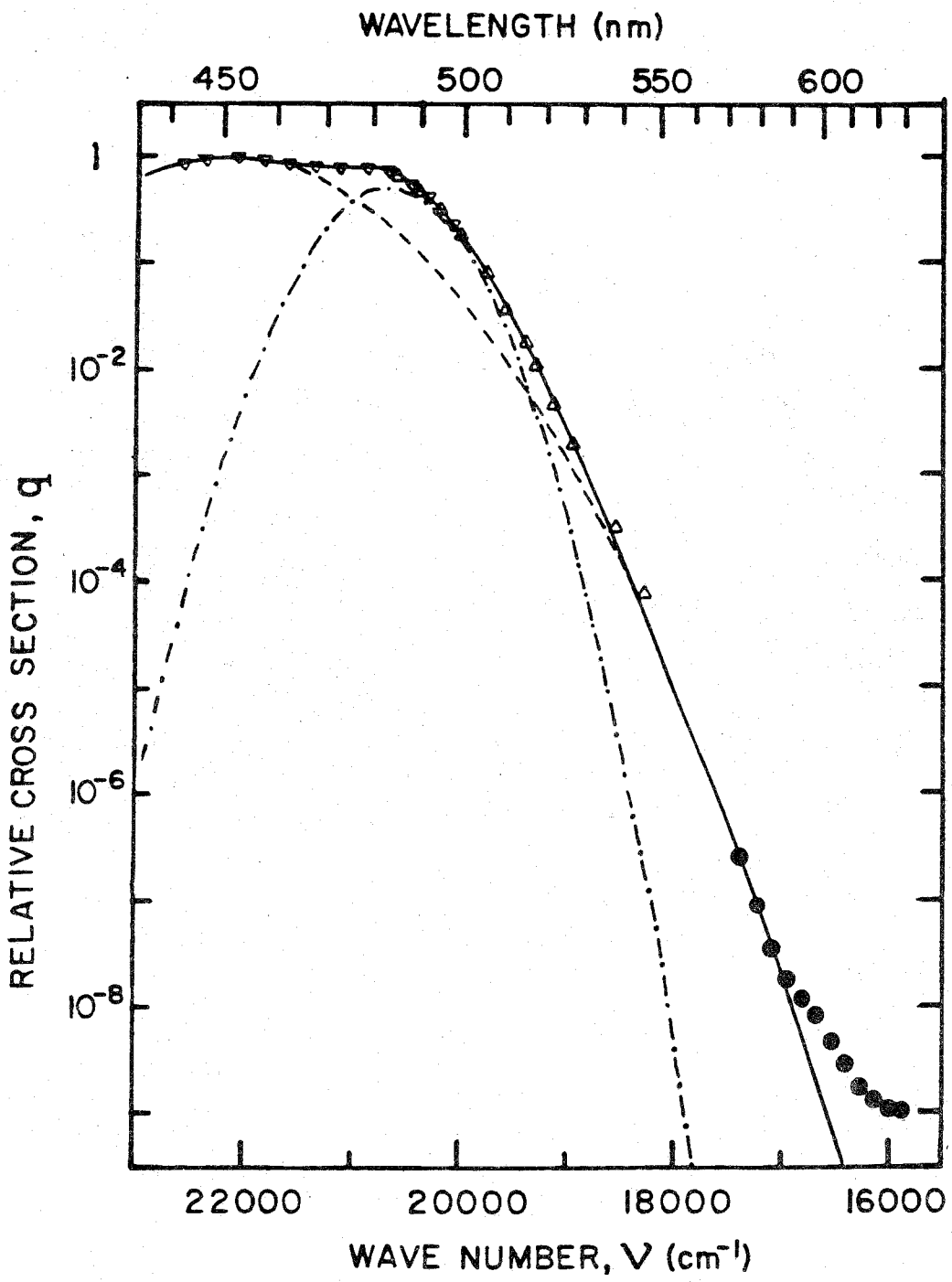
by a factor of 1.7 on the linear scale. In view of the excellent fit in slope and the approximate fit in absolute value, it seems reasonable to trust equation IV-1 farther out as representing the long wavelength tail of the strong transitions of the receptor pigment. The experimental values beyond 585 nm deviate conspicuously from equation IV-1. They represent the sum of the strongly allowed action spectrum bands and an exceedingly weak new band. Subtracting the allowed cross section (equation IV-1) from our data, the new band is isolated. The difference is plotted linearly in figure IV-4. It shows a single peak at about 595 nm, a sharp cut-off on the blue side at 585 nm, and a tail on the red side, extending beyond 630 nm, the longest wavelength available to us from the rhodamine 6G dye laser. The location of this new action peak is consistent with that of the corresponding transition from the ground state S_0 of riboflavin to the lowest triplet state T_1, since the phosphorescence spectrum of riboflavin shows an emission maximum at about 615-620 nm (Sun et al., 1972).

We can obtain an oscillator strength for this new action band relative to the strongly allowed action bands in the blue region of the spectrum by computing the areas under their respective curves. To obtain an oscillator strength for the blue light action bands, we will want to integrate equation IV-1. Thus, (converting, for numerical convenience, from cm^{-1} to μm^{-1} for the frequency units)

$$f_{blue} = f_{450} + f_{483} \propto \int_0^\infty \exp\left[-\frac{1}{2}\left(\frac{\nu - 2.2222}{0.0877}\right)^2\right] + 0.5 \exp\left[-\frac{1}{2}\left(\frac{\nu - 2.0704}{0.0447}\right)^2\right] d\nu$$

$$= \int_0^\infty e^{-65(\nu - 2.2222)^2} d\nu + 0.5 \int_0^\infty e^{-250(\nu - 2.0704)^2} d\nu \qquad (IV-2)$$

Figure IV-4

Action spectrum peak near 595 nm. Here q_{total} is the experimental cross section, $q_{singlet}$ is a plot of equation IV-1 and $q_{triplet} = q_{total} - q_{singlet}$.
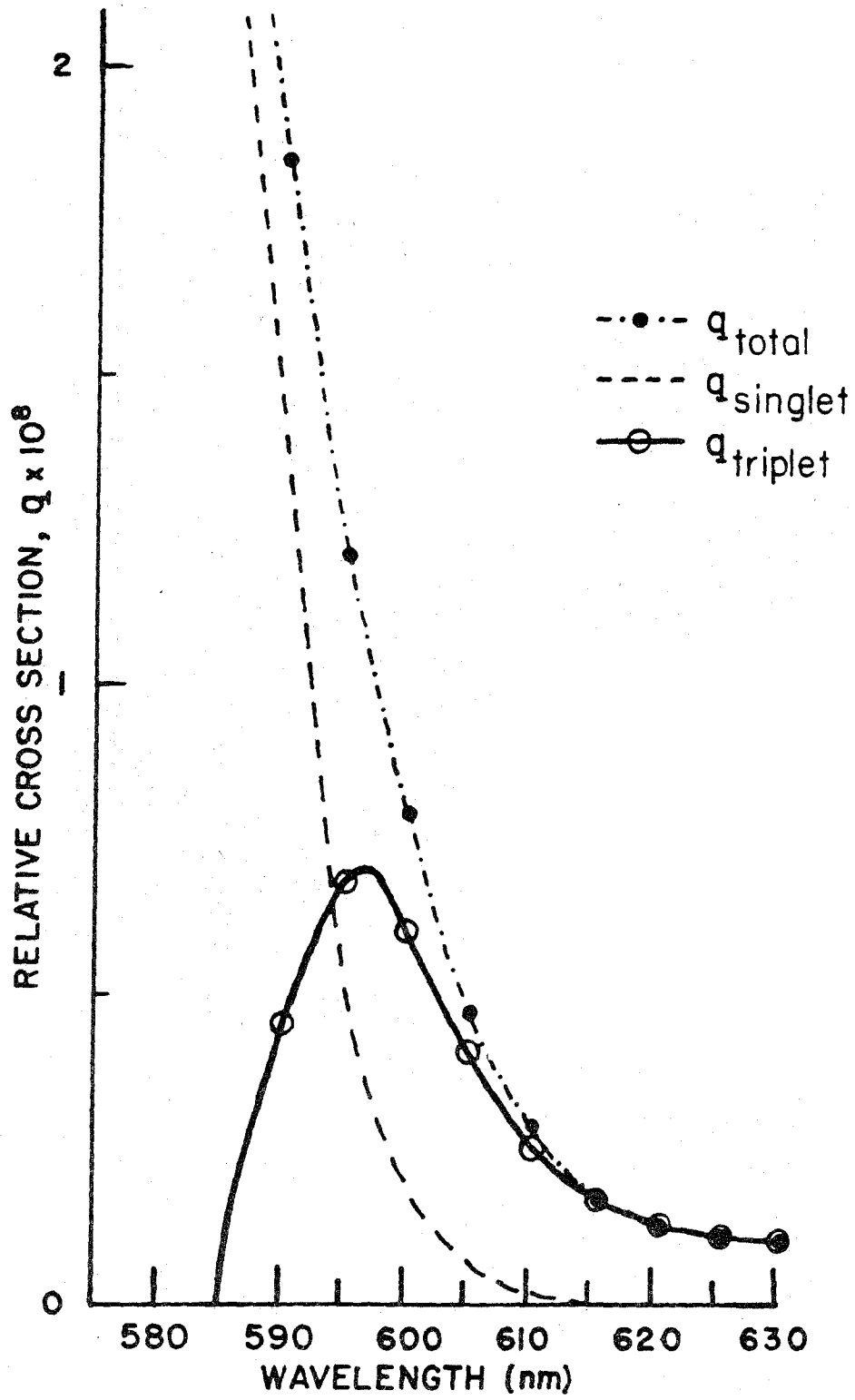
The Gaussian functions being integrated in equation IV-2 are narrow enough so that virtually all the area under their curves is above the positive ν axis. Indeed, 99.74% of the Gaussian's area lies within ± 3 standard deviations from the mean, corresponding to ± 0.263 μm^{-1} and ± 0.134 μm^{-1} for the first and second Gaussians, respectively. Thus the integrals are (for all practical purposes) unaffected (but easier to evaluate) if the curves are displaced so that their maxima occur at $\nu = 0$ and the integrations are extended to include the negative ν axis as well. We then obtain:

$$f_{blue} \propto \int_{-\infty}^{+\infty} e^{-65\nu^2}\, d\nu + 0.5 \int_{-\infty}^{+\infty} e^{-250\nu^2}\, d\nu$$

$$= \sqrt{\frac{\pi}{65}} + 0.5\sqrt{\frac{\pi}{250}}$$

$$= 0.276 \tag{IV-3}$$

To obtain an oscillator strength for the new orange light action band, we must compute the area under the peak in figure IV-4. Thus,

$$f_{orange} \propto \int q_{triplet}\, d\nu \tag{IV-4}$$

This integral may be evaluated by partitioning the region between 585 and 625 nm into four 10 nm sections and approximating the integral over each section by Simpson parabolic integration. Using the Simpson integration rule

$$\int_{\nu_0}^{\nu_2} q\, d\nu \simeq \frac{<\Delta\nu>}{3}\left(q_0 + 4q_1 + q_2 \right) \tag{IV-5}$$

we obtain the result that

$$f_{orange} \propto \int q_{triplet} \, d\nu \simeq 4 \times 10^{-10} \tag{IV-6}$$

The ratio

$$\frac{f_{orange}}{f_{blue}} = 1.45 \times 10^{-9} \tag{IV-7}$$

should be compared to the ratio of the oscillator strengths for the $S_0 \rightarrow T_1$ and $S_0 \rightarrow S_1$ transitions for riboflavin. This ratio is given by (equation AI-28)

$$\frac{f_{S_0 \rightarrow T_1}}{f_{S_0 \rightarrow S_1}} = 3.1 \times 10^{-9} \tag{IV-8}$$

Thus, the f-value of the new action spectrum band relative to that of the blue light bands, 1.45×10^{-9}, is about a factor of two smaller than that estimated, with an uncertainty of at most about 25%, of the relative $f_{S_0 \rightarrow T_1}$ oscillator strength for riboflavin. This agreement is certainly close enough to lend additional support to the conclusion that the new band in the growth response action spectrum for the Phycomyces sporangiophore represents the direct optical excitation of riboflavin's lowest triplet state. This suggests that the relevant photochemistry for the Phycomyces growth response can occur with a high quantum yield from the flavin lowest triplet state. However, can singlet state photochemistry be ruled out?

Delayed fluorescence has been observed from riboflavin imbedded in a plastic matrix at room temperature (Sun et al., 1972); the interpretation of this phenomenon is that the fluorescent first excited singlet state can be populated

via thermally excited intersystem crossing from the lowest triplet state. The

quantum yield for $T_1 \to S_1$ intersystem crossing (and thus also for delayed fluores-

cence) is proportional to the Boltzmann factor for thermal activation over an

energy gap $\Delta E = E(S_1) - E(T_1)$. For riboflavin we have $E(S_1) \simeq 475$ nm $= 21053$ cm^{-1}

and $E(T_1) \simeq 600$ nm $= 16667$ cm^{-1}; the energy difference is thus $\Delta E = 4386$ cm^{-1} $=$

9×10^{-20} joules $= 12.9$ kcal mole^{-1}. Therefore, at room temperature (298°K)

$$\phi_{T_1 \to S_1} \quad \alpha \quad e^{-\Delta E/kT} \simeq 4 \times 10^{-10} \qquad \qquad (IV-9)$$

which is not a very large number. Nevertheless, because the rigid environment

of a plastic matrix presumably decreases the nonradiative decay of the triplet

state and therefore increases its steady state population, the delayed fluorescence

observed from riboflavin in such an environment occurs with a yield of only several

hundred times less than that of normal fluorescence (P.-S. Song, personal communi-

cation, 1978). The environment of a chromophore in a protein may also be rigid

enough to increase enormously the probability of delayed fluorescence. (This

should be compared with delayed fluorescence which has been measured from

molecules (proflavin, eosin, anthracene) in the nonrigid environment of liquid

solution (Parker and Hatchard, 1961, 1962; Parker, 1964). It was found that delayed

fluorescence at room temperature occurred with yields ranging from about 150

to several thousand times less than those for normal fluorescence. The corresponding

$S_1 - T_1$ energy differences in these molecules are in the range of 2700 to 3400 cm^{-1},

a thousand or more wave numbers less than the $S_1 - T_1$ energy difference in ribo-

flavin.) Even if one assumes that the singlet state S_1 may be populated from

the triplet state T_1 with a higher efficiency when the flavin is present in a rigid

protein environment, it is not likely that $\phi_{T_1 \to S_1}$ would be large enough to account

for the light responses of the <u>Phycomyces</u> sporangiophore to 600 nm light via a $S_0 \to T_1 \to S_1$ type of mechanism. Thus, triplet state photochemistry remains the most reasonable interpretation of the new peak in the <u>Phycomyces</u> action spectrum. <u>It should be stressed, however, that there is no theoretical or experimental evidence that singlet and triplet mechanisms are mutually exclusive; both may contribute to a photochemical reaction</u> (Birks, 1976).

The light responses of the <u>Phycomyces</u> sporangiophore are eminently quantifiable, thereby making such highly precise measurements (as the action spectrum described above) possible and permitting the detection of relatively small effects with low noise levels. The detection of a secondary action peak 10^{-9} times the size of the main peak is an event unique in photobiology and rare in photochemistry. (One photochemist commented to us that one might use our data as evidence supporting a Gaussian nature for an absorption tail, a hypothesis which has little justification other than the central limit theorem.) The experiment would hardly be feasible without the spectral purity, high intensity and tunability of the dye laser, as well as the high sensitivity and low noise level of the organism. Especially, the fact that the laser intensities used were high enough to elicit the highly forbidden $S_0 \to T_1$ transition but not high enough to make <u>Phycomyces</u> uncomfortable is due to the high sensitivity of the organism.

Chapter Five

LIGHT-INDUCED OPTICAL ABSORBANCE

CHANGES IN PHYCOMYCES

If indeed the photoreceptor for the physiological responses of Phycomyces (and various other organisms) to light is riboflavin, one would like to know the nature of that step in the transduction chain which follows the photo-excitation of the flavin. Light-induced optical absorbance changes may furnish an approach to the study of these early steps in the sensory transduction chain.

Several years ago, observations of light-induced optical absorbance changes were reported in whole cell preparations of the fungi Dictyostelium, Phycomyces and Neurospora (Poff and Butler, 1974; Muñoz et al., 1974; Muñoz and Butler, 1975). The optical absorbance changes indicated the reduction of a b-type cytochrome and action spectra for the production of the absorbance changes indicated that a flavin was probably playing the role of photoreceptor. (The photochemical act would be the reduction of the excited flavin by an unknown donor. This would probably be a one-electron reduction to yield a flavin radical. The reduced flavin would then transfer an electron to cytochrome b.) On the basis of action spectra alone, the cytochrome absorbance changes were associated with the physiological responses of these organisms to blue light and this conclusion has been widely accepted. Although the implication is that both the cytochrome absorbance changes and the physiological responses are mediated by flavins, the crucial question remains as to whether these optical absorbance changes represent part of the normal transduction chain or whether they constitute a spurious photochemical event brought about by the action of high intensity light on flavins not necessarily involved in the photoreceptor system for the physiological responses.

To help resolve this question, we undertook to examine mutants of Phycomyces, representing genes along the sensory pathway for phototropism. The simplest result that would strongly support the hypothesis that these observed

light-induced optical absorbance changes are related to the photoresponse system of the organism would be the absence or distortion of such light-induced optical absorbance changes in a photomutant preparation. Such a result would suggest that the light-induced optical absorbance change was suppressed or altered along with the physiological photoresponse because of a defect in the common photo-receptor system.

Methods

The strains of Phycomyces used in this work each carry a mutation in the gene carA (Ootaki et al., 1973). These are albino mutants containing very little β-carotene, thus eliminating the screening effects of this pigment. The strain C2 has photophysiology identical to wild type. The other four strains (S131, S132, C148 and S134) carry mutations in genes involved in the sensory pathway for phototropism (figure II-1) and thus possess abnormal photophysiology (Bergman et al., 1973; Ootaki et al., 1974). For in vivo studies of Phycomyces mycelium, the strains were grown in plates containing SIV agar medium. The cultures were started from a small piece of mycelium and incubated for four days under room light. Samples were prepared by first plucking away the sporangiophores and then removing a mycelial layer from the agar surface. The mycelium was scraped free of agar and cut into strips which were then layered evenly above the window of a cylindrical cuvette to yield a uniformly dense sample approximately 4 mm thick. Fresh samples were used for all spectrophotometric measurements.

HeLa cells were grown by a standard method consisting of a suspension in so-called "modified Eagle's medium" (see references in Amaldi and Attardi, 1968). HeLa cells derive from a human cervical carcinoma of one Henrietta Lacks

(anonymous, 1976). The cells were isolated in the early 1950's (Gey et al., 1952) and have been maintained in culture since that time. Sample preparation consisted of suspending approximately 4×10^7 whole cells in 1 ml of 0.13 M NaCl, 0.005 M KCl, 0.001 M $MgCl_2$. Here, too, fresh HeLa cell suspensions were used for all spectrophotometric measurements.

The spectrophotometer used for measuring light-induced absorbance changes incorporates several features of earlier systems (Chance et al., 1971; Butler, 1972). The system operates in two modes: a single-beam mode which scans wavelength for the measurement of difference spectra, and a dual-wavelength mode for more sensitive measurements and for following the kinetics of recovery after an actinic exposure. Samples were contained in cylindrical aluminum cuvettes with Pyrex windows (1 cm^2 in area) at the bottom. Both the actinic and measuring beams were incident from above and passed through the sample in the vertical direction. A photomultiplier tube (EMI 9656B) was located below the cuvette. The actinic light source was a 500 watt xenon arc lamp. In front of the lamp housing was a shutter which remained closed except during actinic irradiation. In most of the experiments, the actinic light passed through two heat filters (a 3 cm path of 15% aqueous $CuSO_4$ and a Schott KG-1 infrared cut-off filter) and a Corning 5-61 blue filter. This combination produced an actinic intensity at the sample of 240 W/m^2 "broad-blue" light. For the dose-response measurements, neutral density filters were used to attenuate the beam. For the action spectrum measurements, the $CuSO_4$ and the Corning 5-61 blue filter were removed and replaced by interference filters of 10 nm bandwidth. The combination of one interference filter and the Schott KG-1 heat filter produced actinic intensities at the sample in the range of 10-40 W/m^2. All absolute intensities were measured

with a Hewlett-Packard 8330A radiant flux detector. The light source for the
measuring beams was a 200 watt tungsten-iodine-quartz lamp. Light was focused
by lenses onto the entrance slits of two quarter-meter monochromators (model
82-410, Jarrell-Ash Division, Fisher Scientific Company, Waltham, Massachusetts)
located at right angles. The light from each monochromator was directed by
way of lenses and mirrors. The intensities could be adjusted independently by
means of irises and neutral density filters. The two beams were reunited at a
motor-driven chopper wheel which in the dual-wavelength mode alternated the
two beams at a frequency of 145.7 hertz. After the chopper, the beams were
focused onto the sample. A lock-in amplifier (model 120, Princeton Applied Research,
Princeton, New Jersey) was used to detect absorbance changes between the two
wavelengths. The inputs to the lock-in amplifier consisted of a reference, which
provided the instantaneous phase of the chopper, and a signal from the photomul-
tiplier via a preamplifier. The output of the lock-in amplifier was recorded on
a strip chart recorder. The intensity of the measuring beams, about 5 mW/m^2,
was far below that of the actinic beam and insufficient to induce measurable
absorbance changes in the samples.

The procedure in the dual-wavelength mode was to calibrate the system
on a low sensitivity range of the lock-in amplifier with one measuring beam blocked.
Then, after the beams were balanced, a suitably sensitive measuring range was
chosen. During actinic exposures, the photomultiplier was blocked by a shutter.
The chart recorder showed the absorbance change due to the actinic exposure
and the subsequent recovery in the absence of actinic light.

In the scanning mode, only one of the monochromators was used. The
wavelength was scanned in 10 seconds from 625 to 375 nm by means of a stepper

motor. The photomultiplier anode current was converted to a voltage by means
of an operational amplifier and then processed by a logarithmic amplifier so that
the spectra would be recorded directly in absorbance units. Up to four scans could
be stored in the memory of a Fabri-Tek Instrument Computer (model 1062, Nicolet
Instruments, Madison, Wisconsin). Difference spectra were computed by subtraction
of the memory quarters and were plotted on an x-y recorder.

Absolute spectra shown are actually the difference between scans of
the sample and a scattering reference of comparable density, composed either
of a slurry of Al_2O_3 in water or of several thicknesses of tissue paper (Kimwipes).
For the light-minus-dark difference spectra, the sequence consisted of a scan
from 625 to 375 nm, followed by a 30-second exposure to actinic light, followed
immediately by another wavelength scan.

Results and Discussion

The absorption spectrum of a mycelial sample of the albino strain C2
is shown in figure V-1. It exhibits the characteristic absorptions of reduced cyto-
chromes. The peak at 602 nm is the α-band of one or more cytochrome a species,
while that at 552 nm represents the α bands of cytochromes b and c. The main
peak at 422 nm is due to the Soret bands of b and c type cytochromes. The small
shoulder at 440 nm is due to the Soret band of cytochromes a. Identical spectra
were observed for mycelial samples of the phototropically abnormal mutant strains.
Muñoz and Butler (1975) measured a similar spectrum for a mycelial preparation
from an albino strain from Neurospora.

In figure V-2 are shown light-minus-dark difference spectra for C2 and
for four phototropically abnormal mutants, S131, S132, C148 and S134. The

Figure V-1

Absorption spectrum of a mycelial sample of Phycomyces albino strain C2. Identical spectra were observed for mycelial samples of the albino photomutant strains (S131, S132, C148, S134). This spectrum exhibits the characteristic absorptions of reduced cytochromes. The peak at 602 nm is the α-band of one or more cytochrome a species, while that at 552 nm represents the α-bands of cytochromes b and c. The main peak at 422 nm is due to the Soret bands of b and c type cytochromes. The small shoulder at 440 nm is due to the Soret band of cytochrome a. The plateau between 470 and 495 nm may be due to small amounts of β-carotene in the sample.
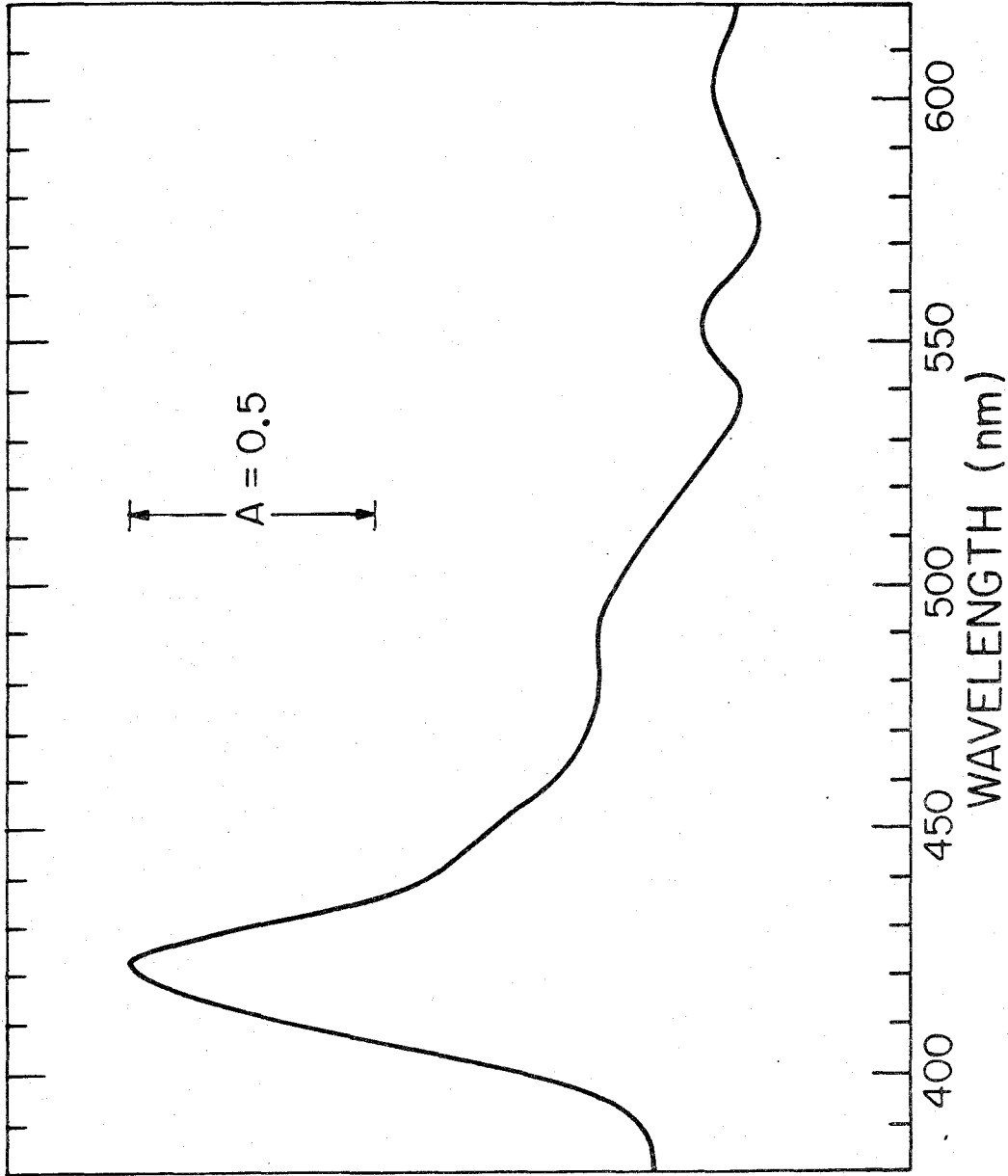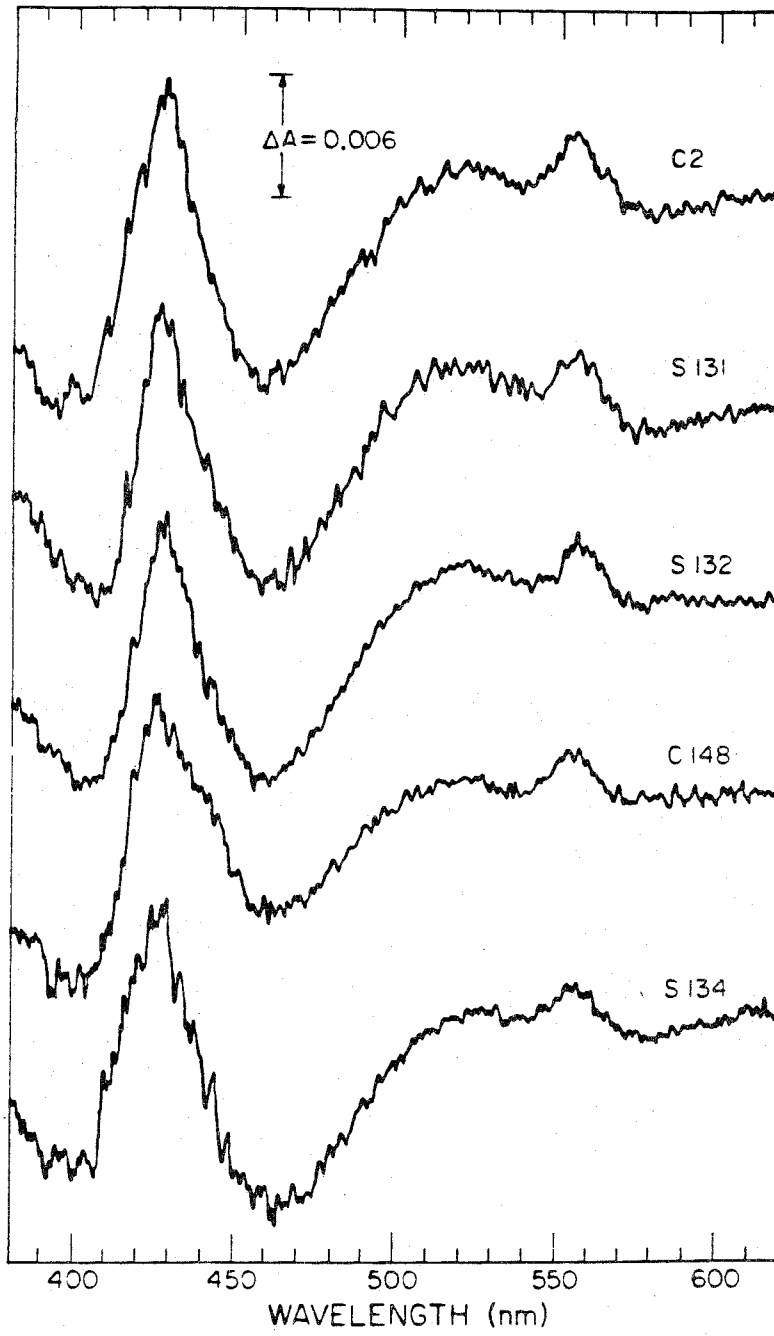
A = 0.5

WAVELENGTH (nm)

Figure V-2

Light-minus-dark difference spectra of mycelium from photophysiologically normal (C2) strains and photomutant strains (S131, S132, C148, S134) of <u>Phycomyces</u>. The actinic irradiation was 240 W/m^2 of broad-blue light for 30 seconds, thus giving a dose of 7200 $joules/m^2$.

light-induced absorbance changes found in C2 are definitely present in all four

of the mutants, and are substantially the same in all cases. These difference

spectra are similar again to those observed in Neurospora by Muñoz and Butler

(1975). The absorbance changes indicate the reduction of a cytochrome b, according

to the peaks at 427 nm and 556 nm, and the bleaching of riboflavin, according

to the drop in the spectrum at wavelengths below 500 nm.

Thus, none of the Phycomyces mutants studied showed any difference from

a phototropically normal strain with respect to the blue-light-induced cytochrome

absorbance changes. Since it is not known whether the mutants used are defective

at the initial point of the signal transduction pathway, no definite conclusions may be

drawn regarding the relationship of the absorbance changes with the physiological

blue-light receptor. There might still be undiscovered photomutants that also

exhibit defects in the light-induced cytochrome absorbance changes. All that can be

said now is that one of the best approaches to showing a connection between

Phycomyces blue light physiology and the blue-light-induced cytochrome absorbance

changes, namely the use of known photomutants, has yielded a negative result.

In figure V-3A is shown the absolute spectrum of a sample of whole HeLa

cells in suspension. The spectrum is very similar to the Phycomyces mycelium

spectrum shown in figure V-1. A light-minus-dark difference spectrum for the

same sample is shown in figure V-3B. This spectrum, too, is remarkably similar,

both qualitatively and quantitatively, to the corresponding spectra for Phycomyces

in figure V-2. Thus, similar light-induced optical absorbance changes appear in

cells derived from a human cervical carcinoma where it is highly unlikely that

light would play any physiological role. This demonstrates that these blue-light-

induced cytochrome absorbance changes are not specific to organisms with blue-

Figure V-3

(A) Absorption spectrum of HeLa cells in suspension. Like the Phycomyces spectrum in figure V-1, this spectrum also exhibits absorptions characteristic of reduced cytochromes.

(B) Light-minus-dark difference spectrum of HeLa cells in suspension. The actinic irradiation was the same as that for the Phycomyces samples in figure V-2, that is, 240 W/m^2 broad-blue light for 30 seconds.

A

A = 0.25

WAVELENGTH (nm)

B

ΔA = 0.003

WAVELENGTH (nm)
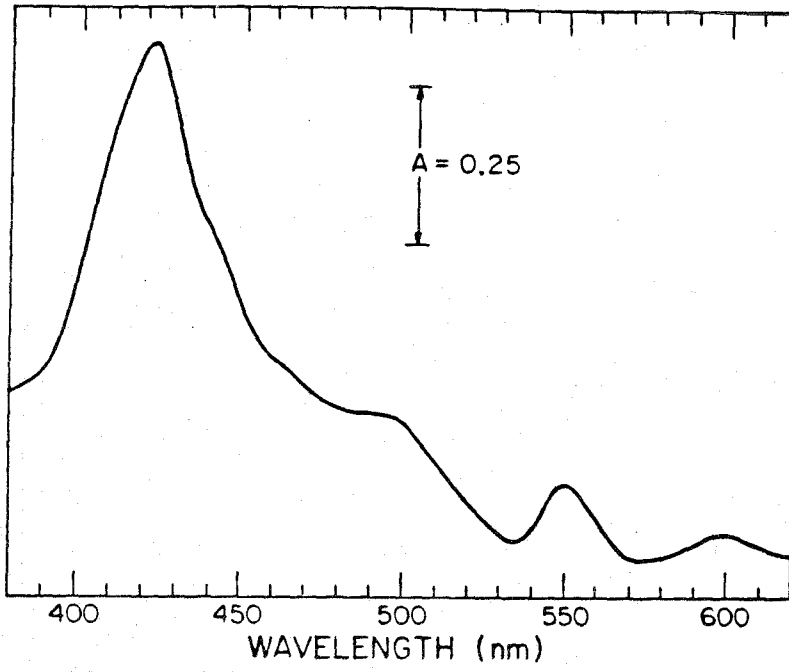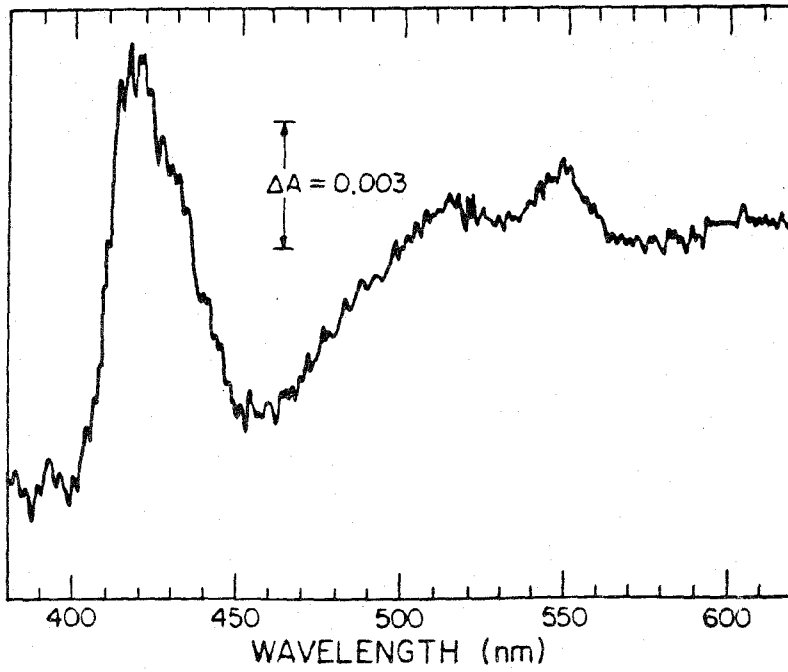
light physiological responses. It should also be noted that although Dictyostelium

shows a light-induced absorbance change indicative of a flavin-mediated reduction

of cytochrome b (Poff and Butler, 1974), this organism is not known to possess

any blue-light-induced physiological function (Poff and Butler, 1975). Dictyostelium

does, however, show phototaxis to green light and one can measure corresponding

green-light-induced absorbance changes (Poff et al., 1973). Further evidence

for the nonspecificity of these blue-light-induced optical absorbance changes

is provided by the in vitro flavin-cytochrome studies of Schmidt and Butler (1976),

which showed that flavins and cytochromes existing in free solution can undergo

a variety of mutual redox reactions, including blue-light-induced reduction of

cytochrome.

The dependence of the light-induced optical absorbance change upon

the intensity and duration of the actinic light was quantified as follows. The dual-

wavelength mode of the spectrophotometer was used to follow changes in the

absorbance at 427 nm relative to 457 nm (the cytochrome Soret region). This

relative change will be denoted by $\Delta A_{427} - \Delta A_{457}$, or, for brevity, by $\Delta \Delta A$. The

"response" is taken as the absorbance change during the course of the actinic

irradiation. It is deduced from the relative change in transmitted intensity at

the two wavelengths just after and before the actinic exposure. For a mycelial

sample, the absorbance change regenerated in the dark with a time constant of

$\tau = (100 \pm 10)$ seconds (mean plus-minus the standard deviation of the mean for

8 determinations).

The filled circles in figure V-4 represent the dependence of the optical

absorbance change upon the intensity of the broad-blue actinic irradiation, with

a fixed exposure time of 30 seconds. The crosses show similarly the dependence

Figure V-4

Dose-response data for the production of light-induced cytochrome optical absor-
bance changes in mycelium of Phycomyces strain C2. The response is the increase
in absorbance in the cytochrome Soret region. The actinic dose is given by $I_o \Delta t$,
where I_o (W/m^2) is the incident intensity of broad-blue light at the top of the
sample and Δt is the exposure time. The filled circles arose from a series of
measurements with $\Delta t = 30$ seconds and I_o ranging from 8 W/m^2 to 240 W/m^2.
The crosses arose from measurements with $I_o = 240$ W/m^2 and Δt ranging from
4 seconds to 360 seconds. Each data point represents the average from three
to six measurements. The curve is the result of a nonlinear least-squares fit to
equation V-3 using both series of data. In general, according to equation V-3,
reciprocity would not hold between intensity and exposure time. However, over
the region where the data overlap, it was found to hold reasonably well. The
full curve should be considered valid for $I = 240$ W/m^2.
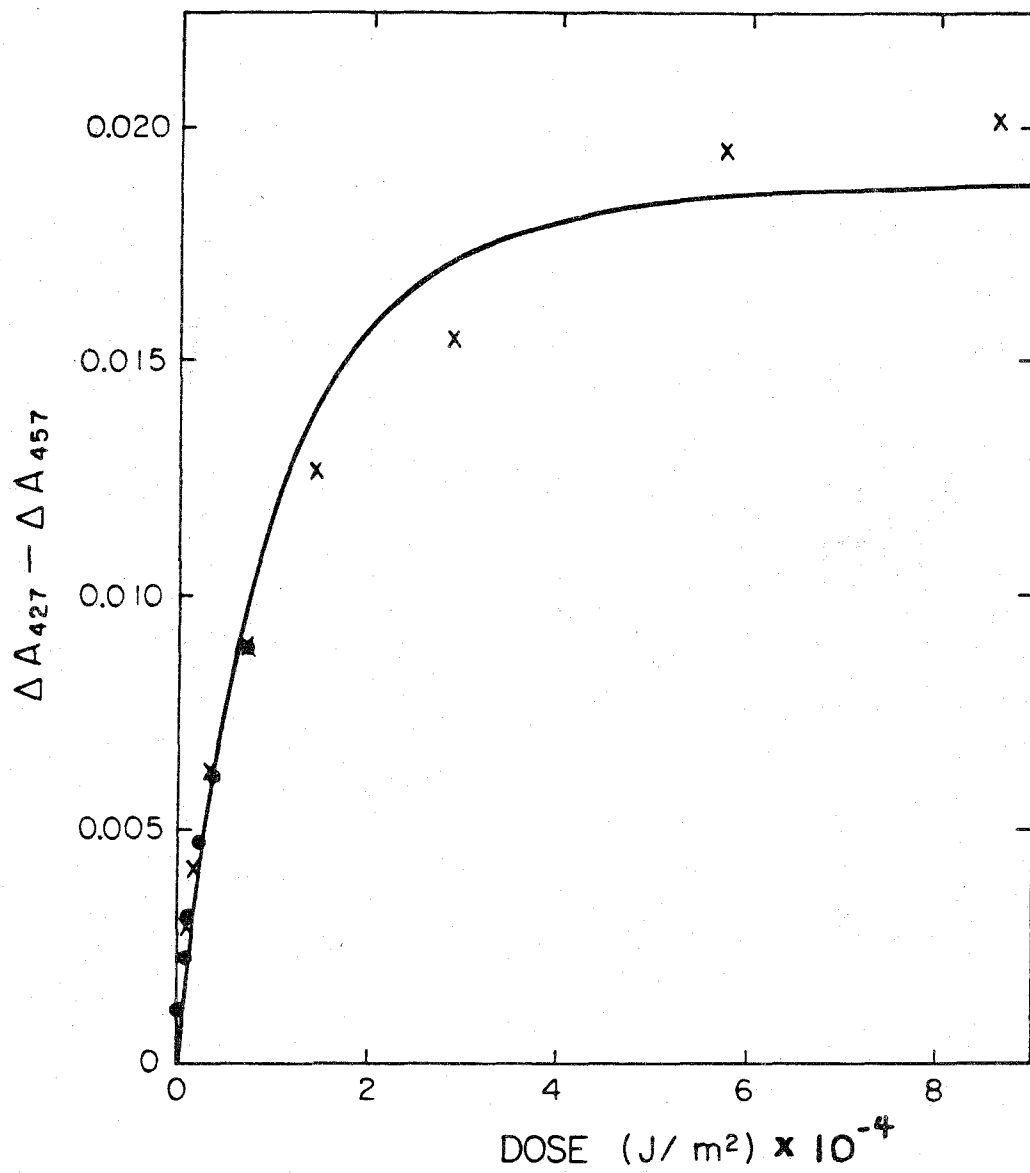
upon the exposure time for a fixed incident intensity of 240 W/m^2 broad-blue

light. These data were obtained on a single dense sample of C2 mycelium with

absorbance of 2.8 at 427 nm and 2.2 at 457 nm. The actinic exposures were spaced

with 10 minute dark intervals between them, in order to allow the sample to fully

regenerate. Each data point represents from three to six measurements spread

randomly through the experiment. In a given sample, the magnitude of the blue-

light-induced cytochrome absorbance changes vary somewhat as a function of

time, presumably due to drift in the oxidation state of the sample. To correct

for this during dose-response measurements which extended over a long period

of time (e.g., several hours), a "standard dose" was given for every fifth actinic

dose and the responses to these standard doses were used to construct a baseline

to which all other dose-response data were normalized. Although we sometimes

experienced variations of as much as 25% in the response to a standard dose, we

never experienced the supposed critical dependence of the magnitude of the cyto-

chrome absorbance changes on the age of the sample as reported for <u>Neurospora</u>

by Muñoz and Butler (1975). In general, the light-induced cytochrome absorbance

changes in <u>Phycomyces</u> were found to be eminently reproducible.

These dose-response data may be used to obtain an estimate of the quantum

yield of the presumed flavin photoreceptor for mediating these cytochrome absorbance

changes measured between 427 and 457 nm. In order to do this, we shall assume

that the inactivation and regeneration kinetics of the system are governed by

the first-order monomolecular photochemical equation (Appendix II),

$$\frac{dp}{dt} = -\tilde{\sigma} Ip + k(1 - p) \tag{V-1}$$

where p is the fraction of active photoreceptor pigment, $\tilde{\sigma}$ is the cross section for

inactivation and k is the regeneration rate constant. Before an actinic exposure,

I = 0 and p = 1. It will be <u>assumed</u> that the absorbance change $\Delta \Delta A$ is proportional

to the fraction of receptor pigment rendered inactive by light at the end of the

actinic pulse, that is, to 1 - p. For an actinic exposure with intensity I and duration

Δt, equation V-1 then yields the solution (equation AII-11).

$$\Delta \Delta A = A_o \frac{1 - \exp[-k\,\Delta t\,(1 + I/I_c)]}{1 + I_c/I} \qquad (V-2)$$

where $I_c = k/\bar{\sigma}$. The above solution is strictly valid only for a very thin sample.

For a thick sample of thickness h, the intensity will vary through the sample.

Then one may generalize the above equation to

$$\Delta \Delta A = A_o \int_0^h \frac{dz}{h} \frac{1 - \exp[-k\,\Delta t\,(1 + I(z)/I_c)]}{1 + I_c/I(z)} \qquad (V-3)$$

If, for example, the intensity diminished exponentially from a value I_o at the top

to a value I_h at the bottom, then one would have $I(z) = I_o \exp(-\alpha z)$ with $\alpha = (1/h)\ln$

(I_o/I_h). One may easily measure I_h and I_o and thus obtain an expression for $I(z)$

insofar as the approximation of exponential attenuation is valid.

A more accurate estimation of $I(z)$ was determined using the theory

of Kubelka (1948). This theory is concerned with the scattering and absorption

of light in dense turbid samples and corrects for the fact that the intensity near

the top of the sample includes not only the incident intensity but also the scattered

light directed upwards from lower parts of the sample. Parameters in the theory

may be derived by measuring the absorbance as a function of sample thickness

(Butler, 1962). We carried out such measurements on mycelial samples with 1

to 22 layers. The resultant numerical $I(z)$ computed according to Kubelka theory

was, in fact, very close to exponential (except near the bottom of the sample where the intensity was so small that it made little difference). Application of the Kubelka (1948) theory to our sample also yielded the result that the effective intensity just inside the top of the sample included contributions from both the incident light and light scattered back from within the sample and in fact was equal to about 1.6 times the incident intensity. Using this numerical form of $I(z)$ in the above equation for $\Delta \Delta A$, we have fit the data in figure V-4 by numerical integration and a nonlinear least-squares method.

From the least-squares fit, one obtains the estimates $A_o = 0.064 \pm 0.009$ and $I_c = (104 \pm 25)$ W/m^2. The curve in figure V-4 represents the results of the fit, i.e., the evaluation of equation V-3 with these parameters.

For the purposes of estimating the extinction coefficient ε from the critical intensity I_c, it is appropriate to consider monochromatic light of 455 nm, near the peak of the physiological action spectrum for the photoresponses of Phycomyces (figure I-1) and of the absorption spectrum of riboflavin (figure I-2). It was shown that for our filter combinations a given intensity of broad-blue light is equivalent to 455 nm light of 0.6 times that intensity. Thus the above value for I_c may be adjusted to 455 nm by multiplying by this factor yielding (63 ± 15) W/m^2 at 455 nm. In terms of quantum flux, this critical intensity corresponds to $(1.4 \pm 0.4) \times 10^{20}$ m^{-2} sec^{-1} at 455 nm. From the decay following absorbance changes, we have determined $k = (0.010 \pm 0.001)$ sec^{-1}. Thus using $\tilde{\sigma} = k/I_c$ and converting to conventional units for extinction coefficients according to $\varepsilon = 2.62 \times 10^{20} \sigma$, we obtain $\varepsilon_{LIAC} = (1.9 \pm 0.5) \times 10^2$ liter mole^{-1} cm^{-1}. The subscript LIAC indicates that this is a partial extinction coefficient for producing light-induced optical absorbance changes. The quantum yield for this process is thus given by

$\phi_{LIAC} = \varepsilon_{LIAC}/\varepsilon$, where $\varepsilon = 1.25 \times 10^4$ liter mole^{-1} cm^{-1} assuming a flavin receptor pigment. Finally, the estimate for the quantum yield is $\phi_{LIAC} = 0.015 \pm 0.004$.

Most biological photoreceptor systems have evolved for high sensitivity and so have quantum yields near unity for transduction of the energy resulting from the absorption of a photon. In the case of Phycomyces' light-growth response, the extinction coefficient of the physiological photoreceptor has been estimated by modeling the kinetics of inactivation and regeneration and has been found to match that of riboflavin (Lipson, 1975). If the physiological photoreceptor in Phycomyces is indeed a flavin, that result would indicate a quantum yield of near unity for the light-growth response. In contrast, the results of our work indicate a quantum yield for the presumed flavin-mediated absorbance changes in Phycomyces of only about 0.015. This low value for the quantum yield speaks further against the relevance of these blue-light-induced absorbance changes to the blue-light receptor for physiological responses.

An action spectrum was measured for the optical absorbance change induced by different wavelengths of actinic light. The action spectrum shown in figure V-5 is an "equal-response" action spectrum, subject to the assumption that the dose-response curve of figure V-4 is applicable for all wavelengths studied.

The sample was not the same one as that used for figure V-4 but was prepared similarly. A set of control measurements on the new sample between a standard wavelength (455 nm) and broad-blue illumination established the appropriate normalization of intensity to permit the use of the dose-response curve in figure V-4. The relative sensitivity was then determined as follows. Suppose a 30-second actinic exposure of intensity I_λ (in units of quanta m^{-2} sec^{-1})

Figure V-5

Action spectrum for the production of the increase in absorbance in the cytochrome

Soret region (absorbance change at 427 nm relative to 457 nm) in a mycelial sample

of the Phycomyces strain C2.
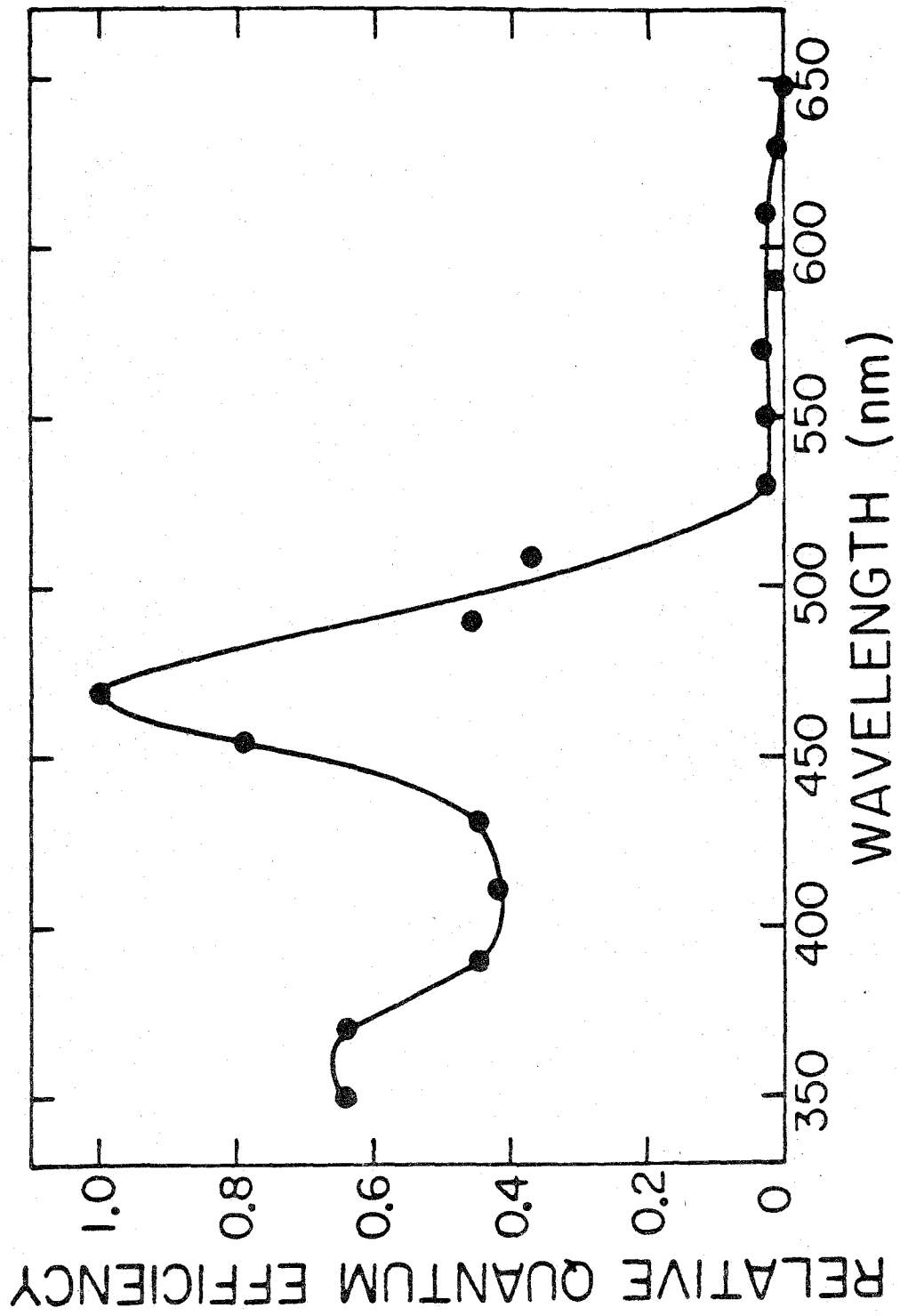
at a particular wavelength λ gave an absorbance change $\Delta \Delta A$. Then from the

dose-response curve, one found that an intensity $I_{B,\lambda}$ of broad-blue light would

have produced the same magnitude $\Delta \Delta A$. Then the relative quantum efficiency

would be proportional to $I_{B,\lambda}/I_\lambda$. The results, normalized to unity at the peak

of 469 nm, are shown in figure V-5. This action spectrum for the light-induced

optical absorbance changes is basically similar to those determined for Neurospora

(Muñoz and Butler, 1975) and for Dictyostelium (Poff and Butler, 1974) and indi-

cates a flavin as the photoreceptor responsible for these absorbance changes.

The slight effectiveness of wavelengths beyond 520 nm is not expected for a flavin

photoreceptor. However, this long-wavelength tail that appears to cut off around

600 nm may indicate that absorption by the cytochrome itself, and not only by

the flavin, can effect the reduction of the cytochrome. It should be noted that

Poff and Butler (1974) indicated slight sensitivity in Dictyostelium samples at

560 nm. Muñoz and Butler (1975) unfortunately did not show any data beyond

520 nm for their action spectrum. On the whole, though, we have verified that

the light-induced cytochrome optical absorbance changes, at least in the Soret

region, are produced with a typical blue-light action spectrum, indicating a flavin

as the photoreceptor.

Assuming the decrease in absorbance between 450 and 470 nm to be

due to the photobleaching of riboflavin, we may obtain a rough estimate of the

photoreceptor concentration. From figure V-2, this decrease in absorbance is

seen to be about 0.01 absorbance units. Thus $\Delta A = 0.01 = \epsilon c x$, where $\epsilon_{riboflavin} \approx$

10^4, c is the concentration of the receptor in moles per liter and x is the path

length of light through the sample. Even though the mycelial samples were only

about 0.5 cm thick, the actual path length of light through the samples is probably

between 1 and 10 cm because internal scattering increases the path length of light enormously (Butler, 1962). We thus obtain a value of 10^{-6}-10^{-7} molar for an estimate of the concentration of the flavin photoreceptor which mediates the light-induced absorbance changes in Phycomyces mycelium.

Retrospect and Prospect

Blue-light-induced cytochrome absorbance changes have been studied as a function of fractionation in Neurospora and, less reliably, in Zea mays (corn) (Brain et al., 1977). It was found that in Neurospora the absorbance changes are greatest in a fraction characterized as enriched in plasma membrane. A similar plasma membrane enriched fraction from Zea mays has been found to contain a b-type cytochrome (Jesaitis et al., 1977). Moreover, b-type cytochrome has also been detected in plasma membrane enriched fractions from Phycomyces, Neurospora and Dictyostelium (Schmidt et al., 1977a); the photoreducible b-type cytochrome from Dictyostelium has recently been purified by Manabe and Poff (1978). Although the plasma membrane fraction of Schmidt et al. (1977a) from Phycomyces also contained flavin, these authors detected no blue-light-induced cytochrome absorbance changes. They hypothesize that their failure to observe light-induced absorbance changes was due to most of the flavin associated with the plasma membrane in vivo becoming washed off during the isolation procedure. If there is any physiological significance to the flavin-mediated cytochrome absorbance changes, an even more likely possibility is that the blue-light sensitive photosystem had not yet developed in the immature (grown only 15 hours after inoculation of spores) specimens studied by Schmidt et al. (1977a). We also looked for blue-light-induced absorbance changes in Phycomyces grown from spores for 24 hours and found only insignificant and irreproducible effects.

The question of whether the flavin-mediated cytochrome absorbance changes observed in these several organisms are indeed related to physiological photochemistry is still unresolved. The facts that they occur with a low quantum yield in Phycomyces, that they occur in human cervical carcinoma HeLa cells, and that they occur in Dictyostelium, an organism which does not possess any known blue-light photophysiology (Poff and Butler, 1975), might argue against their being physiologically relevant. The observed absorbance changes may be due to light-stimulated electron transfer within an enzyme or enzymes unrelated to the physiological photoreceptor. Enzymes are known which contain both flavins and hemes, for example, the flavocytochrome b_2 from yeast (Appleby and Morton, 1954). Furthermore, flavins and cytochromes exist in close proximity within the mitochondrial respiratory chain. Indeed, irradiation of flavin-supplemented respiratory enzyme complexes from beef heart mitochondria has been found to result in the reduction of cytochrome b (Ninnemann et al., 1977). Moreover, model studies with flavins and cytochromes in solution have shown a variety of mutual redox reactions, including blue-light-induced reduction of cytochrome (Schmidt and Butler, 1976). These facts serve to point out the nonspecificity of light-induced cytochrome absorbance changes. (The nonspecific photoreactivity of riboflavin shows itself again in the observation that endogenous flavins act as photoreceptors for oxidative damage to human lung fibroblasts in culture (Pereira et al., 1976) and possibly for production of abnormal swimming behavior in the bacteria Escherichia coli and Salmonella typhimurium [Macnab and Koshland, 1974; Taylor and Koshland, 1975].) However, the facts that the absorbance changes are greatest in plasma membrane enriched fractions and that the plasma membranes from the fungi in question contain cytochrome b might argue in favor of the absorbance

changes being physiologically relevant. These findings implicate the involvement of a nonmitochondrial cytochrome b and place the site of action at the plasma membrane, a very reasonable place for the physiological blue-light receptor to be.

Finally, we should note the indications (Brain and Briggs, 1976; Brain et al., 1977) that the so-called poky mutation in Neurospora, which causes a deficiency of cytochrome b, is accompanied by a deficiency in the light-induced cytochrome absorbance changes and by an impairment of physiological sensitivity as measured by entrainment of the circadian rhythm of conidiation. This is an interesting observation and if substantiated will provide the first connection between the light-induced cytochrome absorbance changes and blue-light photophysiological response.

In view of the photoreactivity of flavins and of their ubiquity among living organisms, as long as the only connection between the receptor for physiological responses to blue light and the receptor for blue-light-induced cytochrome absorbance changes is the similarity of their respective action spectra (that is, both indicative of a flavin), correlation of the two cannot be made with any degree of confidence.

Chapter Six

TWO ATTEMPTS TO PROBE THE PHOTORECEPTOR:
ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY AND
FLUORESCENCE LIFETIME SPECTROSCOPY

Introduction: Electron Paramagnetic Resonance

Atoms or molecules having unpaired electrons possess an intrinsic magnetic moment and are said to be paramagnetic. The unpaired electrons may be detected by electron paramagnetic resonance spectroscopy (EPR). The substance to be studied is placed in a magnetic field; this forces the magnetic moment of the unpaired electrons to assume one of a small number of orientations which have different energies relative to each other. These energy differences are in the range of microwave quantum energies and thus transitions between different magnetic energy levels may be induced by irradiating the sample with microwaves of appropriate energy. The microwave absorptions that are measured in EPR spectroscopy can provide information about the nature and environment of the species having the unpaired electrons.

Most of the electrons in atoms and molecules do not give rise to EPR signals. This is because most electrons are paired so that the net spin magnetic moment of the species is zero. Substances having unpaired electrons (paramagnetic substances) include organic free radicals, transition metal ions and various inorganic molecules such as O_2 and NO.

If disruption of paired electron spins is part of the photochemical act of the Phycomyces photoreceptor, then EPR spectroscopy may be able to serve as a probe of the nature of the reacting species. The ground state of riboflavin has all electron spins paired and thus gives rise to no EPR absorption. However, flavin raised to the lowest triplet state by the action of light gives rise to an EPR signal (figures VI-1, 2). Furthermore, one possible photochemical pathway for a flavin would be to accept a single electron to form a flavin radical (also called semiquinone); such a radical would give rise to an EPR signal.

Figure VI-1

Electron paramagnetic resonance (EPR) spectra of riboflavin in H_2O at $77^\circ K$ (~ 0.25

mM concentration). Riboflavin in ethanol and riboflavin tetrabutyrate in ethanol

yielded spectra identical to the ones shown. A) Spectrum of unirradiated sample.

Any features, such as the broad absorption at high field, are due to impurities in

the cavity and dewar. B) Spectrum of sample continuously irradiated for 20 minutes

with broad blue light. The signal at $g = 4.12$ is due to the riboflavin triplet state.

It rapidly builds up to a maximum upon irradiation and decays with a lifetime of

0.185 second when irradiation is stopped (figure VI-2). The signal at $g = 2.01$ is

due to one or more free radical species which build up very slowly upon irradiation

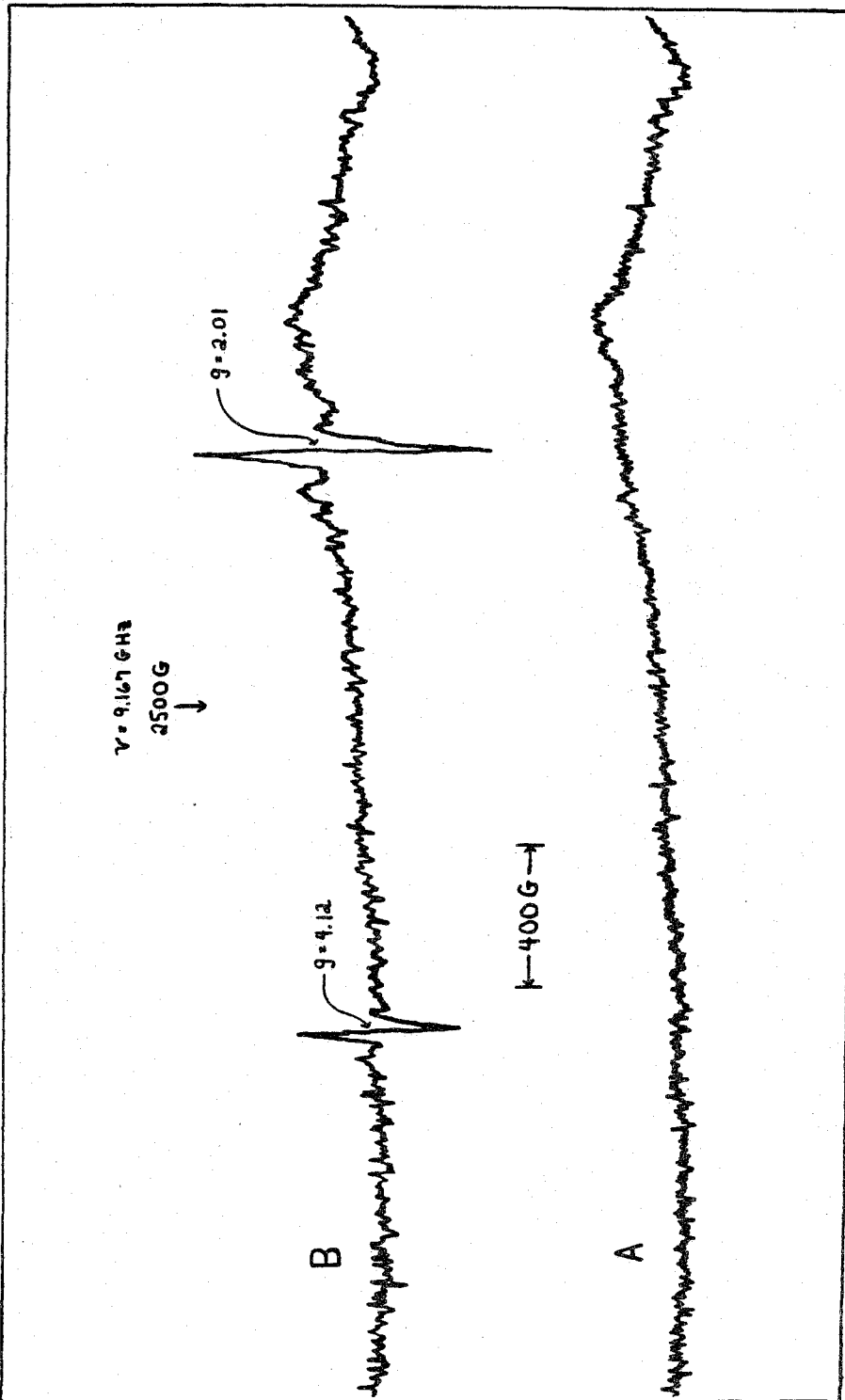and do not decay when irradiation is stopped.

Figure VI-2

Build up and decay of the riboflavin triplet state as irradiation is started and stopped.
Triplet state population was monitored by measuring the magnitude of the g = 4.12
EPR signal from riboflavin tetrabutyrate in ethanol at 77^OK. The e-fold lifetime
for the decay of the triplet EPR signal is 0.185 second. (This agrees well with
the phosphorescence lifetime of riboflavin measured by Sun et al. [1972] of 0.17
second.) The curve shown is the sum of 100 one second irradiations and decay curve
measurements so as to improve the signal-to-noise ratio.

LIGHT OFF

LIGHT ON

0.25 sec
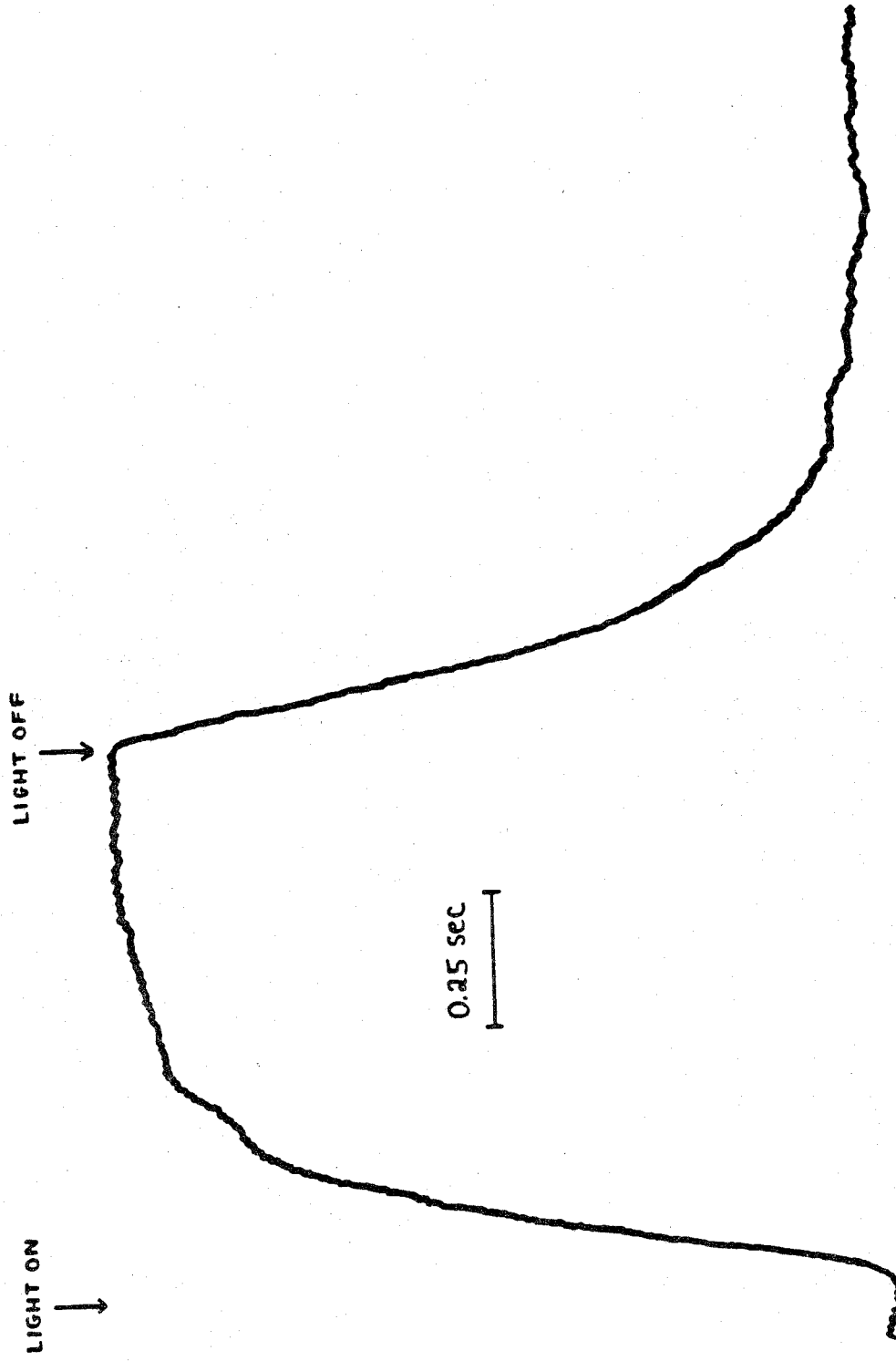
Methods: EPR

The energy separation between magnetic energy levels is given by

$$E = h\nu = g\beta H$$

where h is Planck's constant, ν is the microwave frequency at resonance, g is

the so-called g-value of the unpaired electron system (g = 2.0023 for a single

free electron), β is the electronic Bohr magneton (e h/4π m$_e$c = 9.273 x 10^{-21}

erg/gauss) and H is the magnetic field strength. In taking EPR spectra, the micro-

wave frequency is held constant and the magnetic field is scanned. EPR absorptions

for unpaired electron systems having various g-values will then appear at various

magnetic field strengths. To improve resolution, the magnetic field is rapidly

modulated with a small amplitude as it is scanned. This results in spectra being

displayed as derivatives of absorptions rather than directly as absorptions.

EPR spectra were taken on a Varian E-line spectrometer operating with

X-band ($\sim$9 GHz = $\sim$9 x 10^9 Hertz) microwaves and employing 100 kHz field

modulation. All spectra shown were taken using an 8 gauss modulation amplitude

and a microwave power of 10 mW. The spectrometer was equipped with an Air

Products LTD-3-110B liquid transfer system for low temperature studies.

All EPR spectra were measured at liquid nitrogen temperature, 77°K

(-196°C). Low temperature spectroscopy was necessary for several reasons.

Measurements in aqueous solution (and thus most measurements of biological

materials) are hampered by the large absorptions of water in the microwave region,

thus causing a huge loss of microwave power; ice does not have this drawback.

Moreover, spectra are sharper and more intense at low temperature, due to in-

creased populations in the states from which absorption occurs and decreased

efficiency of various relaxation processes. Low temperature should not affect the efficiency of the primary photochemistry of the Phycomyces receptor. For example, the photoinduced transfer of an electron from chlorophyll to the primary acceptor quinone in photosynthetic bacteria has been studied using EPR spectroscopy at temperatures as low as $2^{O}K$ (Okamura et al., 1975). The quantum yield of this process is one and has been found to be independent of temperature between $300^{O}K$ and $5^{O}K$ (Clayton and Yamamoto, 1976).

Samples for EPR spectroscopy were prepared in cylindrical quartz tubes of 4 mm inner diameter and 5 mm outer diameter.

When desired, samples were irradiated directly while inside the EPR cavity. The light source was a 200 watt xenon-mercury arc lamp running at 150 watts. Light from the xenon-mercury lamp was passed through 14 cm of 15% $CuSO_4$, two Schott KG-1 infrared cutoff filters, two ultraviolet cutoff filters and a Corning 5-61 blue filter and focused with a glass lens. This combination effectively removed the infrared and ultraviolet wavelengths from the light source and produced an actinic intensity of approximately 10 W/m^2 "broad blue" light at the sample.

The carA Phycomyces strain C2 was used for EPR studies. Mycelium and sporangiophores (stage I) were grown from spores for 5-6 days in the light on SIV agar medium. Mycelial samples for EPR spectroscopy were prepared by peeling about 400 mm^2 of mycelium from the surface of the agar, scraping off any agar which might be clinging to the mycelium, cutting the mycelium into pieces and stuffing it into an EPR sample tube. Sporangiophore samples were prepared by plucking about 200 stage I sporangiophores and stuffing them into an EPR sample tube. Samples were prepared, inserted into the EPR cavity and cooled to $77^{O}K$

in the dark or under red light so that the initial spectra of unirradiated samples do in fact represent spectra in the complete absence of (blue) light.

Results and Discussion: EPR

EPR spectra of Phycomyces mycelium and stage I sporangiophores are shown in figures VI-3 and VI-5. The principle features of these spectra are due to iron and manganese. Iron gives rise to a prominent absorption at $g \simeq 4.3$. Such a signal is known to occur in preparations of the iron storage protein ferritin; however, the signal is not thought to be due to the core iron in ferritin, but instead to Fe(III) either free or loosely bound to ferritin outside the core (Rollie Myers, personal communication, 1977). Phycomyces is rich in ferritin (David and Easterbrook, 1971; David, 1974).

Manganese, Mn(II), produces a structured signal centered at $g \simeq 2$ and having a total width of about 600 gauss. The structure of the Mn(II) signal is due to interactions of the unpaired electrons with the magnetic moment of the spin 5/2 ^{55}Mn nucleus. Comparison of the pure Mn(II) spectrum in figure VI-4B with the manganese signal in the Phycomyces spectrum in figure VI-4A indicates that the concentration of manganese in Phycomyces mycelium is more than 10^{-5} M. This represents an accumulation of at least a factor of ten over the concentration of Mn(II) in the SIV growth medium. The reason (if any) for this accumulation of manganese by Phycomyces is unknown. One possibility was that manganese was being taken up by ferritin in lieu of iron, but this was ruled out by growing Phycomyces on SIV medium enriched with 20 µg/ml iron (versus 0.2 µg/ml in regular SIV medium), well above the concentration known to saturate Phycomyces ferritin with iron (David, 1974). The resulting EPR spectrum showed a much larger $g \simeq 4.3$

Figure VI-3

EPR spectra of <u>Phycomyces</u> mycelium at 77°K. A) Spectrum of unirradiated (indeed, absolutely dark) sample. The absorption at g = 4.3 is due to iron. The structured absorptions centered at g = 2 are due to manganese (see figure VI-4). B) Light-minus-dark difference spectrum of mycelium. The sample was continuously irradiated with broad blue light for approximately 20 minutes. The growth of the g = 2.01 radical peak occurs very slowly upon irradiation and does not decay when irradiation is stopped. In order to improve the signal-to-noise ratio, the difference spectrum was computed by subtracting the sum of 30 dark sample spectra from the sum of 30 irradiated sample spectra.
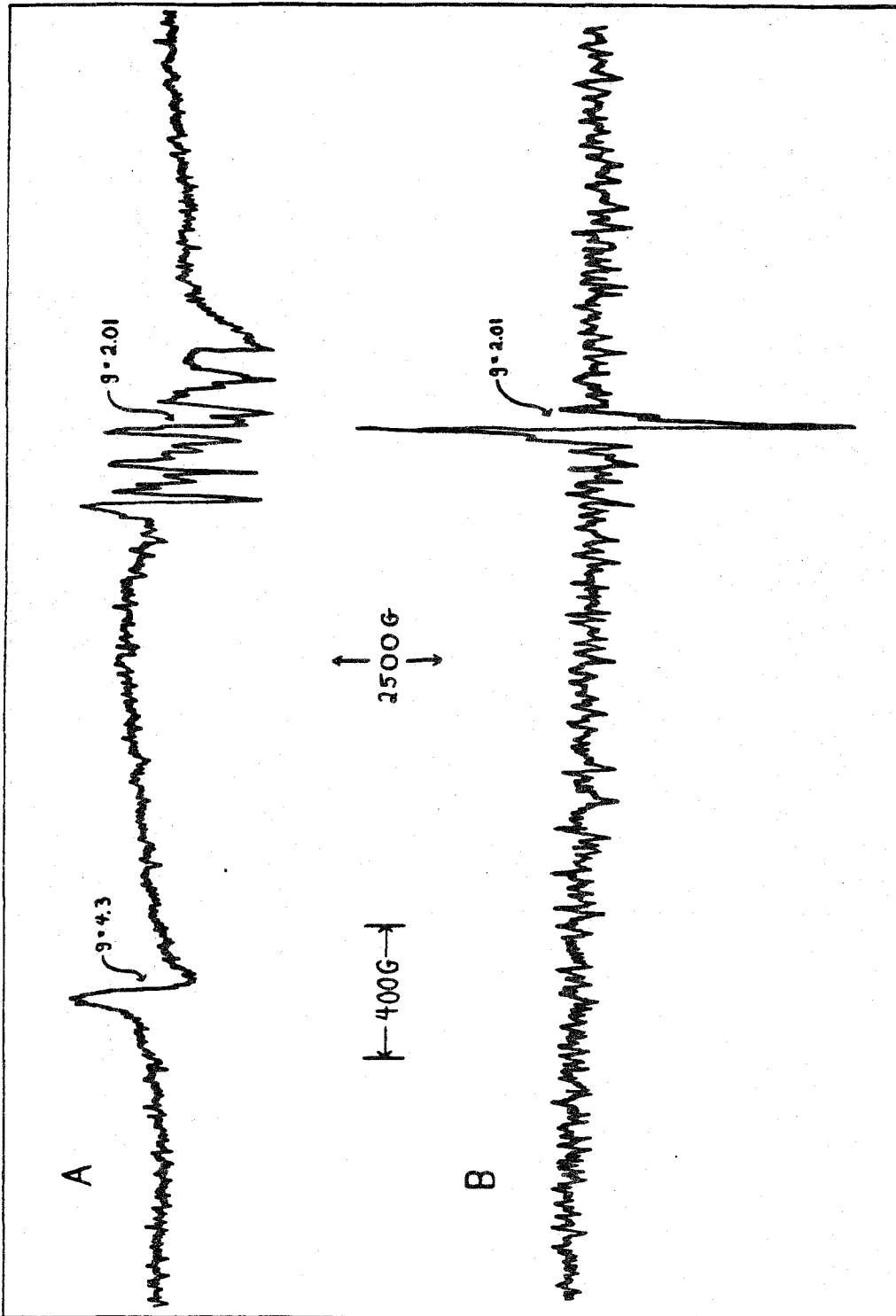
Figure VI-4

A) EPR spectrum of (unirradiated) <u>Phycomyces</u> mycelium at $77^{\circ}K$. The spectrum was taken at a microwave power of 10 mW and a receiver gain of 3.2×10^4.

B) EPR spectrum of $MnCl_2$ in H_2O (0.3 ml of 5×10^{-5} M) at $77^{\circ}K$. The spectrum was taken at a microwave power of 10 mW and a receiver gain of 10^4.
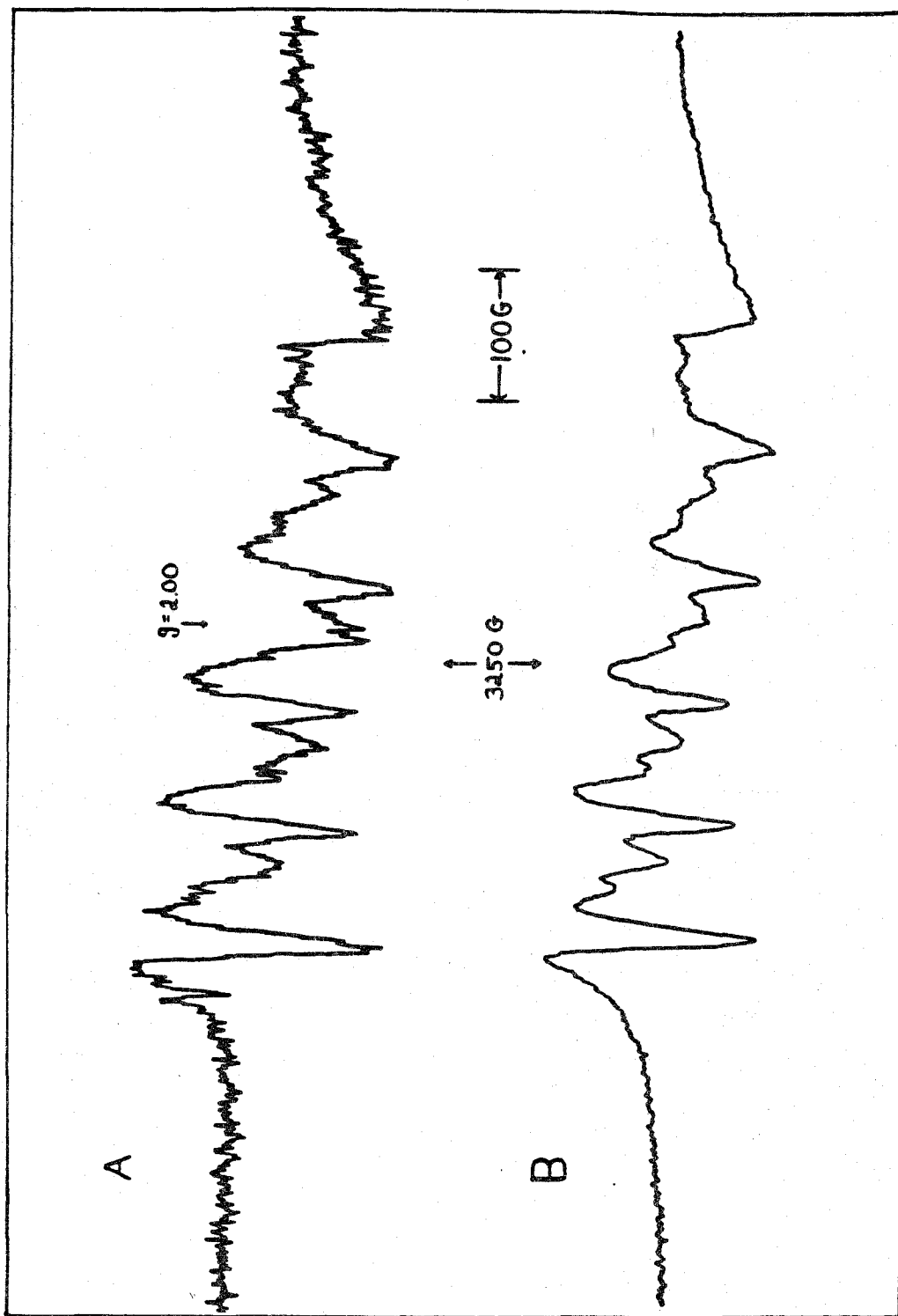
Figure VI-5

EPR spectra of <u>Phycomyces</u> sporangiophores at $77^{\circ}K$. A) Spectrum of unirradiated (indeed, absolutely dark) sample. Like mycelium, sporangiophores possess the g = 4.3 iron absorption peak and the structured absorptions centered at g = 2 due to manganese. In addition, unirradiated sporangiophores possess a g = 2.01 free radical peak. B) Spectrum of sample continuously irradiated for 20 minutes with broad blue light. The only difference between the irradiated and unirradiated sample spectra is an increase in the g = 2.01 radical peak. The growth of the radical peak occurs very slowly upon irradiation and does not decay when irradiation is stopped.
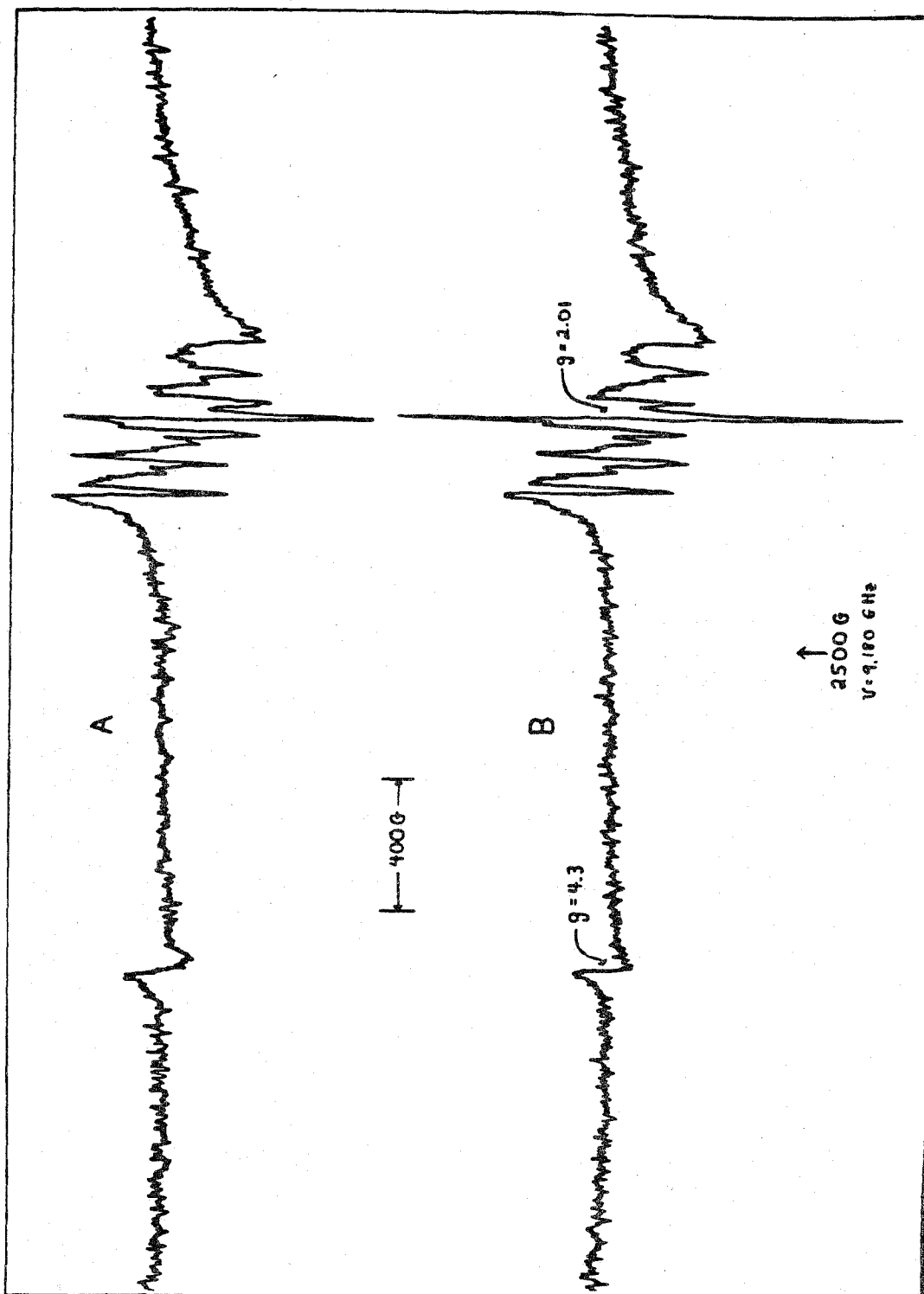
signal but no change in the size of the manganese signal. To check for any obvious

physiological effects of manganese in Phycomyces, SIV medium was prepared

lacking manganese altogether. Growth of Phycomyces on this manganese-free

medium was indistinguishable from growth on regular medium. The EPR spectrum

of Phycomyces grown this way showed no manganese signal. Thus, presence or

absence of trace amounts of manganese does not appear to affect the growth

of Phycomyces.

With respect to the manganese and iron peaks, the spectra of mycelium

and sporangiophores are identical. However, in contrast to mycelium, unilluminated

sporangiophores possess a g = 2.01 free radical peak of unknown origin.

No changes in EPR spectra were observed when samples were irradiated

with broad blue light for short periods of time (<0.5 minute). However, irradiation

of mycelium with broad blue light for a relatively long time (>5 minutes) causes

the appearance of a g = 2.01 free radical peak in the EPR spectrum (figure VI-3).

Likewise, irradiation of sporangiophores causes the already present free radical

peak to grow in size (figure VI-5). The growth of these radical peaks occurs only

very slowly upon irradiation, indicative of a very low quantum yield for their

formation. Furthermore, the free radical peaks do not decay when illumination

is stopped but remain present as long as the samples are kept near $77^{o}K$. Because

of the extremely low induction efficiency, these light-induced EPR free radical

signals do not appear to represent any efficient primary photochemistry which

may be associated with the Phycomyces blue light receptor. The signals might

in fact be due to the production of flavin radicals by light, for there would be

many flavins in the whole cell preparations of mycelium and sporangiophores being

studied. It has been known for two decades that blue light can induce the formation

of radical species in flavoproteins (see, for examples, Commoner and Lippincott, 1958; Massey and Palmer, 1966; Watari et al., 1966; Palmer et al., 1971).

The absence of triplet and free radical EPR signals which are efficiently produced by blue light in samples of Phycomyces should not be construed as negative evidence of any sort with regard to the nature of the photochemistry performed by the Phycomyces photoreceptor. A major shortcoming of the EPR spectroscopy applied to this problem is that of concentration. Although the concentration of the Phycomyces photoreceptor in vivo is unknown, an estimate of 10^{-7} M has been suggested for sporangiophores by Meissner and Delbrück (1968). Similarly, the concentration of the flavin photoreceptor which mediates the light-induced cytochrome optical absorbance changes in Phycomyces mycelium (and in HeLa cells) appears to be about 10^{-7} M (Chapter V). Using this number for the photoreceptor concentration and multiplying by the size of the sample which receives irradiation in the EPR cavity, one arrives at a figure of about 10^{11} for the number of receptor molecules which are exposed to excitation light. This is at or below the lower limit for detection of EPR signals by the spectrometer. Attempts to concentrate the photoreceptor by fractionating Phycomyces mycelium and looking at the membrane-enriched fraction resulted in complete loss of the blue-light-induced EPR signal.

Thus, EPR spectroscopy does not serve as a useful probe for the blue light receptor, at least at this time. It is quite probable that once an assay better able to discriminate the photoreceptor flavin from other flavins is available, EPR spectroscopy might be used as a useful probe of the receptor's primary photochemistry.

Introduction: Fluorescence

While free flavins are quite fluorescent, flavoproteins in general are
not because the flavin excited state is quenched through interactions with aromatic
amino acid residues in the protein (McCormick, 1977). The photoreceptor flavin
should not have its excited state quenched by chromophore-protein interactions
since it will need to use excitation by light to initiate signal transduction. Thus,
it is possible that the photoreceptor flavin <u>might</u> be at least slightly fluorescent.
However, its fluorescence lifetime might be shortened relative to that of free
flavin, indicative of a depopulation of the fluorescent excited state via an efficient
photochemical mechanism. Thus, the fluorescence lifetime of the photoreceptor
flavin <u>might</u> provide the basis for an assay whereby the photoreceptor could be
followed through procedures which result in its eventual purification.

Methods: Fluorescence

<u>Phycomyces</u> wall-membrane and membrane-enriched samples were pre-
pared according to the fractionation method detailed in chapter II. Samples for
spectroscopy, being quite turbid, were contained in triangular quartz cells so that
emission was collected from the same surface where the excitation beam fell.

Fluorescence lifetimes were measured by phase-modulation fluorometry
using a SLM model 480 subnanosecond phase-modulation fluorometer (SLM Inc.,
Champagne-Urbana, Illinois). The excitation source was a 1000 W xenon arc lamp
filtered through a 442 nm interference filter (10 nm bandwidth). Emission was
monitored through a Corning 3-71 yellow filter in order to block the excitation
light.

In a phase-modulation fluorometer, the excitation light is modulated in amplitude at a frequency of about 30 MHz. The fluorescence emission from the excited species will thus also be modulated in amplitude. However, because the emitting species has a finite fluorescence lifetime, the modulation of the fluorescence emission will lag behind that of the excitation light. It is possible to determine the lifetime of the emitting species either by a measurement of the phase lag between excitation and emission (phase method) or by a measurement of the relative amplitude of modulation of excitation and emission (modulation method) (Spencer and Weber, 1969; Spencer, 1970). For a homogeneous sample (i.e., one consisting of a single emitting species), the lifetime as measured by the phase and modulation methods will be the same. However, for a heterogeneous emitting population, the lifetime measured by the modulation method will be longer than the weighted average of the component lifetimes, whereas the lifetime determined by the phase method will be shorter than the weighted average of the component lifetimes (Spencer, 1970).

Results and Discussion: Fluorescence

Fluorescence emission spectra were measured for several Phycomyces samples consisting of wall-membrane preparations from the wild-type NRRL1555, the albino strain C2, and the mad mutant strains C47 (madA) and C114 (madB). Emission spectra were identical for all these samples (figure VI-6) and showed a characteristic flavin fluorescence with an emission maximum at about 525 nm. Emission spectra of plasma membrane-enriched fractions from NRRL1555 and C2 were identical in shape to those of the wall-membrane fractions (figure VI-6) but appeared to have their emission maximum shifted to about 518 nm.

Figure VI-6

Fluorescence emission spectrum of wall-membrane fraction of <u>Phycomyces</u> (spectrum

is uncorrected for the spectral response of the detector). The sample, contained

in a triangular quartz cell, was excited with 442 nm light (10 nm bandwidth inter-

ference filter). Fluorescence was observed through a Corning 3-71 yellow filter

in order to block the excitation light. The fluorescence spectra of the wall-membrane

preparation from all <u>Phycomyces</u> stains studied were identical, with emission maxima

at about 525 nm. Fluorescence spectra from the membrane-enriched preparations

were identical in shape to one shown but had their emission maximum shifted to
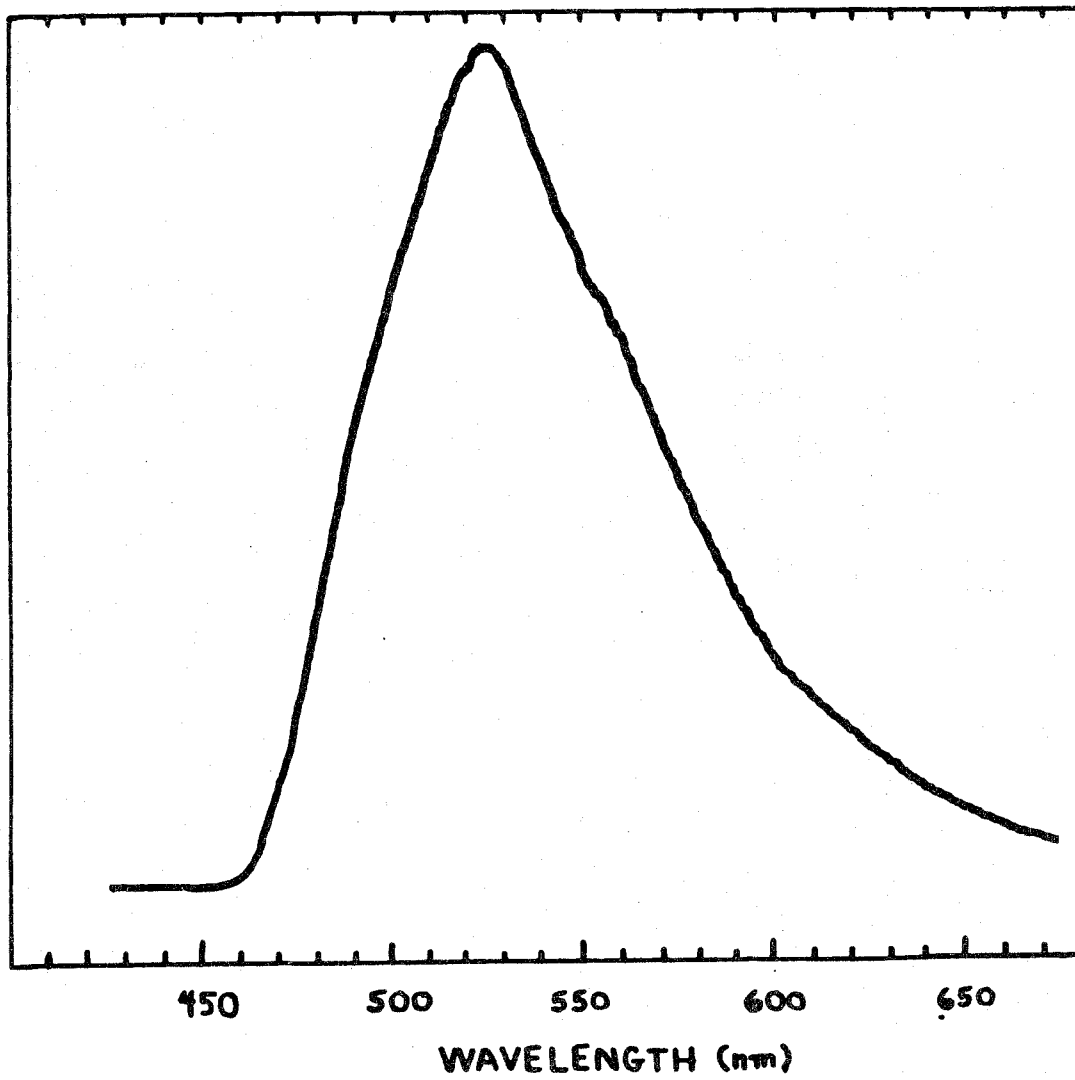
about 518 nm.

WAVELENGTH (nm)

Table VI-1 shows the fluorescence lifetimes measured for the various

Phycomyces samples. Each entry is the average of approximately 30 lifetime

measurements. As explained in the Methods section of this chapter, the fact that

the phase and modulation values differ indicates the samples are heterogeneous,

i.e., that two or more fluorescent substances having different lifetimes are emitting.

The species with the shortest fluorescence lifetime can be expected to have a

lifetime shorter than the phase value for the heterogeneous mixture, and thus

the data in table VI-1 suggest the presence of a fluorescent species in most of

the samples having a lifetime less than about 2 ns. Measurement of the fluores-

cence decay by pulse spectroscopy also suggests the presence of a species having

a fluorescence lifetime of approximately 1 ns in the wall-membrane and plasma

membrane-enriched preparations from NRRL1555 and C2. However, interpretation

of pulse spectroscopic data of heterogeneous samples is very ambiguous at best.

Table VI-2 gives the fluorescence lifetimes of several flavin species.

All that can be said at this time is that the Phycomyces preparations exhibit flavin-

like fluorescence appearing to possess a lifetime shorter than those of most other

flavin species.

Whether such a short-lifetime flavin is associated with the photoreceptor

cannot be said. No test is yet available to specifically distinguish the photorecep-

tor's excited state properties in vitro. Even the hypothesis that the photoreceptor

flavin should be fluorescent and possess a relatively short fluorescent lifetime

involves several, perhaps unwarranted, assumptions. For example, free riboflavin

has a high quantum yield ($\sim$0.7) for intersystem crossing from the excited singlet

manifold to the lowest triplet state; if the photochemistry leading to physiological

response occurs exclusively from the triplet state, then a quantum yield of 0.7

Table VI-1

Fluorescence lifetimes for wall-membrane and

membrane-enriched preparations of <u>Phycomyces</u>

Preparation	Fluorescence lifetimes	
	by phase	by modulation
NRRL1555 Wall-membrane	1.7 ± 0.2	3.3 ± 0.3
NRRL1555 Membrane-enriched	1.8 ± 0.3	2.8 ± 0.3
C2 Wall-membrane	2.5 ± 0.2	4.6 ± 0.2
C2 Membrane-enriched	1.9 ± 0.3	3.8 ± 0.2
C2 Sporangiophores wall-membrane	3.4 ± 0.3	5.9 ± 0.3
C47 (<u>madA</u>) wall-membrane	2.0 ± 0.2	4.8 ± 0.2
C114 (<u>madB</u>) wall-membrane	2.3 ± 0.1	4.5 ± 0.2

Table VI-2

Fluorescence lifetimes of flavin species

Flavin	Fluorescence lifetime (nanoseconds)	Reference
Lumiflavin in pH 7 buffer	4.8	1
Riboflavin in ethanol	5.6	2
FMN in H_2O or pH 7 buffer	4.6	1, 3
FAD in H_2O or pH 7 buffer	2.3	1, 3
D-amino acid oxidase in pH 8.2 buffer	2.1	3
Lipoamide dehydrogenase in pH 7.2 buffer at $21^\circ C$	0.8, 3.4	4, 5

Fluorescence lifetimes listed above were measured by phase spectroscopy (which agrees with modulation for homogeneous samples). An exceptional case is that of lipoamide dehydrogenase; it contains two FAD chromophores which fluoresce with different lifetimes, 0.8 and 3.4 ns as measured by pulse spectroscopy.

References:

(1) Spencer and Weber, 1969

(2) Fugate and Song, 1976

(3) Song et al., 1978

(4) Wahl et al., 1975

(5) Veeger et al., 1976

should be sufficiently large to maintain a high triplet population; thus

a decrease in the lifetime of the fluorescent singlet state (because of an increase

in the intersystem crossing quantum yield) may not appear. If the photochemistry

occurs from the singlet state, it might or might not have a major effect on fluores-

cence; however, if it does affect fluorescence, it might well quench it so completely

that only high-resolution photon counting spectroscopy of purified receptor complex

would be capable of detecting fluorescence (such is the case with bacteriorhodopsin

[Lewis et al., 1976]). Furthermore, a flavoprotein exhibiting fluorescence with

a lifetime shorter than that of free flavins is by no means indicative of the photo-

receptor. For example, the flavoprotein lipoamide dehydrogenase contains two

FAD chromophores, one of which fluoresces with a lifetime of about 0.8 ns. Lipo-

amide dehydrogenase is part of the pyruvate dehydrogenase multienzyme complex

leading into the tricarboxylic acid cycle in the inner compartment of the mito-

chondria. It is certainly far-removed from being a photoreceptor.

The motivation behind carrying out fluorescence spectroscopy experiments

with Phycomyces comes from similar studies conducted by Song et al. (1978) on

the so-called plasma membrane-enriched fraction from corn coleoptiles. These

authors claim to measure a short-lifetime (<1 ns) fluorescent flavin which they

associate with the photoreceptor for phototropism in corn coleoptiles. However,

their data are just as unconvincing as are those for Phycomyces. The upshot is

that there is no clear indication that fluorescence lifetime spectroscopy can yet

be used as a useful probe of the receptor's excited state properties in vitro or

as the basis for an assay to distinguish the photoreceptor flavin.

Addendum: Roseoflavin Fluorescence

In an attempt to see if the riboflavin analogue roseoflavin (in which the

8-methyl group in riboflavin is replaced with a dimethyl-amino group) was being

incorporated into membrane-associated flavoproteins of Phycomyces grown on

medium which contained the analogue, fluorescence emission spectra were taken

for the wall-membrane and membrane-enriched fraction of the wild-type NRRL1555

grown in both the presence and absence of roseoflavin. (Growth in the absence

of roseoflavin was as described in chapter II. Growth in the presence of roseo-

flavin was according to the same methods except that cultures were grown in

the dark [to prevent photodegradation of roseoflavin] for 8 days; the longer time

was necessary since roseoflavin inhibits mycelial growth somewhat.) Since the

fluorescence emission maximum of roseoflavin (and/or possible photodegradation

products of roseoflavin) is red-shifted about 20 nanometers relative to that of

riboflavin, one would expect to see at least some red-shift in the fluorescence

emission spectrum of membrane preparations of roseoflavin-grown Phycomyces

if the analogue is indeed incorporating into the flavin photoreceptor as the results

of Jayaram, Hamilton and Delbrück (unpublished, 1978) suggest. However, no

difference was detected in the emission spectra of membrane preparations of

Phycomyces grown in the presence and absence of roseoflavin. This may be due

to the possibility that it is the monomethyl-amino photolysis product of roseoflavin

(Matsui and Kasai, 1976) which is actually incorporating into the photoreceptor

and that none of this photolysis product was produced since the Phycomyces used

for spectral analysis was grown in the dark. Alternatively, it could mean that

the fluorescence spectra of membrane preparations contain no contributions from

the photoreceptor.

Chapter Seven

LIGHT-INDUCED CAROTENE SYNTHESIS IN PHYCOMYCES

It has long been known that visible light, in the presence of molecular oxygen and so-called photosensitizers, can damage various cells. The excited singlet species of molecular oxygen is now thought to be responsible for at least a large part of this damage (Foote, 1968). The commonly accepted sequence is as follows:

$$^1S \longrightarrow {}^1S* \longrightarrow {}^3S* \xrightarrow{\;{}^3O_2\;} {}^1S + {}^1O_2^* \longrightarrow \text{nonspecific oxidation}$$
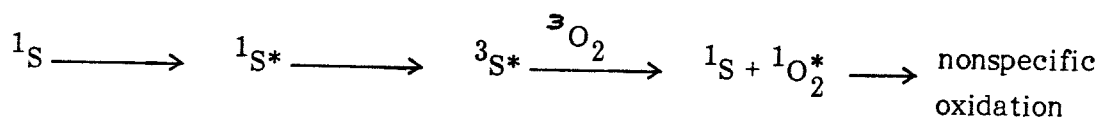
The photosensitizer, S, is a molecule which absorbs visible light and then transfers the excitation to its triplet state; the triplet species, $^3S*$, then reacts with molecular oxygen (which has a triplet ground state) to produce singlet oxygen, a powerful oxidizing agent which can create havoc in cells. In photosynthetic organisms, the role of the photosensitizer is filled admirably by chlorophyll. In nonphotosynthetic organisms, other molecules, such as riboflavin (Pereira et al., 1976), may play the role of endogenous photosensitizers.

Carotenoid pigments are widespread in nature, occurring among bacteria, protists, fungi, plants and animals. One of the major functions of carotenoids is that they confer upon the organism protection against the damaging effects of light and molecular oxygen (Krinsky, 1968, 1971; Burnett, 1976). They do this both by reacting with singlet oxygen and quenching its excited state (the oxygen is returned to the ground state species and the carotenoid dissipates the excitation energy as heat) and, in photosynthetic organisms, reacting with and quenching triplet chlorophyll (before singlet oxygen can be formed).

Since carotenoids serve such a photoprotective function, it is not altogether surprising that the synthesis of these pigments is often stimulated by light. Where there is light, there is need for protection by carotenoids. In some organisms,

for example the fungi <u>Fusarium</u> <u>aquaeductuum</u> and <u>Neurospora</u> <u>crassa</u>, production

of colored carotenoids appears to be strictly photoinduced; that is to say, only

very small quantities of colored carotenoids are produced in the dark. In other

organisms, such as the fungi <u>Phycomyces</u> and <u>Mucor</u>, substantial amounts of colored

carotenoids are produced even in the dark; however, carotenoid synthesis is further

enhanced in the light.

β-carotene is the principal carotenoid in <u>Phycomyces</u>. Only relatively

small amounts of other carotenoids are present in mycelium and sporangiophores

of the wild type. Why <u>Phycomyces</u> produces and accumulates such large quantities

of β-carotene is unknown. The pigment serves as a biosynthetic precursor to

trisporic acid-type molecules which are believed to be mating-type specific chemi-

cal messengers in the sexual interaction (Sutter, 1975, 1976). It also serves as

a precursor to sporopollenin, a polymer of β-carotene which occurs in the cell

walls of zygospores and sporangiophores (Furch and Gooday, 1978). However,

these roles should not require the large quantities of β-carotene present in

<u>Phycomyces</u>. β-carotene probably also plays a small photoprotective role in

<u>Phycomyces</u>.

Methods

<u>Phycomyces</u> was grown on petri plates containing SIV minimal medium

agar and was maintained so that a culture possessing a mycelial growing edge

was always available for use as a "mother" culture. For photoinduction studies,

5 cm petri plates containing SIV agar were inoculated in the center with a small

piece of mycelium from the growing edge of a "mother" culture. These plates

were incubated for 55 hours in the dark at 21-22°C. At the end of this incubation

period, the mycelium has covered the entire surface of the agar in a thin layer; no sporangiophores have yet been produced.

After 55 hours of incubation in the dark, culture plates were irradiated in a specially-built box designed to provide uniform blue-light irradiation to about 10 culture plates simultaneously. The light source consisted of sixteen 40 W tungsten-filament incandescent light bulbs (run at ∿100 volts). The incandescent light was directed through a sheet of diffusing plastic, a sheet of blue plastic (Rohm and Haas 2424 blue plexiglas: visible light transmission maximum at 465 nm, bandwidth ∿100 nm; also transmitted infrared), and a 15% aqueous solution of $CuSO_4$ to absorb heat. The resulting intensity of broad-band blue light at the place where the plates were irradiated was 0.065 W/m^2 (measured using two 2 mm thick Schott KG-1 infrared blocking filters over the photodiode). During the irradiation, the lids of the petri plates were removed in order to expose the cultures to air. If lids of the plates were left on during irradiation, erratic and irreproducible light-induced β-carotene synthesis (LICS) was obtained. Leaving lids off during the post-irradiation incubation period seemed to have little effect on Phycomyces LICS. The absence of oxygen during irradiation and/or during the post-irradiation incubation period is known to result in reduced LICS in the fungi Neurospora crassa (Zalokar, 1954) and Fusarium aquaeductuum (Rau, 1969, 1971). Lids of the unirradiated dark controls were also removed for ∿15 minutes in order to make light the only variable in the experiments. All manipulations of plates during the irradiation procedure were carried out either in the dark or under dim red light.

Following irradiation, plates were incubated again in the dark for 23 hours at 21-22°C. At the end of this incubation period, the β-carotene content of the cultures was measured using an in vivo spectrophotometric assay.

The spectrophotometric assay was carried out by passing a beam of white light (produced by a fan-cooled 250 W Sylvania tungsten-halogen lamp) first through an interference filter (455 nm for blue light, 633 nm for red light, 10 nm bandwidths; the intensities of the blue and red lights so produced were 0.3-0.4 W/m^2) and then through the culture plate to be assayed. After passing through the culture plate, the light was collected by a pin 10DP photodiode which produced a current proportional to the intensity of light being detected. The photocurrents produced by the blue and red lights after passing through a culture plate will be denoted by i_{bp} and i_{rp}, respectively. The photocurrents produced by the blue and red lights when no culture plate was present in the beam were also measured and will be denoted by i_b and i_r, respectively. (As the light source was powered through a line voltage stabilizer, this intensity did not change throughout the assay.) The absorbance of a culture at 455 nm (near the peak of the β-carotene absorption spectrum) is then given by

$$A_{blue} = \log \frac{i_b}{i_{bp}} \qquad (VII-1)$$

Similarly, the absorbance of a culture at 633 nm (where β-carotene has no absorbance, thus a measure of the scattering density of the culture) is given by

$$A_{red} = \log \frac{i_r}{i_{rp}} \qquad (VII-2)$$

A_{blue} is always greater than A_{red} since A_{blue} contains all the scattering components which gives rise to A_{red} plus an additional component due to the absorbance of β-carotene. (A_{blue} actually contains more contributions from scattering than does A_{red} since 455 nm light is scattered almost 4 times more effectively than

633 nm light. However, this scattering difference is constant and need not be further mentioned.) The difference

$$\Delta A = A_{blue} - A_{red} \qquad (VII-3)$$

isolates the component due to β-carotene absorbance.

In order to measure the amount of β-carotene induced by irradiation of Phycomyces, we must subtract off the absorbance due to the relatively large amount of β-carotene produced by Phycomyces even when grown in the dark. Thus, the relevant quantity to measure for the light-induced carotene synthesis (LICS) response is

$$A_{LICS} = \Delta A_{irradiated} - \Delta A_{dark\ grown} \qquad (VII-4)$$

Results and Discussion

Previous studies of β-carotene photoinduction in Phycomyces have used continuous lighting of cultures over a period of several days (Bergman et al., 1973). The resulting photoinduced β-carotene was assayed by extracting the culture with acetone and measuring the carotenoid content of the extract spectrophotometrically (see chapter III, Methods). Thus these procedures employ both ill-defined light doses and cumbersome extraction methods. A main result of the work described in this chapter was the development of a reproducible procedure for obtaining quantitative light-induced β-carotene synthesis (LICS) to well-defined, relatively small doses of blue light. In addition, a simple in vivo spectrophotometric assay for photoinduced β-carotene in Phycomyces was developed.

Figure VII-1 shows the time course of β-carotene synthesis in wild-type Phycomyces. From this, one sees that there is considerable β-carotene synthesis

Figure VII-1

The results of two experiments which measured the time course of β-carotene synthesis in mycelium of <u>Phycomyces</u> wild type NRRL1555. Plates were inoculated with mycelium, incubated for 55 hours in the dark, and then either irradiated or not irradiated with blue light. The abscissa represents incubation time (in hours) following irradation at t = 0. The ordinate represents carotene absorbance according to equation VII-3. Absorbance is normalized to 0 at t = 0 (55 hours after inoculation). Triangles, --- Δ---, represent plates irradiated for 3 minutes with 0.065 W/m^2 blue light (11.7 J/m^2) at t = 0 (photoinduced β-carotene synthesis plus dark synthesis); splotches, ---ᛘ---, represent unirradiated plates (dark synthesis); and filled circles, —— ● ——, represent the difference between irradiated and unirradiated plates (LICS). Each point is the average of 5 or more plates.
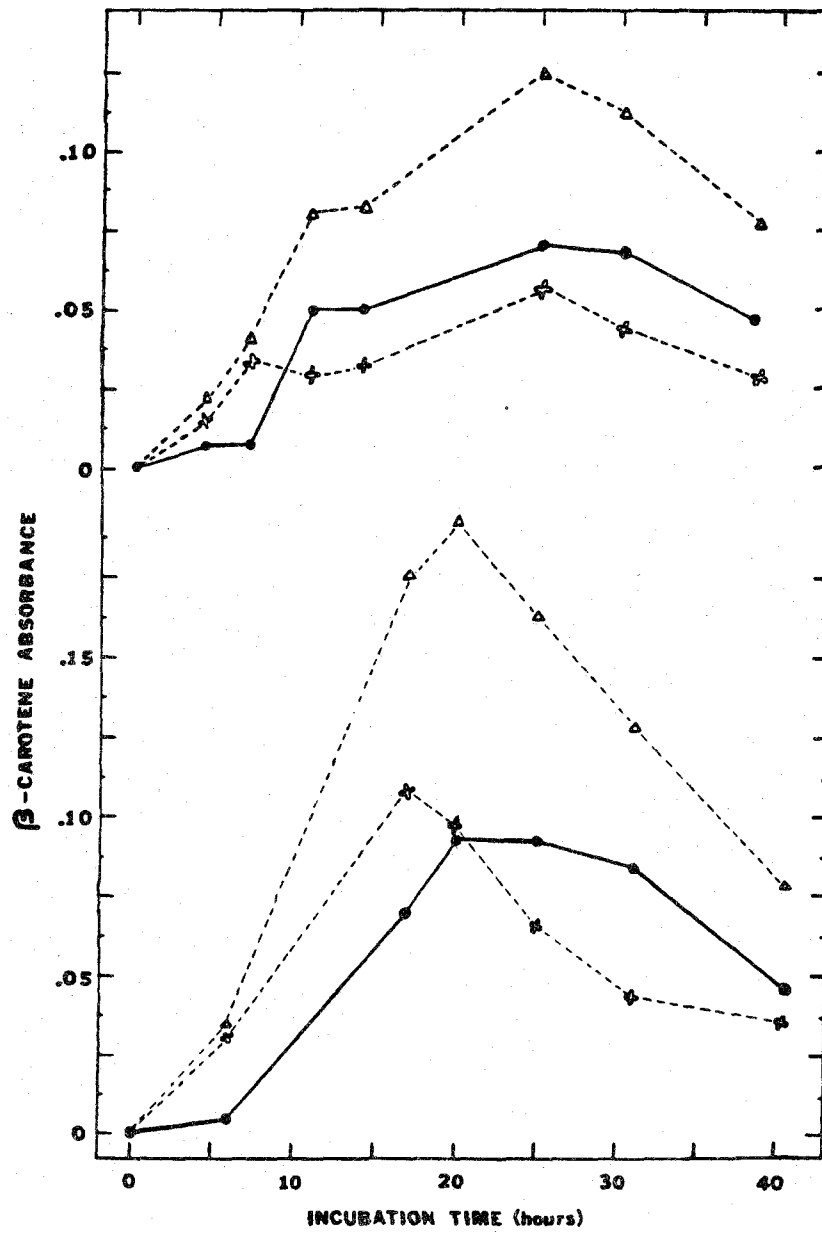
even in unirradiated cultures. One also sees that the light-induced β-carotene

synthesis (LICS) reaches a maximum approximately 20-25 hours after irradiation

of the culture. Thus, for the dose-response studies, carotene content was assayed

23 hours after the irradiation dose was administered. Figure VII-2 shows LICS

dose-response data for irradiation of wild-type mycelium with 0.065 W/m^2 blue

light; duration of irradiation varied between 0 and 30 minutes. The light-induced

carotene synthesis is seen to increase dramatically with increasing small doses

of light and then level off somewhat with further increase in dose. Unfortunately,

without a way to measure the regeneration rate of the photoreceptor mediating

LICS, it is impossible to estimate a receptor cross-section using the pigment

kinetics model outlined in Appendix II. The data shown in figure VII-2 may even

represent several components of induction. Indeed, experiments of Jayaram

(unpublished, 1978) have indicated that light-induced carotene synthesis in

Phycomyces mycelium occurs in two distinct components: a short irradiation

time (small dose) component which is insensitive to cycloheximide and actinomycin

D treatment (thus does not require new protein and RNA synthesis) and a long

irradiation time (large dose) component which is sensitive to cycloheximide and

actinomycin D (thus requires synthesis of new protein and RNA).

Figure VII-3 shows the dose-response data for LICS in four phototropically

defective mutant strains of Phycomyces, representing three complementation

groups: C21 madA, C47 madA, C114 madB and C68 madD. It is seen that LICS

in C68 is very similar to that in the wild type shown in figure VII-2. This is not

surprising since the madD mutation of C68 affects the output side of the sensory

pathway of phototropism (figure II-1) and thus would not be expected to have

altered mycelial responses. The data for the other three mutant strains, C21,

Figure VII-2

Dose-response data for the induction of β-carotene synthesis in wild (NRRL1555)
type <u>Phycomyces</u> mycelium by blue light. The response, A_{LICS}, is the absorbance
due to light-induced carotene synthesis (LICS) according to equation VII-4.
A_{LICS} was assayed 23 hours following irradiation. Zero represents the absorbance
of unirradiated mycelium. The dose is given by $I\Delta t$ where Δt is the duration of
irradiation (irradiation time) and $I = 0.065$ W/m^2 is the intensity of the broad blue
radiation at the surface of the mycelium; thus, for example, one minute of
irradiation equals a dose of $(0.065$ W/m$^2)$ $(60$ sec$) = 3.9$ J/m^2. Each point represents
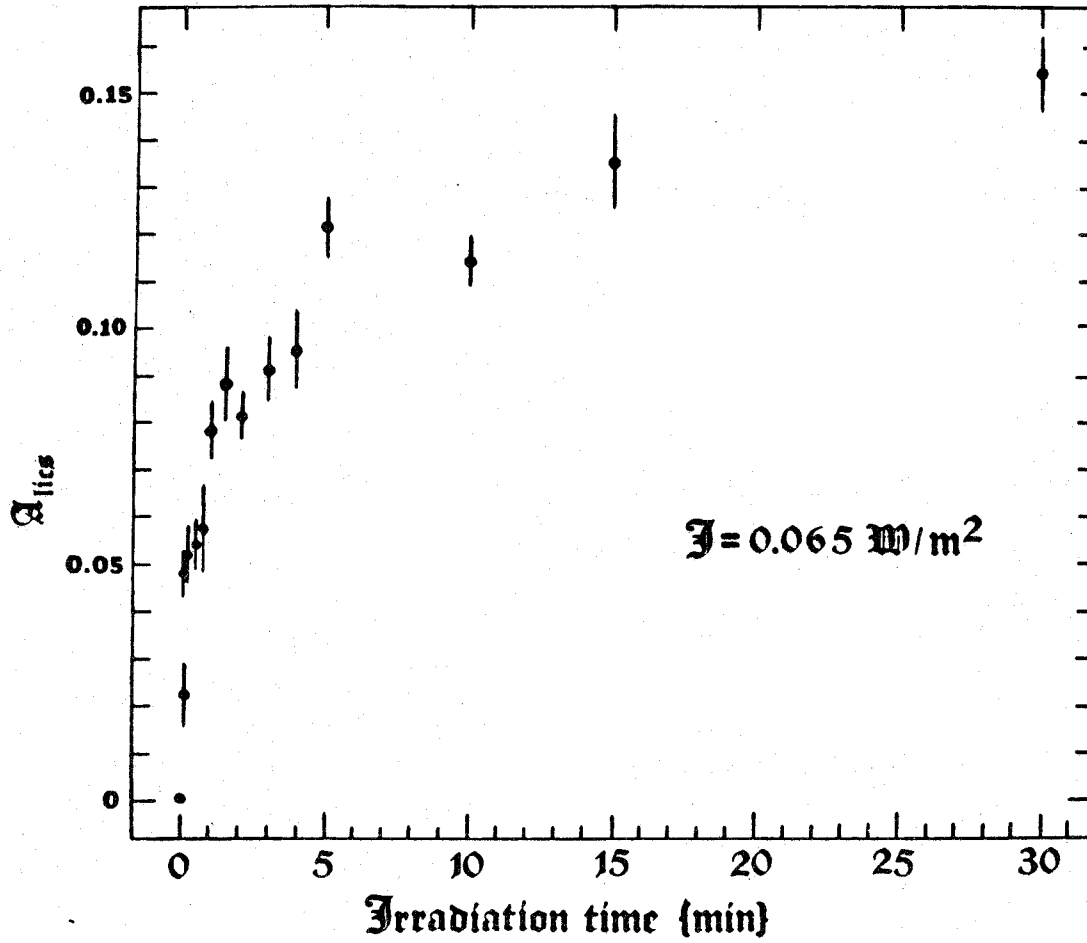the average of from 5 to 25 plates. Error bars are $\pm$ the standard deviation of
the mean.

Figure VII-3

Dose-response data for the induction of β-carotene synthesis in four phototropically

defective strains of <u>Phycomyces</u>: C21 <u>madA</u>, C47 <u>madA</u>, C114 <u>madB</u>, and C68 <u>madD</u>.

Conditions were the same as those in figure VII-2. The abscissa represents the

duration of irradiation with 0.065 W/m^2 broad blue light. The ordinate represents

A_{LICS} according to equation VII-4. Each point is the average of 10 or more plates.

Standard deviations of the mean for the data points are approximately 0.01 absorbance

unit.
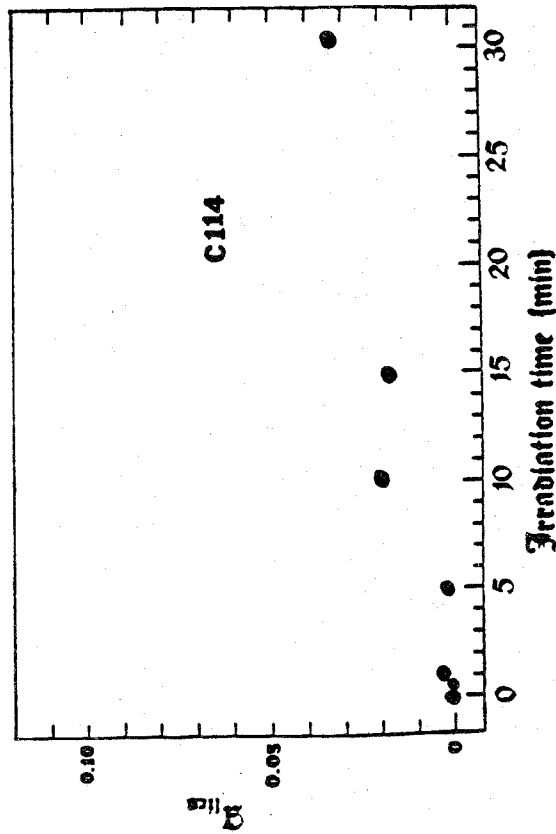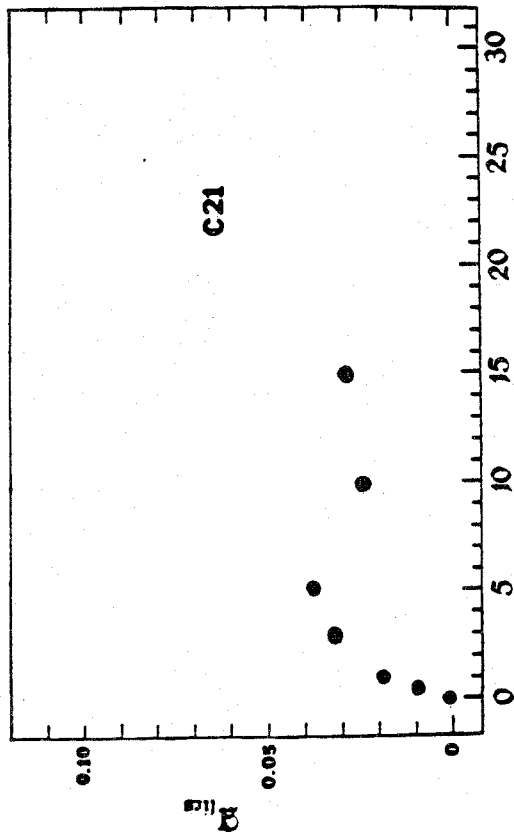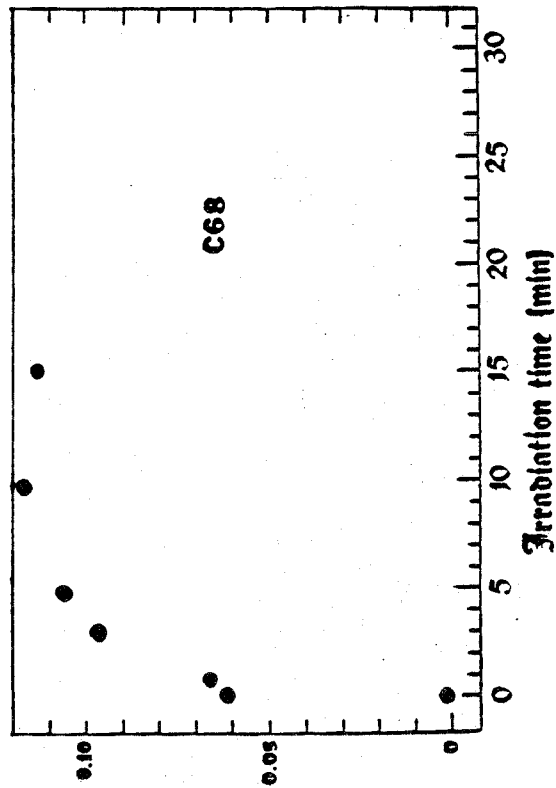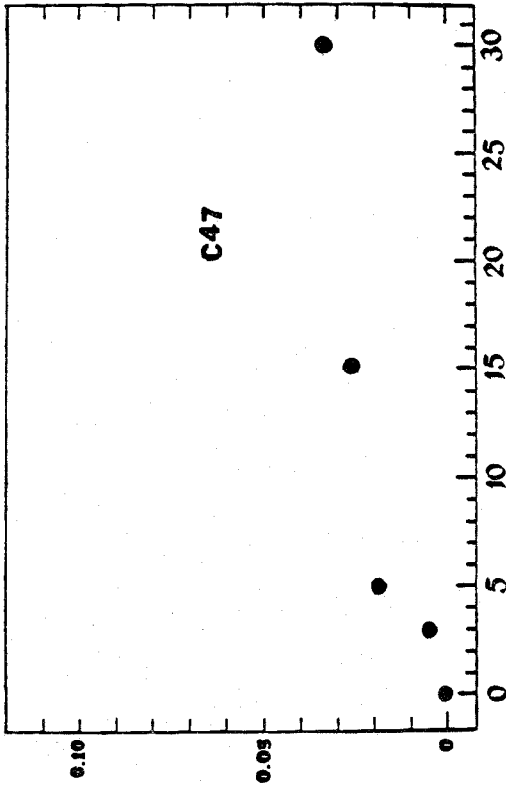
C47 and C114, clearly show these strains to be defective in light-induced synthesis of β-carotene. This is in accord with the tentative sensory pathway outlined in figure II-1 where madA and madB are shown as genes which play roles in the early stages of the transduction chain; thus mutations in these genes affect all the responses of Phycomyces to blue light. The C114 madB strain especially shows absolutely no indication of LICS to small doses of light.

It is not known at what point in the Phycomyces carotenoid biosynthetic pathway (figure III-1) photoinduction operates. It may be that the synthesis of the polyene precursor phytoene is photoinduced, or perhaps it is the synthesis and/or activity of the dehydrogenase and cyclase enzymes necessary to convert phytoene into lycopene and β-carotene. There is some evidence for light-induced phytoene synthesis in carB type mutants (phytoene dehydrogenase mutants) of Phycomyces (Eslava and Cerdá-Olmedo, 1974) and Neurospora (Lansbergen et al., 1976); phytoene concentration in cultures of these fungi grown under continuous illumination for 3-4 days showed an increase of approximately 40% in Phycomyces and approximately 130% in Neurospora over cultures grown in the dark. However, in Neurospora it is also known that phytoene is synthesized in the dark and that the dehydrogenation of phytoene to form colored carotenes is photoinduced (Zalokar, 1954), Finally, in the bacterium Mycobacterium species, at least one phytoene precursor and the enzyme necessary for its synthesis have been shown to be photoinduced (Gregonis and Rilling, 1973; Johnson et al., 1974). Thus, photo-induction appears to be capable of affecting a variety of steps in the biosynthetic pathway of carotenoids.

Addendum: Photoprotection in Phycomyces?

In order to check for a photoprotective role for β-carotene in Phycomyces, growth of wild type (NRRL1555), carA (C2) and carB (C5) strains was examined under moderately intense white light ($\sim$7 W/m^2 provided by two 40 W fluorescent lights placed about 3 inches away from the culture plates; the air near the light was cooled by a fan in order to maintain an ambient temperature of $\sim$21°C, equal to the temperature in the absence of the intense light) and compared with growth under ordinary room light ($\sim$0.5 W/m^2 white fluorescent light) and growth in the dark. Approximately 50 spores were inoculated per 10 cm petri plate containing SIVYCA medium. Colonies were counted after 3 days and after 6 days of growth. Results are presented in table VII-1. It is seen that the wild type strain grew approximately equally well whether in the dark or in the light. Similarly, the carotene-deficient mutants C2 carA and C5 carB grew equally well in the dark and under ordinary room light. However, growth under white light of high intensity clearly inhibited both spore germination and subsequent mycelial growth. Sunlight, which is a factor of 10 more intense than the highest intensities used in these experiments, can be expected to be even more inhibitory to growth in strains lacking β-carotene. Thus, β-carotene appears to play at least a small photoprotective role in Phycomyces.

Table VII-1

Colony counts of <u>Phycomyces</u> grown from spores in the dark, under ordinary room light (~ 0.5 W/cm^2) and under intense white light (~ 7 W/m^2). Colonies were counted at 3 days after inoculation and (given in parentheses) at 6 days after inoculation. Each entry represents the mean of 5-10 plates $\pm$ the standard deviation of the mean.

Strain	Dark	Room Light	Intense Light
Wild type NRRL1555	55 ± 3	59 ± 8	51 ± 5 (54 ± 3)
C2 <u>carA</u>	72 ± 8	72 ± 10	47 ± 8 (54 ± 5)
C5 <u>carB</u>	59 ± 5	59 ± 5	13 ± 5 (33 ± 3)

Appendix One

SOME RAMBLINGS ON PHOTOPHYSICAL PARAMETERS

WITH APPLICATIONS TO RIBOFLAVIN

The Einstein transition probabilities per unit time for induced absorption, induced emission and spontaneous emission between an upper state u and a lower state ℓ are denoted by $B_{\ell u}$, $B_{u\ell}$ and $A_{u\ell}$, respectively (Einstein, 1917). The number of induced absorptions per second is proportional to $N_\ell B_{\ell u}$ and is given by $N_\ell B_{\ell u}\, \rho(\nu_{u\ell})$, where N_ℓ is the population of the lower state ℓ and $\rho(\nu_{u\ell})$ is the density of radiation of frequency $\nu_{u\ell} = (E_u - E_\ell)/h$. Similarly, the number of induced emissions per second is given by $N_u B_{u\ell}\, \rho(\nu_{u\ell})$. For a system in equilibrium, the rates of absorption and emission must be equal. Thus

$$N_\ell B_{\ell u}\, \rho(\nu_{u\ell}) = N_u B_{u\ell}\, \rho(\nu_{u\ell}) + N_u A_{u\ell} \qquad \text{(AI-1)}$$

The ratio of the populations of the two states ℓ and u is given by the Boltzmann expression

$$\frac{N_\ell}{N_u} = \frac{g_\ell e^{-E_\ell/kT}}{g_u e^{-E_u/kT}} = \frac{g_\ell}{g_u} e^{h\nu_{u\ell}/kT} \qquad \text{(AI-2)}$$

where g_ℓ and g_u are the degeneracies of the ℓ and u states, respectively. Thus

$$\frac{g_\ell}{g_u} e^{h\nu_{u\ell}/kT} \rho(\nu_{u\ell}) B_{\ell u} = \rho(\nu_{u\ell}) B_{u\ell} + A_{u\ell} \qquad \text{(AI-3)}$$

The Einstein A and B coefficients are intrinsic quantities of the atomic or molecular system under consideration and do not depend upon the values of the temperature T and the radiation density ρ. We may obtain a relation between $B_{\ell u}$ and $B_{u\ell}$ by letting ρ and T become very large, whereupon we find that

$$g_\ell B_{\ell u} = g_u B_{u\ell} \qquad \text{(AI-4)}$$

Substituting for $B_{\ell u}$ in equation AI-3 and solving for the radiation density yields

$$\rho(\nu_{u\ell}) = \frac{A_{u\ell}}{B_{u\ell}} / (e^{h\nu_{u\ell}/kT} - 1) \qquad (AI-5)$$

According to the Planck expression for the density of radiation in equilibrium with matter, we have

$$\rho(\nu) = \frac{8\pi h\nu^3}{c^3} / (e^{h\nu/kT} - 1) \qquad (AI-6)$$

whence

$$\frac{A_{u\ell}}{B_{u\ell}} = \frac{8\pi h\nu_{u\ell}^3}{c^3} \qquad (AI-7)$$

and thus

$$A_{u\ell} = \nu_{u\ell}^3 \, B_{u\ell} \qquad (AI-8)$$

The oscillator strength, f, of an electronic transition is a measure of the total absorption intensity of the transition. It is proportional to the integral of the extinction coefficient (which is a measure of the absorption intensity at a single frequency or wavelength), ϵ, over all frequencies which contribute to the transition

$$f \, \alpha \int \epsilon \, d\nu \qquad (AI-9)$$

Thus, the f of a transition is proportional to the area under its spectroscopic absorption curve. One would expect there to be a simple relationship between the oscillator strength $f_{\ell \to u}$ of a transition and the probability per unit time $B_{\ell u}$ for such a transition to be induced by light. Since the extinction coefficient

is defined in terms of light intensity (thus energy) and $B_{\ell u}$ in terms of light quanta, the appropriate relationship is obtained by dividing ε by ν (since energy per quantum $= h\nu$) and integrating over the absorption band (Strickler and Berg, 1962)

$$B_{\ell u} \alpha \int \frac{\varepsilon}{\nu} \, d\nu \tag{AI-10}$$

If no other decay processes exist, a population of electronically excited species will decay radiatively to the ground state by spontaneous emission. If N_u is the population of the excited state, then

$$\frac{dN_u}{dt} = - A_{u\ell} N_u \tag{AI-11}$$

where $A_{u\ell}$ is the number of times per second that the excited state u spontaneously emits a photon and returns to the ground state ℓ; that is, $A_{u\ell}$ is the Einstein coefficient for spontaneous emission. Thus

$$N_u(t) = N_u(o) e^{-A_{u\ell} t} \tag{AI-12}$$

We can therefore define a radiative lifetime

$$\tau^o = \frac{1}{A_{u\ell}} \tag{AI-13}$$

which is the time for the excited state to radiatively diminish to $1/e$ of its initial population. The radiative lifetime τ^o of an excited state would be equal to the actual lifetime only if decay were to occur exclusively by spontaneous radiative emission.

Combining equations AI-4, AI-8, AI-10 and AI-13, we find for the radiative lifetime of an excited state u which decays to a ground state ℓ

$$\tau^o \ \alpha \ \frac{g_u}{g_\ell <\nu>^3 \int \frac{\varepsilon}{\nu} d\nu} \qquad\qquad \text{(AI-14)}$$

where $<\nu>$ is the mean frequency (energy) separation between the excited state

u and the ground state ℓ. Mean frequency is used because electronic transitions

in molecules are broadened by vibrational effects.

It is important to make a clear distinction between the radiative lifetime

τ^o and the actual lifetime τ of an excited species.

In order to facilitate carrying out the integrations in chapter IV, we will

make the approximation

$$\int \frac{\varepsilon}{\nu} \ d\nu \ \simeq \ \frac{1}{<\nu>} \ \int \varepsilon d\nu = \frac{f}{<\nu>} \qquad\qquad \text{(AI-15)}$$

Equation AI-14 then becomes

$$\tau^o \ \alpha \ \frac{g_u}{g_\ell <\nu>^2 f_{\ell \to u}} \qquad\qquad \text{(AI-16)}$$

This expression is strictly applicable only to atomic systems where electronic

transitions are relatively sharp lines so that ν can be removed from the integral.

However, prior to 1962 when Strickler and Berg pointed this out and derived

equation AI-14 for molecular systems possessing broad transitions, equation AI-16

enjoyed wide use. For our purposes, too, it will suffice. Consider a general kinetic

scheme in which I denotes the rate of absorption of photons by the ground state

singlet S_0 to yield the excited singlet S_1 (i.e., I is the intensity of absorbed radiation),

k_f and k_p are the rate constants for fluorescence (radiative decay of S_1) and

phosphorescence (radiative decay of T_1), respectively, k_{isc} is the rate constant

for intersystem crossing from S_1 to the lowest triplet T_1, and k_{rs} and k_{rt} are

the rate constants for nonradiative (radiationless) decays of S_1 and T_1, respectively. Then

$$\frac{dS_1}{dt} = -(k_f + k_{isc} + k_{rs})\, S_1 \qquad \text{(AI-17)}$$

Also

$$I = (k_f + k_{isc} + k_{rs})\, S_1 \qquad \text{(AI-18)}$$

since, at equilibrium, the rates of formation and destruction of S_1 are equal. The actual lifetime of S_1, as measured, for example, by the decay of fluorescence intensity, is given by

$$\tau_f = \frac{1}{k_f + k_{isc} + k_{rs}} = \frac{1}{k_f}\,\frac{k_f}{k_f + k_{isc} + k_{rs}} = \tau_f^o\, \phi_f \qquad \text{(AI-19)}$$

where $\tau_f^o = 1/k_f$ is the radiative lifetime of the excited state S_1 and

$$\phi_f = \frac{k_f}{k_f + k_{isc} + k_{rs}} = \frac{k_f S_1}{I} = \frac{\text{rate of emission by } S_1}{\text{rate of absorption by } S_o} \qquad \text{(AI-20)}$$

is the so-called quantum yield of fluorescence. In general, the quantum yield of any particular process is a measure of the efficiency of photon usage for that process and is given by the ratio

$$\phi = \frac{\text{number of molecules of product formed}}{\text{number of photons of radiation absorbed}} \qquad \text{(AI-21)}$$

$$= \frac{\text{rate of formation of product}}{\text{intensity of absorbed radiation}}$$

For the case of fluorescence, the product of interest is a fluorescing excited state and ϕ_f expresses the fraction of excited state species which decay by fluorescence emission.

For the triplet state T_1 the rate of formation is given by $k_{isc} S_1$. At equilibrium this will be equal to the rate of destruction; thus

$$k_{isc} S_1 = (k_p + k_{rt}) T_1 \qquad \text{(AI-22)}$$

Similarly, using equation AI-22, the quantum yield of phosphorescence is given by

$$\phi_p = \frac{\text{rate of formation of phosphorescing } T_1}{\text{intensity of absorbed radiation}}$$

$$= \frac{k_p T_1}{I} = \frac{k_p}{I} \frac{k_{isc} S_1}{(k_p + k_{rt})}$$

$$= \frac{k_p}{k_p + k_{rt}} \frac{k_{isc} S_1}{I}$$

$$= \theta_p \phi_{isc} \qquad \text{(AI-23)}$$

where, using equation AI-18,

$$\phi_{isc} = \frac{k_{isc} S_1}{I} = \frac{k_{isc}}{k_f + k_{isc} + k_{rs}} \qquad \text{(AI-24)}$$

is the quantum yield for intersystem crossing and

$$\theta_p = \frac{k_p}{k_p + k_{rt}} \qquad \text{(AI-25)}$$

is the quantum efficiency (to be distinguished from quantum yield) of phosphorescence. θ_p gives the fraction of triplet states which decay by phosphorescence emission. ϕ_p, not θ_p, is the directly measurable quantity.

The measured phosphorescent lifetime of T_1 is given by, using equation AI-23,

$$\tau_p = \frac{1}{k_p + k_{rt}} = \frac{1}{k_p} \frac{k_p}{k_p + k_{rt}} = \tau_p^o \theta_p = \tau_p^o \frac{\phi_p}{\phi_{isc}} \qquad \text{(AI-26)}$$

where $\tau_p^o = 1/k_p$ is the radiative lifetime of the triplet state.

An expression for the ratio of the oscillator strengths (integrated absorptions) of the $S_o \rightarrow T_1$ and $S_o \rightarrow T_1$ transitions may be written using equation AI-16:

$$\frac{f_{S_o \rightarrow T_1}}{f_{S_o \rightarrow S_1}} = \frac{g_{T_1}/g_{S_o} \tau_p^o <\nu_p>^2}{g_{S_1}/g_{S_o} \tau_f^o <\nu_f>^2} = \frac{3\tau_f^o <\nu_f>^2}{\tau_p^o <\nu_p>^2} \qquad (AI-27)$$

where $<\nu_f>$ and $<\nu_p>$ are the mean frequencies of fluorescence and phosphorescence, respectively, and $g_{T_1} = 3$, $g_{S_o} = g_{S_1} = 1$.

The radiative lifetimes τ_f^o and τ_p^o of the S_1 and T_1 states, respectively, of riboflavin may be calculated according to equations AI-19 and AI-26 using the experimental data shown below:

τ_f (298°K)	5.65 x 10^{-9} seconds	(Fugate and Song, 1976)
ϕ_f (298°K)	0.33	(Moore et al., 1977;
		Sun et al., 1972)
τ_p (77°K)	0.17 seconds	(Sun et al., 1972)
ϕ_p (77°K)	0.005	(Sun et al., 1972;
		Moore et al., 1977)
ϕ_{isc} (77°K, 298°K)	0.67	(Moore et al., 1977;
		Grodowski et al., 1977)

We obtain $\tau_f^o = 17.1$ x 10^{-9} seconds and $\tau_p^o = 22.8$ seconds. These radiative lifetimes are intrinsic properties of the molecule and do not vary with temperature. For riboflavin we also have $<\nu_f> = 525$ nm $= 19047$ cm^{-1} and $<\nu_p> = 620$ nm $=$

16129 cm^{-1} (Bowd et al., 1968; Sun et al., 1972). Thus from equation AI-27 we

may estimate for riboflavin

$$\frac{f_{S_o \to T_1}}{f_{S_o \to S_1}} = \frac{3 \, (17.1 \times 10^{-9})(19047)^2}{22.8 \, (16129)^2} = 3.1 \times 10^{-9} \qquad (AI-28)$$

Appendix Two

A FIRST-ORDER EQUATION FOR PHOTOCHEMICAL KINETICS

The number of quanta absorbed by a system of photoreceptor molecules is proportional to pI, where p is the fraction of active photoreceptor and I is the intensity of actinic radiation. Thus the decrease in p with time can be expressed by the differential equation

$$\left(\frac{dp}{dt}\right)_{inactivation} = -\tilde{\sigma} \, pI \qquad (AII-1)$$

where the constant of proportionality $\tilde{\sigma}$ may be called the photochemical cross section. It is a parameter characteristic of the photoreceptor and of the photochemical reaction it performs and is equal to the product of the photoreceptor's absorption cross section (a measure of its efficiency in absorbing light), σ, and the quantum yield, ϕ, of the primary photochemical reaction. If we assume that the inactive pigment regenerates (via a nonphoto reaction) at a rate proportional to its concentration, then the increase in p with time will be described by

$$\left(\frac{dp}{dt}\right)_{regeneration} = +k(1 - p) \qquad (AII-2)$$

where 1 - p is the fraction of photoreceptor which is inactive and k is the regeneration rate parameter. Combining equations AII-1 and AII-2, we obtain an expression for the total rate of change of p with time

$$\frac{dp}{dt} = -\tilde{\sigma} \, pI + k(1 - p) \qquad (AII-3)$$

The photostationary state is defined by the condition that there be no change in p. Setting $\frac{dp}{dt}$ equal to zero in equation AII-3, we obtain

$$p = \frac{1}{1 + \frac{\tilde{\sigma}}{k} I} \qquad (AII-4)$$

A photostationary equilibrium in which half the receptor is active and half inactive

has p = 0.5 in equation AII-4. Solving for I under these conditions one obtains

$I = k/\tilde{\sigma}$. The ratio $k/\tilde{\sigma}$ is called the critical intensity and is denoted by the symbol

I_c. (The origin of the name "critical intensity" is made clear by returning to

equation AII-4. One sees that if in the photostationary state one has $I \gg I_c$, then

p<<1; that is to say, only a small fraction of the photoreceptor remains active

when I is much greater than I_c).

To obtain a solution to the differential equation AII-3, let us rewrite

in the form

$$\frac{dp}{dt} = - (\tilde{\sigma}I + k)\left[p - \frac{k}{\tilde{\sigma}I + k}\right] \qquad \text{(AII-5)}$$

Making the substitution

$$w = p - \frac{k}{\tilde{\sigma}I + k} \qquad \text{(AII-6)}$$

and assuming I to be independent of time, one obtains

$$\frac{dw}{dt} = - (\tilde{\sigma}I + k)w \qquad \text{(AII-7)}$$

Integrating equation AII-7 yields the result that

$$w = w_o \exp[- (\tilde{\sigma}I + k)t] \qquad \text{(AII-8)}$$

Substituting the expression AII-6 for w one obtains

$$p - \frac{k}{\tilde{\sigma}I + k} = \left[p_o - \frac{k}{\tilde{\sigma}I + k}\right]\exp[- (\tilde{\sigma}I + k)t] \qquad \text{(AII-9)}$$

where p_o is the value of p(t) at t = 0 when the actinic irradiation is started. We

may take $p_0 = 1$; that is, all the photoreceptor pigment is to initially be in the

active state.

Thus

$$p = \frac{\frac{k}{\bar{\sigma}I} + \exp[-(\bar{\sigma}I + k)t]}{1 + \frac{k}{\bar{\sigma}I}} \tag{AII-10}$$

The expression for $1 - p$ is

$$1 - p = \frac{1 - \exp[-(\bar{\sigma}I + k)t]}{1 + \frac{k}{\bar{\sigma}I}} \tag{AII-11}$$

If a measured response, R, can be thought of as being proportional to

the fraction, $1 - p$, of photoreceptor inactive at the end of an actinic irradiation

(an assumption to be revised on page 156), then we may write

$$R = R_0 \frac{1 - \exp[-(k + \bar{\sigma}I)t]}{1 + \frac{k}{\bar{\sigma}I}} \tag{AII-12}$$

This equation describes a response which increases from a value of zero at zero

dose to a saturation value of $\dfrac{R_0}{1 + \frac{k}{\bar{\sigma}I}}$ at very large dose. If one has measured

the regeneration rate, k, then equation AII-12 may be applied to dose-response

data to obtain a value for the photochemical cross section, $\bar{\sigma}$, of the photoreceptor

which mediates the response (assuming, of course, that the data for the magnitude

of the response as a function of dose [irradiation intensity, I, and duration of

irradiation, t] is reasonably well described by equation AII-12 for some value of

$\bar{\sigma}$). If the absorption cross section, σ, of the photoreceptor is known, then the

quantum yield of the primary photochemical reaction can be obtained from $\phi = \bar{\sigma}/\sigma$.

A series of light doses can be administered in at least two distinct ways:

(1) a "time series" in which the intensity is kept fixed and the duration of irradiation

is varied and (2) an "intensity series" in which the duration of irradiation is kept

fixed and the intensity is varied. We shall deal with the two cases separately.

If the actinic intensity can be readily determined (i.e., if integration of the response

expression [equation AII-12] as a function of intensity through a thick sample

is not required), then a simple and elegant graphical method can be used to obtain

a value for σ from dose-response data.

Time series dose-response data give the response, R, as a function of

duration of irradiation, t. For small values of t equation AII-12 reduces to

$$R = R_o \sigma I t \qquad\qquad \text{(AII-13)}$$

Thus for small values of t (i.e., small light doses), the response is linearly propor-

tional to the dose; the proportionality factor is the slope of the initial linear region

of the time series dose-response curve and is given by (from equation AII-13)

$$S_t = \frac{R}{t} = R_o \sigma I \qquad\qquad \text{(AII-14)}$$

For large values of t (i.e., large light doses), the response saturates at a value

given by the large t limit of equation AII-12:

$$R_t = \frac{R_o}{1 + \frac{k}{\sigma I}} \qquad\qquad \text{(AII-15)}$$

From equations AII-14 and AII-15 we obtain

$$\sigma = \frac{1}{I}\left(\frac{S_t}{R_t} - k\right) \qquad\qquad \text{(AII-16)}$$

The values of S_t and R_t can be obtained from a graph of the time series dose-response

data and I is a known experimental quantity. Thus if the regeneration rate, k,

can be measured, equation AII-16 yields a value for the photochemical cross section,

σ. The graphical parameters S_t and R_t in equation AII-16 may be simplified still

further.
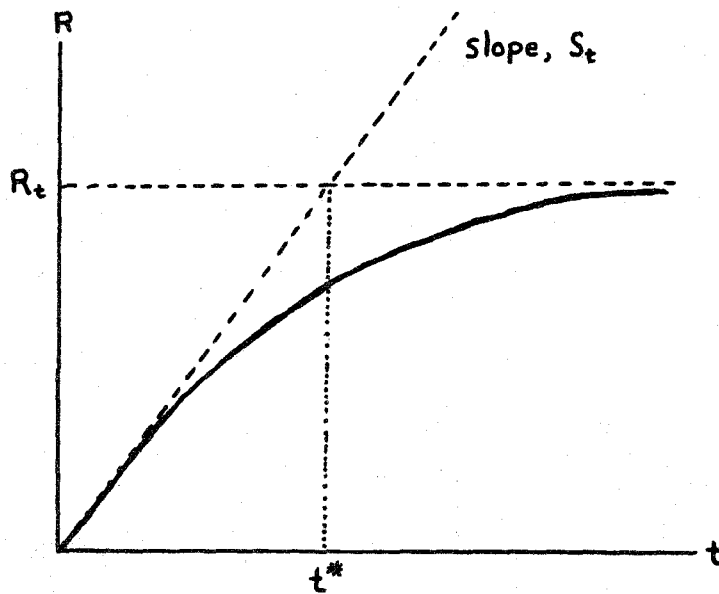
One sees that $t^* = R_t/S_t$ and thus equation AII-16 becomes

$$\tilde{\sigma} = \frac{1}{I} \left[\frac{1}{t^*} - k\right]$$ (AII-17)

or, in terms of a dose characteristic $D_{t^*} = It^*$,

$$\tilde{\sigma} = \frac{1}{D_{t^*}} - \frac{k}{I}$$ (AII-18)

Note that t^* represents the dose of actinic irradiation for which the response equals $1 - e^{-1} \simeq 0.632$ of its saturation value.

Intensity series dose-response data give the response as a function of the intensity, I, of actinic radiation. For small values of I equation AII-12 reduces to

$$R = \frac{R_o \tilde{\sigma} I}{k} (1 - e^{-kt})$$ (AII-19)

Thus for small doses (i.e., small values of I), the response is linearly proportional

to the dose; the proportionality factor is the slope of the initial linear region of

the intensity series dose-response curve and is given by (from equation AII-19)

$$S_I = \frac{R}{I} = \frac{R_o \tilde{\sigma}}{k} (1 - e^{-kt})$$ (AII-20)

For large values of I, the response saturates at a value given by the large I limit

of equation AII-12; this saturation value is $R_I = R_o$. Combining this result with

equation AII-20, one may write

$$\tilde{\sigma} = \frac{S_I}{R_I} (\frac{k}{1 - e^{-kt}})$$ (AII-21)

As with the time series case, the values of S_I and R_I can be obtained from a graph

of the intensity series dose-response data.
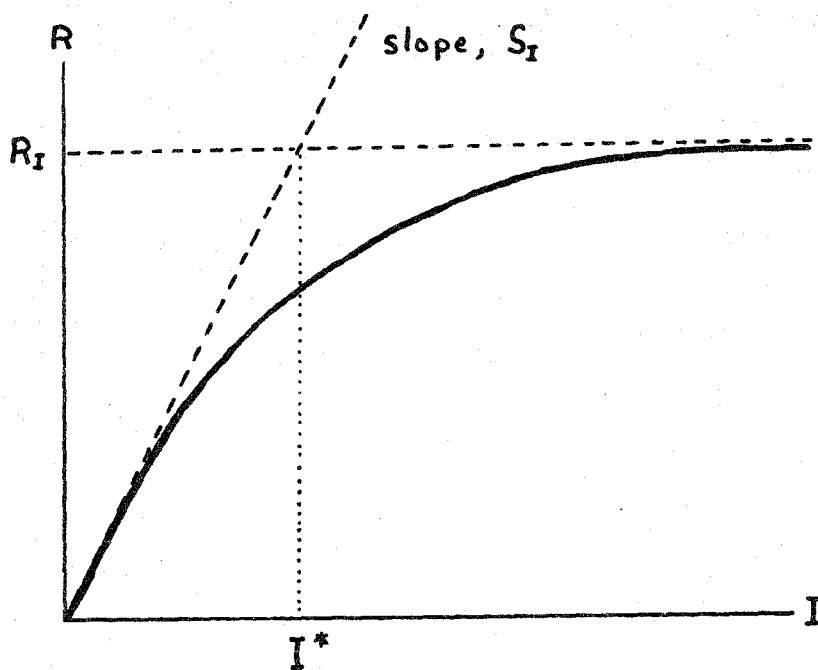
From the above graph one sees that $I^* = R_I/S_I$ and thus equation AII-21 may be written

$$\tilde{\sigma} = \frac{1}{I^*} \left(\frac{k}{1 - e^{-kt}} \right)$$

$$= \frac{1}{D_{I^*}} \left(\frac{kt}{1 - e^{-kt}} \right) \tag{AII-22}$$

where $D_{I^*} = I^* t$ represents a characteristic dose. For small t (i.e., small actinic pulse width), specifically when $t << \frac{1}{k}$, equation AII-22 can be approximated by $\tilde{\sigma} \simeq 1/D_{I^*}$.

Having carried out these derivations for $\tilde{\sigma}$, we now stop to remind ourselves that they depended upon the assumption (page 152) that the measured response, R, is proportional to the fraction, 1 - p, of receptor pigment remaining inactivated by light at the end of an actinic irradiation. However, if appreciable recycling of photoreceptor (i.e., inactivation by light with concomitant signal transduction, regeneration, again inactivation by light, and so on) occurs during the actinic irradiation, then this is a somewhat stupid assumption. Within the framework of the first-order pigment kinetics model, a far better measure of the response would be the total amount of pigment inactivated by light during the actinic irradiation; this is given by the integral of the pigment inactivation rate over the time during which the actinic dose is administered. Thus, we assume that

$$R = R_0 \int_0^t \tilde{\sigma} \, Ip \, dt \tag{AII-23}$$

Substituting for p according to equation AII-10 and carrying out the integration, one finds that

$$R = \frac{R_o \tilde{\sigma} I}{k + \tilde{\sigma} I} \left[kt + \frac{\tilde{\sigma} I}{k + \tilde{\sigma} I} \left(1 - \exp[-(\tilde{\sigma} I + k)t]) \right) \right] \qquad \text{(AII-24)}$$

If we consider the application of this expression to time–series dose–response

data, we see that it has a linear region for small values of t and a linear asymptote

for large values of t. These linear limits are given by

$$R \simeq R_o \tilde{\sigma} It \qquad \text{(AII-25)}$$

for small t and

$$R \simeq \frac{R_o \tilde{\sigma} I}{k + \tilde{\sigma} I} \left[kt + \frac{\tilde{\sigma} I}{k + \tilde{\sigma} I} \right] \qquad \text{(AII-26)}$$

for large t. A shortcoming of the response function in equation AII-24 is that

it does not saturate; that is to say, as the duration of the actinic irradiation in-

creases, the response also increases without bound. This physically unreasonable

aspect of this expression for the response could be corrected by adding a damping

term which prevents the expression from increasing without bound. However,

for our purposes here, it is sufficient to work with the expression as it is.

Let us denote by t* that value of t for which the two straight lines repre-

sented by equations AII-25 and AII-26 intersect.
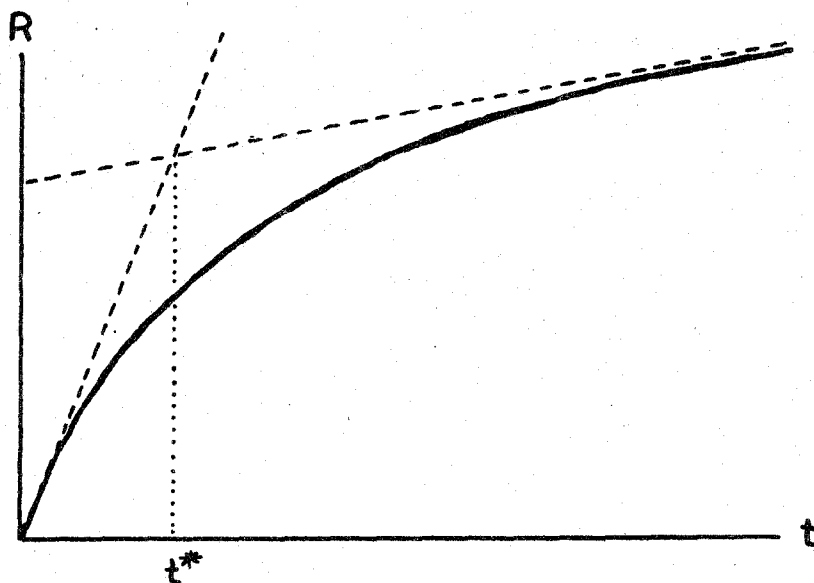
For t = t* the two expressions in equations AII-25 and AII-26 are equal. Solving for $\tilde{\sigma}$, one obtains

$$\tilde{\sigma} = \frac{1}{I} \left[\frac{1}{t*} - k \right] \tag{AII-27}$$

which is formally identical to the previous result obtained in equation AII-17. In terms of a characteristic dose, $D_{t*} = It*$, equation AII-27 becomes formally identical to equation AII-18.

In each case treated above, the value of the photochemical cross section, $\tilde{\sigma}$, is simply the inverse of a characteristic dose, which may be obtained from a plot of the dose-response data, plus a correction for the regeneration rate parameter, k. In every case, the characteristic dose occurs approximately where the dose-response curve makes a big bend from an initial low dose more-or-less linear region to another more-or-less linear region at high doses.

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The ruler of the South Sea was called Light; the ruler of the North Sea, Darkness; and the ruler of the Middle Kingdom, Primal Chaos. From time to time, Light and Darkness met one another in the kingdom of Primal Chaos, who made them welcome. Light and Darkness wanted to repay his kindness and said, "All men have seven openings with which they see, hear, eat, and breathe, but Primal Chaos has none. Let us try to give him some." So every day they bored one hole, and on the seventh day, Primal Chaos died.

Chuang Tzu

Wisdom consists of knowing when to avoid perfection.

Kilgore Trout