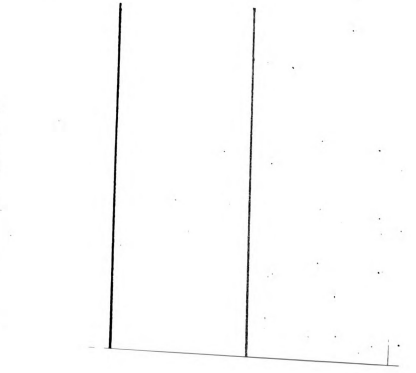

INHIBITORS OF PHOTOTROPISM IN *ZEA MAYS*

By

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ABSTRACT

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Plants exhibit a wide variety of physiological and morphological responses when irradiated with blue light. The identification of the chromophore has remained controversial mainly because action spectra resemble the absorption spectra of both flavins and carotenoids. Using the assay described by Schmidt, *et al*. (Plant Physiol 60: 736-738), the nature and tentative location of blue light photoreceptor pigment responsible for corn seedling's phototropism were examined. Compounds known to affect either flavins or carotenoids were tested for their ability to specifically inhibit phototropism using geotropism as a control.

Phenylacetic acid (PAA), which photoreacts with flavins as well as stimulates auxin related growth will specifically inhibit phototropism. Using analogues of PAA, this inhibition was found to be related primarily to PAA's photoreactivity with flavins and not its auxin activity. Estimates indicate that a substantial percentage of the photoreceptor pigment must photoreact with PAA to induce specificity.

Thus, the photoreceptor pigment must be more highly photoreactive with or more accessible to PAA than other cellular flavins.

Using the carotenoid synthesis inhibitor, SAN 9789 [4 chloro-5-methylamino-2-(α,α,α, trifluoro-m-tolyl)-3(2H) pyridazinone), the role of carotenoids was examined. Reductions in carotenoid content by 99% with SAN 9789 treatment did not specifically affect phototropism toward 380 nm light as compared to geotropism and did not shift the threshold intensity required to elicit phototropism using either 380 or 450 nm light. However, results from experiments using 450 nm light indicate that, even though bulk carotenoids are not the photoreceptor pigment, they are involved in light perception, acting as a screening pigment necessary for the plant to detect light direction. As a result, the action spectrum for phototropism in corn coleoptiles is a function of the absorption spectra of screening pigments (carotenoids) as well as a function of the absorption spectrum of the photoreceptor pigment (flavin).

Using iodide and xenon, the nature of the flavin excited state involved was investigated. Xenon and iodide will significantly quench the flavin triplet state <u>in vitro</u> while, at higher concentrations, iodide will also quench the flavin singlet state (>1 mm). Because xenon did not affect corn seedling's phototropism and because iodide was effective only when used at high concentrations, it was concluded that the flavin singlet state is more likely involved in phototropism.

Because PAA will photoreact with a significant percentage of the photoreceptor pigment, leaving a benzyl residue covalently linked to the flavin, PAA was used as a photoaffinity label in an effort to locate the photoreceptor pigment. Membrane fractions from corn coleoptiles were assayed for in vivo light-induced PAA binding using radioactively labelled PAA. A majority of such labelling was found to correlate with enzymatic markers specific for the plasma membrane after fractionation by either differential, isopycnic or rate-zonal centrifugation. Importantly, this binding did not correlate with the flavin content of the membrane fractions. A comparison between the in vivo labelling and the in vitro photoreactivity of PAA with membrane bound flavins suggests that plasma membrane-bound flavins are more accessible to PAA than other membrane bound flavins.

These results indicate that a flavin and not a carotenoid is the chromophore involved in blue light reception for the phototropic response in corn coleoptiles. Association of light-induced PAA binding with the plasma membrane of corn coleoptiles provides a tentative link between the plasma membrane and the blue light photoreceptor pigment.

For their assistance in my thesis work, I would like to thank my guidance committee, Drs. Kenneth Poff, Jan Zeevaart, Norman Good and Philip Filner. I would also like to express my appreciation to the Department of Enery Plant Research Laboratory for my financial support during this work and the people who worked there for providing a stimulating place for learning and research. Special thanks go to my parents, Bernard and Sally Vierstra for nuturing my scientific curiosity and to my wife, Karen, for her moral support during my training and for enduring the highs and lows common in such an experience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
E	Einstein
EDTA	Ethylenediamine Tetraacetic Acid
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide (Riboflavin 5' phosphate)
Hepes	N-2 Hydroxylthylpiperazine-N'-2-ethanesulfonic Acid
IAA	Indole-3-acetic Acid
Mes	2-(N-Morpholino)ethanesulfonic Acid
1-NAA	Naphthalene-1-acetic Acid
2-NAA	Naphthalene-2-acetic Acid
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NPA	Naphthylphthalamic Acid
PAA	Phenylacetic Acid
POPOP	1,4-bis[2(5-Phenyloxazolyl)]-Benzene
PPO	2,5 Diphenyloxazole
SAN 9789	4 chloro-5-(methylamino)-2-(, , -trifluoro-m-tolyl)-3(2H)-pyridazinone
SDS	Sodium Dodecyl Sulfate
SHAM	Salicylhydroxamic Acid
Tris	Tris(hydroxymethyl)amino-methane
UDPG	Uridine 5-diphosphate-D glucose
W	Watt

Chapter 1

General Introduction and Literature Survey

1.1 INTRODUCTION

Blue light stimulates a wide variety of physiological and morphological responses in plants. Due to, in most cases, their inherent sessile habit, and, in the case of green plants, their dependence on light for photosynthesis, plants use such a sensitivity to light to obtain information vital to their growth and development in an ever changing environment. There are two major classes of non-photosynthetic photoresponses in plants. These are characterized by the type of chromophore used; phytochrome, a photoreversible red/far-red absorbing linear tetrapyrrole and a blue-light photoreceptor pigment(s) of uncertain identity (See Presti and Delbrück, 1978; Gressel, 1979; Senger, 1979).

Unlike the blue light photoreceptor pigment(s), the phytochrome chromoprotein has been isolated. Criteria for identification was based on the matching of the photoreversible red/far-red absorption spectrum of the purified protein with red/far-red photoreversible action spectrum (Butler, et al., 1964). Initial detection of the chromoprotein was made easy by the large quantities present in etiolated tissue, the uniqueness of the chromophore absorbing at wavelengths where masking by other pigments is not a problem, and the photoreversibility of the red and far-red absorbing forms. These characteristics allowed the use of

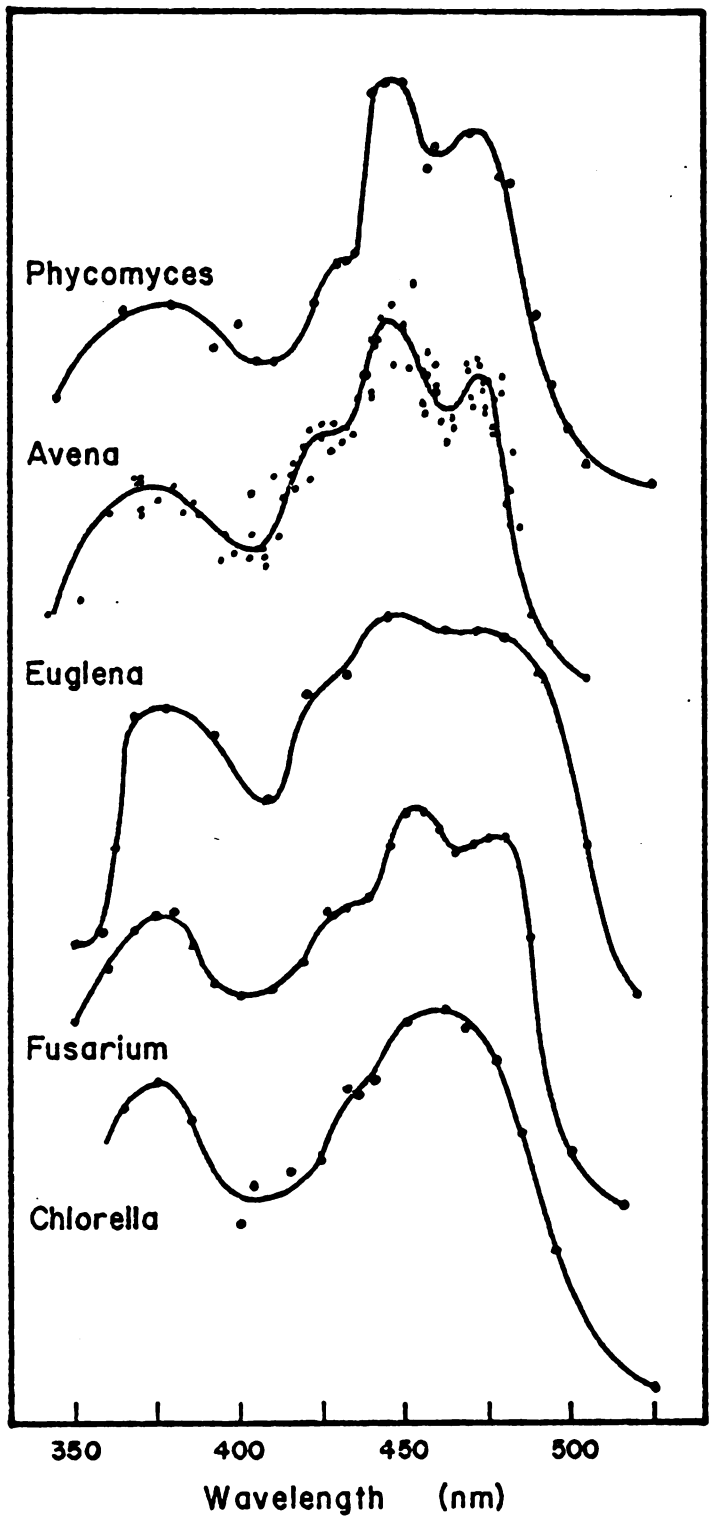
conventional spectroscopic assays for detection. In contrast, the blue light photoreceptor has none of these convenient properties, and as a consequence, neither it nor its sensory transduction pathway (i.e., the chain of events between the excited photoreceptor pigment and the observed photoresponse) have been identified (See Russo, 1979). As will be seen below, unlike phytochrome, action spectra indicate that the blue light photoreceptor pigment is neither unique nor does it absorb at wavelengths where masking pigments are not a problem. Theoretical calculations also suggest that the pigment is present at concentrations too low for spectrophotometric detection (Bergman, et al., 1969; Briggs, 1964) and other than the action spectrum, no specific assay exists.

The so-called "blue-light responses" can be elicited from a plethora of organisms, ranging from the bacteria Salmonella and E. coli (Taylor and Koshland, 1975) to the fruit fly Drosophila (Klemm and Ninnemann, 1976), with the majority of the reported responses occurring in the plant kingdom. They are characterized by an action spectrum with photoactivity between 350 and 500 nm with a prominent action maximum centered around 450 nm, with (when detailed information exists) a subpeak and shoulder at 480 and 420 nm respectively and an important (see below) broad maximum at 370 nm. The blue-light responses so far determined by the aforementioned criteria include: phototropism by the sporangiophores of the fungi, Phycomyces (Curry and Gruen, 1959) and Pilobolus (Page and Curry, 1966), and the seedlings

of Avena (Shropshire and Withrow, 1958; Thimann and Curry, 1961) and Zea mays (Briggs, 1960); polarotropism in the germlings of the liverwort Sphaeorcarpos and the fern Dryoperteris (Steiner, 1967); phototaxis in the protozoan, Euglena (Diehn, 1969); enhancement of respiration in the alga, Chlorella (Pickett and French, 1967); chloroplast rearrangement in the moss, Funaria (Zurzycki, 1972) and in the alga Vaucheria (Fischer-Arnold, 1963; Blatt and Briggs, 1980); stimulation of carotenoid synthesis in the fungi, Neurospora (DeFabo, et al., 1973) and Fusarium (Ray, 1967); entrainment of the circadian rhythms of conidia formation in the fungus Neurospora (Sargent and Briggs, 1967); sporangiophore formation in the fungus, Phycomyces (Bergman, 1972); and stimulation of the opening of stomata from leaves of Vicia faba (Hsiao, et al., 1973) and Avena (Skaar and Johnsson, 1978) (Figure 1.1).

The similarity between action spectra from such diverse photoresponses has suggested to many that there exists a common photoreceptor pigment. However, the identification of the blue light chromophore has remained controversial because of the inherent inadequacies of action spectra when used as the major criterion for determination. For example, the absorption spectrum of a chromophore may depend strongly on its environment in vivo (See Massey, 1979), thus resulting in an action spectrum which deviates from an absorption spectrum in vitro. An action spectrum is also a function of the screening pigments altering it (depending on the nature of the photoresponse) from the absorption spectrum of

Figure 1.1 Examples of action spectra for several photoresponses to blue light: phototropism in Phycomyces (Curry and Gruen, 1959) and Avena (Thimann and Curry, 1961); phototaxis in Euglena (Checcucci, et al., 1976); stimulation of carotenoid synthesis in Fusarium (Rau, 1967); and stimulation of O_2 uptake in Chlorella (Pickett and French, 1967).


Phycomyces
Avena
Euglena
Fusarium
Chlorella
350
400
450
500
Wavelength (nm)

the photoreceptor pigment *in vivo*. Finally, an action spectrum cannot distinguish between two pigments in the organism with very similar absorption spectra. The controversy has centered aroung two prominent plant pigments--flavins and carotenoid (Briggs, 1964)--who absorption spectra match closely the blue light action spectra.

A carotenoid was proposed first as the blue light photoreceptor pigment because the major extractable pigments from etiolated *Avena* coleoptiles were carotenoids and because their absorption spectra matched closely the complex action spectrum for phototropism of such coleoptiles between 400 and 520 nm (Wald and DuBuy, 1936). On the other hand, flavins are not found in large quantities nor do the absorption spectra of flavins exhibit similar detail. The view remained unchallenged until Galston and Baker (1949) proposed that a flavin might be involved based on the observations that flavins could photosensitize the decarboxylation of indole-3-acetic acid (IAA), a plant hormone involved in coleoptile growth. [Earlier it was suggested by Went (1928) and later Briggs (1963) that phototropism was a result of growth differential initiated by a light-induced difference in auxin between the illuminated and shaded sides.] By photoreacting with IAA, flavins would create such a concentration difference by photosensitizing the breakdown of IAA on the illuminated side. This hypothesis was later refuted from observations that light treatments sufficient for phototropic curvature did not significantly reduce the total extractable auxins from such tissue and that the light intensities

sufficient for phototropic induction was too low to cause such a photooxidative loss of IAA (Briggs, et al., 1957).

To resolve the flavin/carotenoid dispute, research then concentrated on exploiting characteristics specific to one or the other chromophore. Detailed action spectra were obtained which extended into the ultraviolet, especially at 370 and 280 nm where flavins had additional maxima while most carotenoids did not. [However, certain carotenoids under special conditions do exhibit a maximum at 350 nm, e.g., lutein (Hager, 1970) and 15,15' cis β-carotene (Vetter, et al., 1971). These two possibilities were eliminated as the blue light photoreceptor pigment when Song and Moore (1974) demonstrated that only the stacked dimer of lutein could absorb at 350 nm making it an unlikely candidate and Presti, et al. (1977) found no detectable amounts of 15,15' cis β-carotene in the sporangiophores of Phycomyces.]

All "blue light" action spectra obtained have maxima at 370 and 280 nm, thus favoring a flavin chromophore. In certain photoresponses, the action maxima at 370 and 280 nm is less effective than would be expected from the absorption spectrum of a flavin. This argument, in addition to the complexity of action spectra between 400 and 500 nm, was used to argue against a flavin chromphore (DeFabo, et al., 1976). But, because positions and heights of action maxima cannot be precisely determined, due to the inherent problems of action spectra (see above), such a conclusion cannot be made. In addition, when flavins are dissolved in non-polar solvents or rigidly held, they display an absorption spectrum

similar to the action spectra with peaks and shoulders at 420, 450 and 480 nm (Song and Moore, 1974) in contrast to their broad aqueous absorption spectrum (See Figure 1.1).

Based on theoretical grounds, a flavin is more likely to be the chromophore than a carotenoid. Biochemically, flavins (flavoproteins) are exceedingly versatile, in that they participate in a wide variety of redox reactions and are found at the heart of key metabolic pathways, e.g., respiratory electron transport. Flavins can also react photochemically, photoreducing or oxidizing a myriad of electron donors and acceptors [e.g. EDTA and cytochrome-c (Hemmerich, 1976)]. In addition, certain flavoenzymes can be light activated, [e.g. nitrate reductase (Aparicio, et al., 1976) and succinate dehydrogenase (Salach and Singer, 1974)] thus providing a plausible mechanism for photocontrol by a flavin photoreceptor pigment.

Although the argument is not conclusive, theoretical calculations based on spectroscopic data (Song and Moore, 1974) indicate that a carotenoid would make a poor candidate because carotenoids possess excited state lifetimes too short to be efficient chromophores. For example, trans β-carotene has a singlet excited state lifetime of 10^{-13} to 10^{-15} seconds with no significant triplet population (little intersystem crossing). If we assume that the photochemistry involved in blue light photoreception is diffusion limited (rate constant of 10^{9} to 10^{10} seconds), an excited state lifetime of 10^{-13} to 10^{-15} seconds would be too short to be efficient. Carotenoids can act as photoreceptors,

e.g., photosynthesis, but a carotenoid is not the primary photoreceptor in this case, but efficiently transfers the excited state energy to another chromophore (chlorophyll) with a longer excited state lifetime (Song, et al., 1976). However, action spectra for the "blue light" responses do not indicate the presence of an energy acceptor for carotenoids in the long wavelength regions.

Flavins, on the other hand, do possess excited state lifetimes long enough to be efficient, 10^{-8} sec and 10^{-4} sec for the singlet and triplet excited states (Oster, et al., 1962). The flavin triplet has been of interest because of its long lifetime, reactivity, and the presence of a significant population through rapid intersystem crossing (Sun and Song, 1972).

Evidence disfavoring carotenoids has come from experiments demonstrating that a reduction in the total carotenoid content, had no significant effect on the organisms' response to blue light. Carotenoid deficiencies were induced by either mutation in Phycomyces (Presti, et al., 1977), Neurospora (Sargent and Briggs, 1967), Euglena (Checcucci, et al., 1976) and Zea mays (Bandurski and Galston, 1951) or by the addition of carotenoid synthesis inhibitors to Avena (Bara and Galston, 1968) and Pilobus (Page and Curry, 1966). However, because in most cases, significant amounts of carotenoids still existed, a carotenoid could not be definitely ruled out. The photoreceptor pigment may be required at very low concentrations [minimum estimates for the photoreceptor pigment concentration is 10^{-9} M for Avena (Briggs,

1964) and 3 x 10^{-7} M for Phycomyces (Bergman, et al., 1969)]. Thus, it is possible the total carotenoid content could be drastically reduced without significantly reducing the photoreceptor pigment concentration. Carotenoids can be unambiguously ruled out only in Phycomyces, where a mutant blocked in all six steps of β-carotene synthesis, had no detectable carotenoids, but still exhibited a normal phototropic response (Presti, et al., 1977). Based on the upper detection limit of the assay, the mutant sporangiophore had a carotenoid content less than 4 x 10^{-5} that of wild type or approximately 2 x 10^{-9} M β-carotene--significantly less than the theoretical minimum photoreceptor pigment concentration.

Schmidt, et al. (1977) reported another approach to the question of chromophore identity using compounds known to interact with flavins. They found that iodide, azide, and phenylacetic acid (PAA) would inhibit phototropism to a greater degree than geotropism while chloride and cyanide would not. Based on the assumption that the geotropic and phototropic responses of Zea mays use similar metabolic pathways except for the primary sensory input step (Juniper, 1976; Dennison, 1979), interpretation of a compound's ability to inhibit phototropism more than geotropism was that it affected the primary sensory input steps of phototropism. Iodide and azide will quench (depopulate) flavin excited states (Song and Moore, 1968) and azide will also block electron transfer by flavins (Schmidt and Butler, 1976). Although iodide will also quench the excited state of other pigments, it does not affect polyenes such as β-carotene

(Song, et al., 1976). PAA will permanently photoreduce flavins forming a benzyl-derivatized flavin (Hemmerich, et al., 1967), thus inactivating the photoreceptor pigment. Because specific inhibition of phototropism occurred, they argued that the photoreceptor pigment must be more accessible to and/or more photoreactive with PAA than other cellular flavins.

Because iodide will quench the excited state of flavins, especially the long-lived triplet state, inhibition of a blue light photoresponse by iodide has been used as evidence that the flavin triplet state is involved. Iodide has been shown to affect phototropism in corn (Schmidt, et al., 1977) and Avena (Meyer, 1969), the photophobic responses in Euglena (Diehn and Kint, 1970; Mikolajaczyk and Diehn, 1975) and light induced chloroplast orientation in Selaginella (Mayer, 1966). Because iodide will also quench the flavin singlet state, albeit at higher concentrations (Oster, et al., 1962), one cannot infer which flavin excited state is affected. However, an inhibition of a photoresponse by iodide can be used to preclude a carotenoid and thus favor a flavin as the photoreceptor pigment.

Delbrück, et al. (1976), using a tunable laser, investigated the action spectrum for the Phycomyces' light growth response and discovered a shoulder at 600 nm approximately 10^{-9} times as effective as that at 450 nm. When the extended action spectrum was compared to the absorption spectrum of riboflavin, a peak was observed between 585 and 600 nm. They concluded that this shoulder represented the direct

excitation of the lowest flavin triplet state. This state is known to absorb maximally in the red region (Sun, et al., 1972) and, due to its forbidden nature, have an extinction coefficient approximately 10^{-8} that of the singlet ground state (Song, et al., 1972). Delbrück, et al. (1976) used this result to further indicate the involvement of the flavin triplet state. However, this evidence should be interpreted with caution because this shoulder could be due also to the excitation of the flavin semiquinone with an absorption maximum around 570 nm (Beinert, 1956) or the direct excitation of the triplet state could convert to the flavin singlet state via intersystem crossing and then initiate the photoresponse.

In Euglena, the blue light photoreceptor pigment is believed to be localized in the paraflagellar body (PFB), a quasi-crystalline structure surrounded by a membrane and attached to the base of the flagellum (Kivic and Vest, 1972). [The stigma, which contains carotenoids (Batra and Tollin, 1964) was originally thought to be the photoreceptor organelle, but mutants with absorption-less stigma were found to be still photoresponsive (Checcucci, et al., 1976)]. Using fluorescence microscopy, the PFB was found to emit a yellow fluorescent light, indicative of flavins (Benedetti and Checcucci, 1975). In vivo microspectrofluorometry of the PFB has revealed a fluorescence emission spectrum similar to that of riboflavin (Benedetti and Lenci, 1977). Recently, an in vivo fluorescence excitation spectrum also demonstrated the presence of a flavin (G. Columbetti, personal communication).

Isolation of the PFB and identification of the flavoprotein enclosed would provide substantial evidence that a flavin is involved in Euglena photomovement.

Another possible approach to the photoreceptor pigment's identity has come from the observations that blue light induces in vivo absorbance changes in Dictyostelium, Phycomyces, Neurospora and corn (Poff and Butler, 1974; Muñoz and Butler, 1975; Brain, et al., 1977). The absorbance change is consistent with the reduction of a b-type cytochrome and the action spectrum as well as reconstitution experiments with exogenous flavins indicate that the chromophore responsible is a flavin. Localization of the light-induced b-type cytochrome reduction has been reported in several organisms. In Dictyostelium, the cytochrome is highly soluble (Poff and Butler, 1975) and has been purified by Manabe and Poff (1978). On the other hand, the greatest photoactivity from Neurospora and corn corresponded with particulate fractions enriched for "plasma membrane" (Brain, et al., 1977). In addition, "plasma membrane" enriched fractions from corn (Jesaitis, et al., 1977), Neurospora, and Phycomyces (Schmidt, et al., 1977) were found to contain similar b-type cytochromes.

Whether or not the flavin-mediated b-type cytochrome photoreduction in these organisms is indeed related to their physiological light responses is unresolved. Both Dictyostelium (Poff and Butler, 1975) and HeLa cells (Lipson and Presti, 1977) contain such photoactivities despite any indication of blue light photophysiology. A flavin is definitely not the photoreceptor pigment for either pseudoplasmodial

or amoebal phototaxis in Dictyostelium (Poff, et al., 1973; Häder and Poff, 1979).

Lipson and Presti (1977) have argued against the physiological relevance of the cytochrome photoreduction in Phycomyces because of the low quantum yield (0.015) for the photoreaction and the results that none of four phototropic mutant groups (Mad) isolated in Phycomyces lacked such activity. Two mutant groups represented genes believed to be associated with early steps of the sensory transduction pathway (Bergman, et al., 1973). It should be noted, however, that the light-induced absorbance changes they measured were in mycelium and not in the phototropic sporangiophores while later observations indicated that no mutants lacking the photoreceptor pigment or proteins involved in the early events of the sensory transduction pathway have been isolated (Russo, 1979). With respect to quantum yields, the photoreceptor pigment should theoretically transfer energy with a quantum yield close to unity, i.e., by highly efficient. But this is not an absolute requirement in all cases. For example, it is possible that a newly evolving photoreceptor pigment system would not have a high quantum yield. Additionally, quantum yields close to unity have been calculated for the b-type cytochrome photoreduction in corn and Neurospora, suggesting that the photoactivity is much more efficient in thses organisms that in Phycomyces (Lipson and Presti, 1980).

Model systems involving flavins and cytochrome-c showed similar light-induced absorbance changes (Schmidt and Butler,

1976) implying that the phenomenon may be non-specific when considering the close proximity of flavins and cytochromes in the mitochondrial electron transport chain. But evidence from absorption spectra and localization studies indicate that there is one particular non-mitochondrial b-type cytochrome involved, suggesting a great degree of specificity (Muñoz and Butler, 1975; Brain, et al., 1977). Reconstitution of the isolated photoreducible b-type cytochrome from Dictyostelium and corn membrane fractions with purified flavins and flavoproteins have demonstrated that not all flavins are equally active suggesting that a particular flavoprotein may be involved in addition to a particular b-cytochrome (Manabe and Poff, 1978; Caubergs, et al., 1979).

Physiological experiments involving the b-cytochrome photoreduction have provided a tentative link to photophysiology. Brain, et al., (1977) reported that the poky mutant of Neurospora, which is deficient in b-type cytochromes (<10% of wild type) showed less b-type cytochrome photoreduction and an impaired blue light sensitivity of its circadian rhythm. In addition to flavins, the photodynamic dye, methylene blue, can also photoreduce the same particulate b-type cytochrome from corn coleoptile membrane fractions using red light instead of blue (Britz, et al., 1979). Inclusion of methylene blue into two blue light sensitive organisms, Trichothecium (Sagromsky, 1956), and Fusarium (Land-Feulner and Rau, 1974) have conferred red light sensitivity on normally blue light sensitive responses. Using artificial dyes with varying redox potentials in addition to

methylene blue, Land-Feulner and Rau (1975) discovered that those dyes with redox potentials capable of photoreducing b-type cytochromes were active in stimulating light dependent carotenogenesis in Fusarium.

The flavin-mediated in vitro photoreduction of the b-cytochrome of corn is sensitive to iodide, PAA, azide, salicylhydroxamic acid (SHAM) and antimycin A (however, antimycin A was required at concentrations in excess of that needed to block mitochondrial electron transport) (Caubergs, et al., 1979). Iodide, PAA and azide would affect the flavin involved as mentioned earlier. Interestingly, SHAM, an inhibitor of cyanide-insensitive respiration (Schonbaum. et al., 1971) and antimycin A, inhibitor of miotchondrial electron transport between cytochrome b and c (Davis, et al., 1973), are affecting the b-type cytochrome. The ability of SHAM to inhibit blue light photoresponses in vivo would add further support to the involvement of a b-type cytochrome as the second link in the sensory transduction pathway.

Demonstrating which flavin (flavoprotein) is the photoreceptor pigment(s) has proven difficult because: flavin auxotrophs are lethal providing an obstacle to mutant analysis of the chromophore; the plethora of flavins and flavoproteins found in a cell makes it difficult to identify possible candidates (this is in contrast with most other photoreceptor pigments so far isolated, phytochrome, chlorophyll, stentorin, rhodopsin, etc.); the photoreceptor pigment is present at such low concentrations that conventional spectroscopy is not possible; despite intensive work, no photoreceptor

pigment mutants have been isolated; and due to the wide variety of photoreactions that flavins are capable of, the immediate bichemical events after the flavin absorbs the photon (sensory transduction pathway), cannot be theoretically assumed with certainty. This makes any one of these possibilities a criterion for isolation precarious.

In conclusion, substantial evidence exists favoring a flavin as the chromophore responsible for the myriad of blue light responses found in plants. As mentioned above, isolation of the photoreceptor pigment has proven difficult, thus interfering with unequivocal determination of the nature of the chromophore. Moreover, caution must be exercised when extrapolating results from one blue light sensitive response to another between different orgainsims and different blue light responses in the same organism. Because flavins were important even in the early stages of evolutionary history (they are found in the most primitive of extant organisms) and are ubiquitous in cell metabolism, it would not be surprising that different blue light responses would have evolved separately using different flavoproteins and sensory transduction pathways. For example, the photoreceptor pigment for light-induced carotenogenesis may be different and/or have a different sensory transduction pathway from that used for phototropism (See Russo, 1979) even though the same chromophore may be involved.

This dissertation is concerned with the identification of the blue light photoreception pigment(s) using the phototropic response of corn seedlings as the blue light sensitive

system. From the facts that the response is insensitive to wavelenths higher than 520 nm and that the fluence response curves are similar to that for Avena (Briggs, 1960), we are assuming that the same type of photoreceptor pigment is involved. Corn provides a number of advantages for the study of the blue light photoreceptor pigment: The light responses have been described in detail (Briggs, 1960; Dennison, 1979) as well as the effects of light on auxin movement (Briggs, 1963; Elliott and Shen-Miller, 1976), and the effect of auxin on coleoptile growth (Ray, et al., 1977). Techniques have been described for organelle isolation (Jesaitis, et al., 1977; Ray, 1977) and localization of light-induced absorbance changes (Brain, et al., 1977); and the use of geotropism as a control permits the identification of inhibitors which are specific for phototropism (Schmidt, et al., 1977).

Fluence response curves for corn and Avena display several unusual characteristics (Dennison, 1979). Using constant exposure time, increasing light intensities will first increase the phototropic curvature to a maximum angle (first positive curvature), then the curvature will decline to a minimum, sometimes away from the light (first negative curvature), and then at higher light intensities, the coleoptile will bend again toward the light (second positive curvature) (Briggs, 1960). First positive curvature follows the reciprocity law (Bunsen-Roscoe law of photochemical equivalence: if a photoresponse is only dependent on the amount of photons absorbed, an equivalent response will occur if the product

of exposure time and light intensity is held constant.) (Zimmerman and Briggs, 1963) but as the light intensity increases toward that which would elicit second positive curvature, reciprocity begins to fail. In contrast, second positive curvature is a function of exposure time, requiring relatively long periods of illumination. Second positive curvature is more likely to be the response involved under natural conditions as the seedling germinates. The two systems are separable using short exposure time but with long exposures, the two fuse together with the concomitant loss of first negative curvature (Dennison, 1979). The mechanism for the convoluted fluence response curves is unknown although the action spectra for first and second positive curvature in Avena are similar, indicating a common photoreceptor pigment (Evert and Thimann, 1968).

Blue light elicits phototropic curvature by its effects on coleoptile growth. An exposure to light inhibits coleoptile elongation termed the "light growth response" (Blaauw, 1909; Thimann and Curry, 1960) so that unilateral illumination slows the growth of the illuminated side relative to the shaded side of the coleoptile. Because the coleoptile is optically dense, a light gradient and hence a growth gradient is established (Reinert, 1953). In contrast, Phycomyces develops a transient acceleration in growth rate with blue light (Delbrück and Shropshire, 1960).

Theories concerning the mechanisms of the light effects on coleoptile growth generally pertain to the photocontrol of auxin transport. Light either stimulates a lateral

transport of auxin away from the illuminated side (Briggs, 1963; Pickard and Thimann, 1964) or inhibits the basipetal transport of auxins from the coleoptile tip (Shen-Miller and Gordon, 1966; Shen-Miller, et al., 1969) in the illuminated side. A lower auxin concentration would translate into a reduced growth rate.

1.2 STATEMENT OF PURPOSE

It is clear that the flavin(s) involved in the blue light photophysiology is not known, nor are details of its location or mode of action available. Schmidt, et al. (1977) described a technique for examining the effects of potential inhibitors on the phototropic response in Zea mays coleoptiles using geotropism as a control. This dissertation concerns itself with the use of this technique to further probe the photosensory transduction pathway in Zea mays and from the results obtained, attempt a localization of the photoreceptor pigment. Specific areas of research include:

1. Nature of the excited state of the flavin chromophores involved.
2. The mechanism for specific inhibition of phototropism in corn seedlings by PAA.
3. The role or carotenoids in the phototropic response of corn seedlings.
4. Possible localization of the flavin photoreceptor pigment in corn coleoptiles using PAA as a photoaffinity label.

Chapter 2

Effects of Iodide and Xenon of the Excited States of Flavins and Phototropism in Corn

2.1 INTRODUCTION

A flavin has been strongly implicated as the chromophore responsible for the myriad of blue light responses found in plants (See Presti and Delbrück, 1978). Moreover, evidence concerning phototropism in corn (Brain, et al., 1977; Schmidt, et al., 1977), Avena (Meyer, 1969) and Phycomyces (Delbrück, et al., 1976), phototaxis in Euglena (Diehn and Kint, 1970; Mikolajazyk and Diehn, 1975), and chloroplast orientation in Selaginella (Mayer, 1966) have been interpreted to suggest that the flavin triplet state is the active species. This interpretation has been based primarily on the observations that potassium iodide (KI), which effectively quenches flavin triplet photoreactions in solution through heavy atom quenching (Song and Moore, 1968), inhibits the photoresponses of corn, Avena, Euglena and Selaginella.

However, relatively high concentrations of KI have been used to obtain significant inhibition (>10 mM for corn, > 50 mM for Euglena and 100 mM for Avena and Selaginella). At these high concentrations, other effects, in addition to the heavy atom quenching of the flavin triplet state complicate any interpretation of an inhibition of a photoresponse by KI. These effects include: i) KI's ability to act as a general inhibitor or metabolic processes involving flavoenzymes and

electron transport (it is not surprising that geotropism in corn and movement in Euglena are also inhibited by KI concentrations grater than 1 mM); ii) the ability of KI to form complexes with flavins [static quenching (Song, et al., 1972)] rather than exclusively quenching the excited state (dynamic quenching) involved in photoreception; and iii) at relatively high concentration (>1 mM) the ability of KI also to affect the flavin singlet state (Oster, et al., 1962). Thus, KI cannot be used to specifically quench the flavin triplet state.

In this chapter, we examine the effects of KI on both the flavin singlet and triplet states in an effort to interpret the ability of KI to inhibit photoresponses in vivo. Additionally, in order to circumvent the complications from the use of KI, we have examined the use of the gas Xenon, as an external heavy atom quencher (atomic number for Xe is 54; 53 for iodide) of the flavin triplet state in solution, and tested its ability to specifically inhibit the phototropic response of corn seedlings. As mentioned previously, because geotropism and phototropism in corn are believed to follow similar metabolic pathways except for the primary sensory input steps, geotropism was used as a control for phototropism. The ability of a compound (Xenon) to inhibit phototropism to a greater degree than geotropism on a percentage basis, implies that the compound is interacting with the primary steps of photoperception (Schmidt, et al., 1977). Xenon is a potentially useful inhibitor due to its chemical inertness (in contrast to KI) and high solubility in water

[24.1 cc/100 ml, 10.4 mM at 273 K and 11.9 cc/100 ml, 4.8 mM at 298 K (Weast, 1973)].

2.2 MATERIALS AND METHODS

2.2.1 Measurements of Flavin Photochemistry in the Presence of Iodide or Xenon. The effect of KI or Xenon on the flavin triplet state was determined using the anaerobic photooxidation of NADH by riboflavin as described by Sun and Song (1973). Solutions of 125 μM NADH and 80 μM riboflavin (1.0 A at 445 nm) in 10 mM sodium phosphate buffer (pH 7.0) with or without KI were flushed with either N_2 or Xe (Airco) for 30 minutes in quartz cuvettes with a ground glass fitting sealed with a rubber septum. The anaerobic solutions were irradiated with 450 nm actinic light from a slide projector (Bell and Howell CP-40) in conjunction with 3 cm of 10% w/v aqueous $CuSO_4$, and appropriate neutral density filter, and a Baird Atomic 450 nm interference filter (half-band width = 8.5 nm). Light intensities used were 50 and 200 $\mu W\ cm^{-2}$ (measured using a Kettering model 65 radiant power meter). At varying time intervals, the absorption spectrum of the riboflavin solution was recorded using a Cary 15 spectrophotometer and the initial reaction rate of riboflavin photoreduction was determined from a plot of absorbance at 445 nm versus irradiation time. Conversion of the initial rate into the units M/min was based on E_{445} = 1.25×10^4, liter/mol·cm for riboflavin (Oster, et al., 1962).

The effect of iodide and xenon on the flavin singlet

state was determined by measuring the fluorescence quenching by KI or Xe of solutions containing 100 uM riboflavin, FMN, or FAD (10 mM sodium phosphate, pH 7.0) ± varying concentrations of KI. The fluorescence intensity of each solution was determined using an Aminco Bowman (model H-8202) spectrophotofluorometer.

The effect of Xe on the flavin singlet population was measured also by the phase-modulation subnanosecond fluorescence lifetime method (courtesy of Dr. Pill Soon-Song), as described previously (Fugate and Song, 1976). Riboflavin was dissolved in distilled H_2O and the solution divided into two matched fluorescence cuvettes. Each cuvette was fitted with a rubber septum and either Xe or N_2 gas was introduced through a needle in the septum after the gas had gone through a small test tube of distilled water to saturate the gas with vapor. The gas exhaust needle was attached to an inverted volumetric flask to monitor the total volume of gas. The flow rate of the gas was 20-21 ml/min and each cuvette was purged for 1 hour in the dark, after which steady-state fluorescence and phase-modulation lifetimes were measured.

2.2.2 Measurement of Tropic Responses of Corn Seedlings in Presence of Xenon. Xenon was also tested for its ability to specifically inhibit phototropism. Specificity was determined by measuring both phototropism and geotropism in the presence of N_2 or Xe. Corn seeds (Zea mays hybrid MS-WFg X Bear 39; Bear Hybrid Seed Co., Decatur, Ill.) were allowed to imbibe overnight in distilled H_2O and sown, embryo up, in trays covered with cellophane, on Kimpak

germinating-paper dampened with distilled H_2O. The trays were kept at 21.5° ± 1° C for four days in complete darkness. On the third and fourth nights after planting, the seedlings were irradiated with red light (0.5 W/m^2, 630 nm, 30 nm half-band width) for 1 hour to inhibit mesocotyl growth (Blaauw, et al., 1968). After five days, straight seedlings (5 cm tall) were placed in 15 ml plastic tubes filled with distilled H_2O under a dim green safelight. A cork was inserted in the opening of the tube with 3.5 cm of the coleoptile projecting through a hole in the cork. Then plastic tubes (each containing a seedling) were inserted into holes fitted with rubber seals in the bottom of the chamber. In this fashion, the coleoptiles could be rotated to lie at a 90° angle to the test stimulus without opening the chamber. Under valve control, the sealed chamber was filled with water, which was then displaced with either 90% N_2, 10% O_2 or 90% Xe, 10% O_2 (v/v). (In preliminary experiments, tropic responses of corn seedlings were inhibited at O_2 concentrations less than 5% v/v. The O_2 concentration was therefore maintained at 10%.)

The phototropic stimulus consisted of a 3 hour unilateral illumination of 2 $\mu W\ cm^{-2}$ blue (450 nm) light from a Besilar slide projector used in combination with a 450 nm Baird-Atomic interference filter (8.5 nm half-band). This dose corresponds to that which would elicit the second positive curvature (Briggs, 1960). The seedlings were turned on their side for 3 hours in complete darkness to elicit a geotropic response. Immediately following the stimulus presentation,

the curvature of each shoot was determined and the mean curvature was calculated for each test group. Seedlings exposed to 90% Xenon were compared to the control group exposed to 90% N_2 on a percentage basis (the mean curvature for seedlings in a 90% N_2, 10% O_2 atmosphere was 40° for phototropism and 65° for geotropism).

2.3 RESULTS

2.3.1 Effect of Iodide and Xenon on Flavin Excited State. Figure 1 demonstrates the kinetics of the anerobic photooxidation of NADH as measured by the photoreduction of riboflavin at 445 nm. The mechanism of this photoreaction has been determined to proceed via the flavin triplet state (Sun and Song, 1972). The quantum yield is 0.09 $\pm$ .01 at the concentration of NADH used (125 μM). From the calculated initial velocity of riboflavin photobleaching, both KI (300 μM) and xenon (saturated, 4.8 mM) significantly quench the flavin triplet state. The kinetic data derived from Figure 2-1 are listed in Table 2-1. The rate of riboflavin photobleaching was reduced 50% and 84% as compared to the rate in N_2 in the presence of xenon and 300 uM KI respectively. KI will significantly inhibit this photoreaction at concentrations $\geq$ 10 μM with a K_i of 23 μM (Figure 2-2). Although xenon is not as effective in depopulating the flavin triplet at KI, relatively small concentrations (4.8 mM) can be effectively used to quench a photoreaction which proceeds via the flavin triplet state.

The effect of xenon on the singlet state was determined by measuring the heavy atom quenching of flavin fluorescence. The majority of such fluorescence arises from the de-excitation of the flavin singlet excited state [phosphorescence

Figure 2.1 The photooxidation of NADH by riboflavin under anaerobic conditions. The photoreaction was monitored spectrophotometrically as a decrease in absorbance of riboflavin at 445 nm due to the reduction of the flavin moiety by NADH. ● ; rate in Xe, ○ ; rate in N_2. Inhibition of the photooxidation by KI (300 μM) is shown in the top curve (■). Initial rates were evaluated from the dashed lines.

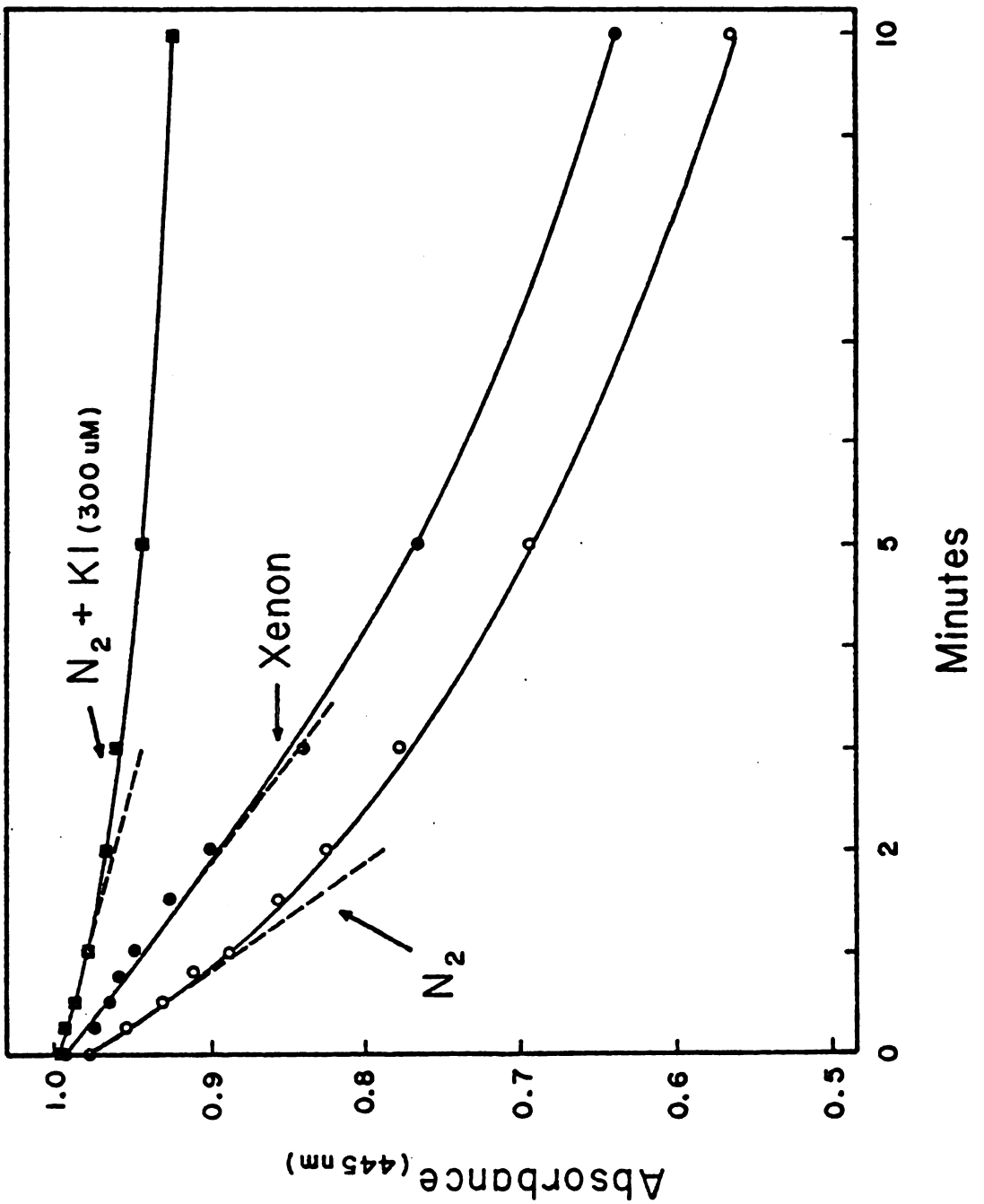

Absorbance (445 nm)
1.0
0.9
0.8
0.7
0.6
0.5
0
2
5
10
Minutes
N_2 + KI (300 uM)
Xenon
N_2

Table 2.1. Effect of xenon on the anaerobic photoreduction of riboflavin by NADH. The initial reaction rate of riboflavin photoreduction was determined from plots of absorbance at 445 nm versus time (Figure 2.1).

Condition	Photoreduction: Initial rate in M/min ($x10^7$)	Percent of reaction rate in N_2
N_2	77*	100
Xe	39*	51
N_2 + 300 µM KI	12	16

*Average of three determinations

Table 2.2. Effect of xenon on fluorescence intensity and fluorescence lifetime of riboflavin. Measurements of fluorescence lifetimes were determined using the phase-modulation subnanosecond fluorescence lifetime method (Courtesy of Dr. Pill Soon-Song).

Condition	Intensity*	Lifetime (ns)			
		By phase shift		By modulation	
		30 MHz	10MHz	30 MHz	10 MHz
N_2	63.3	5.14	5.21	5.23	5.20
Xe	62.6	5.16	5.20	5.19	5.21

*Ub arbitrary unit. Φ_F 525 nm.

and delayed fluorescence arising from the de-excitation of the flavin triplet excited state comprise only a small proportion of the total fluorescence emission (Sun, et al., 1972)]. From the fluorescence measurements both fluorescence intensities and lifetimes were essentially identical in Xe- and N_2- saturated solutions. Thus, it can be concluded that xenon does not alter the flavin singlet state. Alternatively, when KI was used (Figure 2-2), the flavin fluorescence and subsequent flavin singlet state was quenched by KI concentration of 1 mM or greater. The K_i is 19, 34 and 45 mM for riboflavin, FMN and FAD, respectively.

2.3.2 Effect of Xenon on Corn Seedling's Tropic Responses. A 90% xenon, 10% O_2 atmosphere was tested for its effect on the phototropic and geotropic responses in corn using 90% N_2 + 10% O_2 as a control (Table 2-3). Within the experimental error of 10 separate determinations, xenon showed no significant inhibition of phototropism. Moreover, in contrast to iodide (Schmidt, et al., 1977), xenon had no affect on either geotropism or phototropism in corn seedlings.

Figure 2.2 Effects of KI on the singlet and triplet excited states of flavins. The effect of iodide on the triplet excited state was determined by its effect on the anaerobic photooxidation of NADH by riboflavin and is expressed as the percentage of the control initial velocity (-O-). The effect of iodide on flavin singlet excited states was measured from the quenching of flavin fluorescence, riboflavin (-●-), FMN (-■-) and FAD (-▲-) expressed as the percentage of the control fluorescence intensity.

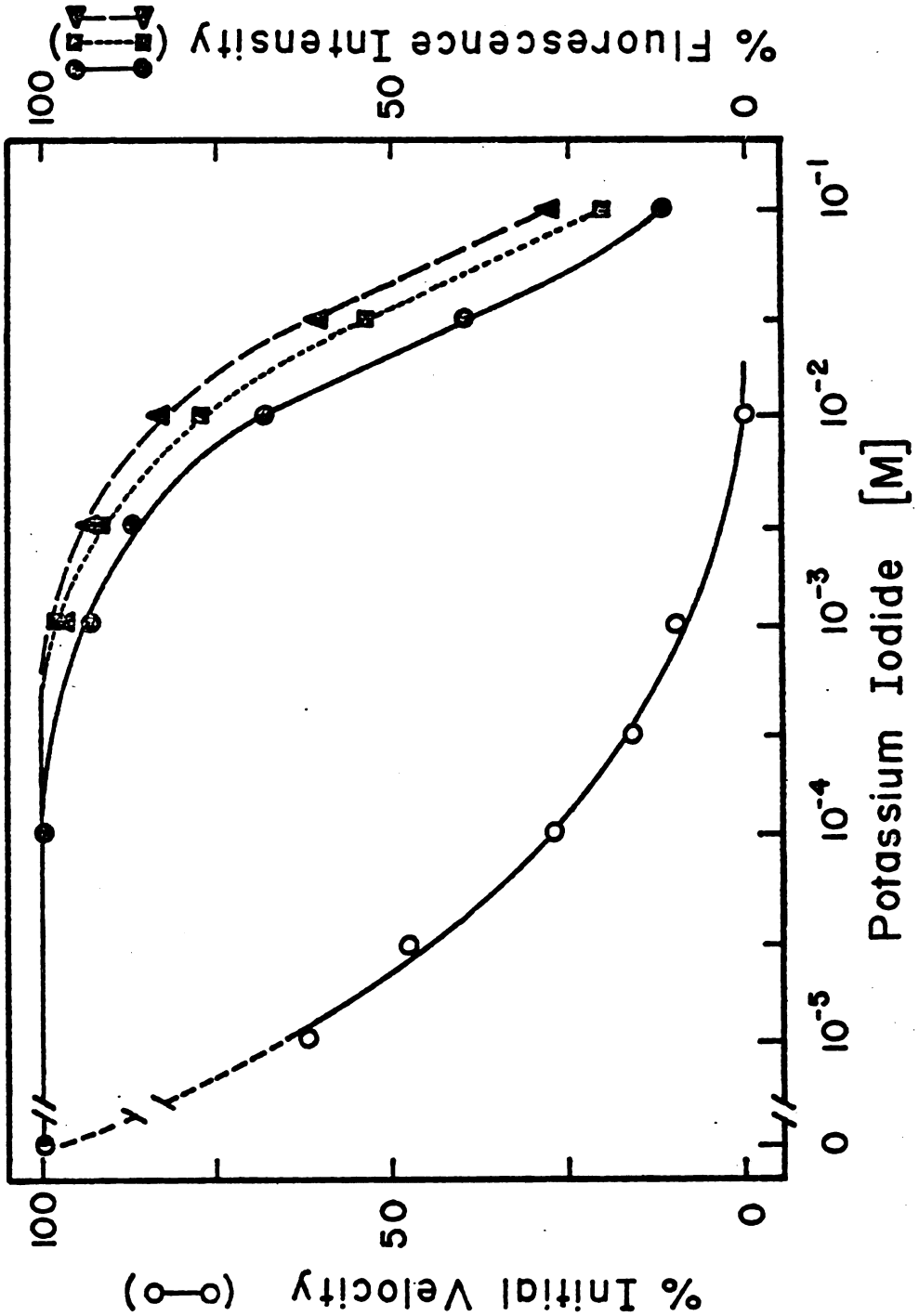

% Initial Velocity (o—o)
100
50
0
% Fluorescence Intensity (▽—▽) (□---□) (●—●)
100
50
0
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Potassium Iodide [M]

Figure 2.2 Effects of KI on the singlet and triplet excited states of flavins. The effect of iodide on the triplet excited state was determined by its effect on the anaerobic photooxidation of NADH by riboflavin and is expressed as the percentage of the control initial velocity (-0-). The effect of iodide on flavin singlet excited states was measured from the quenching of flavin fluorescence, riboflavin (-●-), FMN (-■-) and FAD (-▲-) expressed as the percentage of the control fluorescence intensity.

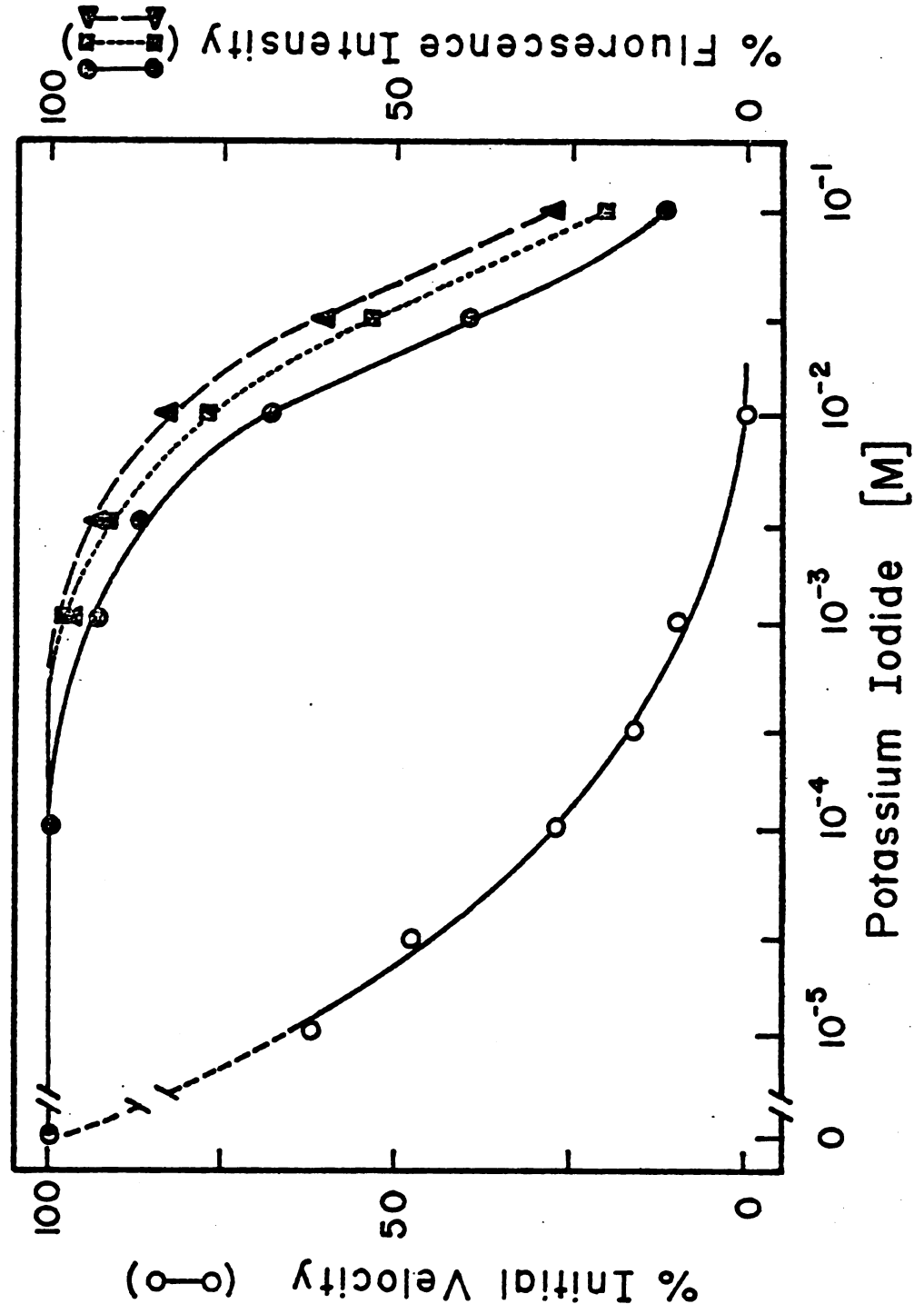

% Initial Velocity (o—o)
100
50
0
0
10^-5
10^-4
10^-3
10^-2
10^-1
Potassium Iodide [M]
% Fluorescence Intensity
100
50
0

Table 2.3. Effect of xenon on the tropic responses in corn seedlings. Phototropic bending was measured after a 2 hour irradiation with 2 $\mu W\ cm^{-2}$ blue light (450 nm). Geotropic bending was measured 2 hours after placing the seedling in a horizontal position.

Response	% response of corn coleoptiles in 90% Xe + 10% O_2, relative to 90% N_2 + 10% O_2
Phototropism	99.7% (6.6 S.D.)
Geotropism	98.7% (3.1 S.D.)

*Average of 10 determinations.

2.4 DISCUSSION

From the results in Figure 2-2, it can be concluded that iodide will affect both the flavin triplet and singlet excited states and therefore that iodide is not a specific quencher of the flavin triplet excited state. The K_i for the quenching of the riboflavin triplet and singlet excited states by KI is 23 μM and 19 mM, respectively. Thus, when using iodide to determine the reactive species in a flavin-sensitized photoreaction, if the flavin singlet excited state is involved, iodide should have little effect at concentrations below 1 mM.

By disregarding the effects iodide has on the flavin singlet state, significant inhibition of a photoresponse by iodide has been used *a priori* to indicate the involvement of the flavin triplet state. For example, *in vitro* studies on a flavin-mediated photoreduction of a *b*-type cytochrome, hypothesized to be involved in corn seedling phototropism, showed that iodide would inhibit the reaction with a K_i of 50 mM (Caubergs, *et al.*, 1979). Widell and Bjorn (1976) also reported that *in vivo* light induced absorbance changes in chopped etiolated wheat coleoptiles required 100 mM KI to inhibit the response by 50%. The conclusion that these photoreactions should proceed *via* the flavin triplet state is not justified considering the high concentration of iodide

needed. Additionally, the flavin triplet state was implicated in the photoresponses of corn (Schmidt, et al., 1979) and Euglena (Diehn and Kint, 1970; Mikolajaczyk and Diehn, 1975) even though KI concentrations greater than 10 mM and 50 mM, respectively, were required for significant specific inhibition, using geotropism and movement as controls. One could argue, in the case of corn seedlings where KI was applied to the roots, that the iodide concentration at the site of the photoreceptor pigment in the coleoptile would be considerably less as a result of transport limitations. However, this argument may not hold in the unicellular Euglena where the barriers prsented to iodide would be substantially less. In Avena (Meyer, 1969) and Selaginella (Mayer, 1966), no measurements of the non-specific effects of iodide were attempted, except that chloride was ineffective. Consequently, the percentage inhibition of these blue light responses attributable to the quenching of flavin excited states is not known. However, the fact that the tissues required immersion in solutions containing 100 mM iodide to inhibit the photoresponse would argue against the involvement of the flavin triplet.

Xenon, although it specifically quenches the flavin triplet state, did not specifically inhibit phototropism in corn seedlings. We assume that xenon has entered the coleoptile cells based on xenon's high solubility in water (incubation of the coleoptiles in xenon for an additional two hours prior to irradiation has no effect on the response), and that the photoreceptor pigment should be accessible to xenon as it is to hydrophilic ions such as iodide,

phenylacetic acid and azide (Schmidt, et al., 1977).

Delbrück, et al. (1976) have described a shoulder at 595 nm in the action spectrum for phototropism in Phycomyces and have interpreted these results to implicate the involvement of the flavin triplet state directly excited from the ground state. There are, however, several difficulties with the flavin triplet as the reactive species of the photoreceptor pigment. These include: i) intersystem crossing of bound flavins is substantially slower than that of free flavins, producing a relatively low population of flavins in the triplet state in vivo (McCormick, 1977; Song, et al., 1980); ii) inhibition of photoresponses in corn, Avena, Euglena and Selaginella requires iodide concentrations that should preclude the flavin triplet state; iii) Xenon, which preferentially quenches the flavin triplet state has no effect on phototropism in corn; and iv) azide and phenylacetic acid, which both affect phototropism in corn, efficiently quench the flavin singlet in the case of azide, and react with the flavin singlet in the case of phenylacetic acid (photodecarboxylation of PAA by riboflavin is uneffected by 1 mM iodide).

The results by Delbrück, et al. (1976) are also open to alternative interpretation of the data. The shoulder at 595 nm could involve the flavin semiquinone known to absorb maximally in that region (Beinert, 1956), or the photoreceptor pigment has a broader absorption spectrum than that of riboflavin used for comparison. It is also possible that direct excitation into the flavin triplet state could be

converted via intersystem crossing into the singlet excited state (such as occurs in delayed fluorescence) thus initiating the photoresponse.

In support of the hypothesis that the flavin singlet state is the active species, highly photoreactive bound flavoprotein(s) from plasma membrane enriched fractions of corn coleoptiles have been recently shown to possess short fluorescence lifetimes characteristic of the singlet excited state (Song, et al., 1980).

In conclusion, I feel that the majority of evidence is consistent with the hypothesis that the flavin singlet state is the reactive species in blue light responses and that caution whould be used in the interpretation of the involvement of the flavin triplet state solely on the ability of iodide to inhibit the response. Additionally, unlike other flavin quenchers, such as iodide, xenon, which is chemically inert, will preferentially quench the flavin triplet state without affecting the singlet state.

Chapter 3

Mechanism of Specific Inhibition of Phototropism in Corn Seedlings by Phenylacetic Acid

3.1 INTRODUCTION

Blue light is known to control many metabolic and morphogenic responses in plants, e.g., phototropism in Phycomyces, Avena and Zea mays (Dennison, 1979), carotenoid synthesis in Neurospora (DeFabo, et al., 1976), photophobic movements in Euglena (Checcucci, et al., 1977), and changes in stomatal aperture in many plant species (Hsiao, et al., 1973). The striking similarities between the action spectra for these responses suggest a common photoreceptor pigment. However, because action spectra resemble the absorption spectra of two prominent plant pigments (flavins and carotenoids) and because the lack of any assay specific for the photoreceptor pigment beyond the action spectrum, the identity of the chromophore remains controversial. Indirect evidence, mainly from the photoresponses of "carotenoidless" mutants of Phycomyces (Presti, et al., 1977), and Neurospora (Sargent and Briggs, 1967), has favored flavins.

Recently, Schmidt, et al. (1977) reported results of another approach to the problem of the chromophore's identity. Using geotropism as a control for phototropism in corn coleoptiles, compounds known to affect flavins [iodide, azide, and phenylacetic acid (PAA)] were found to inhibit phototropism to a greater degree than geotropism. They argued that both tropic responses follow similar

metabolic pathways except for the primary sensory step. For example, because PAA will permanently photoreduce flavins producing a stable photoadduct (the photoadduct would be incapable of photochemical activity), specific inhibition of phototropism would result if the photoreceptor pigment were more accessible to PAA than other flavins.

In addition to forming covalent adducts with flavins, PAA is known also to have auxin-like activity (Jönssen, 1961; Wightman, 1977). Because auxins are involved in the tropic responses in corn (Briggs, 1961), specific inhibition of phototropism by PAA could result from a differential sensitivity of geotropism and phototropim to auxin. We tested carboxylic acids similar to PAA with different degrees of auxin activity and photoreactivity with flavins to determine whether specific inhibition of phototropism by PAA was a hormonal response or based on its photoreactivity with flavins.

3.2 MATERIAL AND METHODS

3.2.1 Plant Material. Corn seeds (Zea mays hybrid MS WFg X Bear 38 from National Starch & Chemical Co., Decatur, Ill.) were allowed to imbibe overnight in distilled H_2O and sown, embryo up, in trays covered with cellophane, on Kimpak germinating paper dampened with distilled H_2O. The trays were kept at 21.5° ± 1° C for four days in complete darkness and exposed to 1 hour of 50 μW cm^{-2} red light (630 nm, 30 nm half-band width) at midnight on the third and fourth nights to inhibit mesocotyl growth (Blaauw, et al., 1968). Only straight seedlings between 4 and 5 cm long were used.

3.2.2 Tropism Experiments. Under a dim green safelight, seedlings were placed in 50 ml test tubes with 2 cm of the coleoptile projecting through a hole in a cork stopper inserted in the opening of the test tube. The roots were immersed in a solution of the potential inhibitor (Sigma) at pH 7.0 (pH adjusted with KOH) for 2 hours prior to the test stimulus (light or gravity). Each experiment consisted of 40 seedlings, 10 immersed in distilled H_2O and 10 each in three different concentrations of the test compound.

To test for phototropism, the seedlings were exposed unilaterally to 2 μW cm^{-2} blue light from a slide projector in combination with a Baird Atomic interference filter (450 nm 8.5 nm half-band width), for four hours. This dose

corresponds to that which would elicit the second positive curvature (Briggs, 1960). Geotropism was induced by holding the seedlings horizontal for 4 hours in complete darkness. Immediately following cessation of the stimulus presentation, the curvature of each shoot was determined and the mean curvature was calculated for each test group. Average curvatures of seedlings exposed to the potential inhibitor were related on a percentage basis to the control group in distilled H_2O. Each point represents between 40 to 80 coleoptiles treated with the potential inhibitor compared to the same number of coleoptiles in distilled H_2O. The mean curvature for control seedlings ranged from 35° to 45° for phototropism and 55° to 65° for geotropism.

A fluence response curve was determined for 450 nm light by irradiating the seedlings immersed in distilled H_2O with various light intensities for 4 hours. The mean curvature was calculated and compared on a percentage basis to a group exposed to 2 $\mu W\ cm^{-2}$ blue light.

3.2.3 PAA Uptake. Transport of PAA into the coleoptile was measured using PAA solutions spiked with 1 µCi/ml [ring 4-^{3}H] PAA (spec. act. 18.5 Ci/mmol, IRE, Belgium). The roots were immersed in the solutions and at various time intervals, the coleoptiles were harvested and dried, and the radioactivity measured by scintillation counting of the samples burned in a Packard Sample Oxidizer.

3.2.4 Riboflavin Photoreduction. The reactivity of each potential inhibitor with flavins was determined by its ability to aerobically photoreduce riboflavin. Reaction

media, consisting of 5 mM potential inhibitor, 0.7 mM riboflavin (A_{445nm} = 0.9) and 10 mM Na phosphate buffer (pH 7.0) were irradiated with 5 mW cm^{-2} blue light from a projector in combination with 3 cm aqueous 10% (w/v) $CuSO_4$ and a Balzers DT Blau filter. Absorption spectra of the solution were recorded at time intervals with a Cary 15 spectrophotometer. Initial reaction rates were calculated from plots of absorbance at 445 nm of the solution versus time. Absorbance maxima of the photoadducts were obtained from riboflavin solutions fully reduced by the potential inhibitor.

3.2.5 Auxin Activity. Auxin activity was measured by the corn coleoptile elongation test similar to that described by Ray, et al. (1977). From 2 cm long corn coleoptiles, 9 mm segments were cut, beginning 3 mm from the top, using two razor blades mounted 9 mm apart. The segments were floated on a 1.5% sucrose solution (w/w) containing 10 mM KH_2PO_4, and 10 mM Na citrate pH 6.3 during cutting and then were transferred to the same buffer solution with the addition of the various carboxylic acids. The length of the coleoptile segments were measured after incubation for 16 hours in the test solutions at 22° C in total darkness by butting at the segments end to end in a v-shaped trough and measuring the total length. The percentage elongation was calculated as the percentage increase in coleoptile length after 16 hours as compared to the initial length.

Light intensities were measured using a Kettering (model 65) radiometer.

3.3 RESULTS

3.3.1 Auxin activity. Using the corn coleoptile bioassay, five known auxin analogues (Jönssen, 1961) (IAA, 1-NAA, 2-NAA, PAA and β-phenylpyruvic acid) were tested for auxin activity. Figure 3.1 shows that both IAA (a natural auxin) and 1-NAA (synthetic auxin) induce a strong growth response in cut coleoptile sections at concentrations greater than 10^{-7} M and 10^{-6} M respectively. PAA and B-phenylpyruvic acid exhibit no effect on growth a concentrations below 10^{-3} M, but with 10^{-3} M, PAA slightly stimulated growth while β-phenylpyruvic acid inhibited growth. 2-NAA (an auxin antagonist) inhibited elongation when applied at concentrations above 10^{-6} M, similar to the concentrations at which its structural analogue 1-NAA stimulated growth.

3.3.2 Photoreactivity with Riboflavin. These compounds with differing auxin activities were assayed for their ability to form aerobically stable photoadducts with riboflavin. When riboflavin solutions containing PAA are irradiated with blue light, they become photo-bleached (Figure 3.2). Hemmerich, et al. (1967), studying details of the reaction, reported that compounds similar to PAA are photodecarboxylated with the remaining benzyl residue attaching covalently to the N-5 or C-4a position of the flavin nucleus. Such photo-adducts can be stable in the presence of oxygen. Of

Figure 3.1 Growth response of corn coleoptiles to auxin and various auxin analogues. The percentage elongation was determined by an increase in segment length after 16 hours when incubated with the compound as compared to the initial length. Control response represents growth in buffer solution alone. Each point represents the measurement of 10 segments.

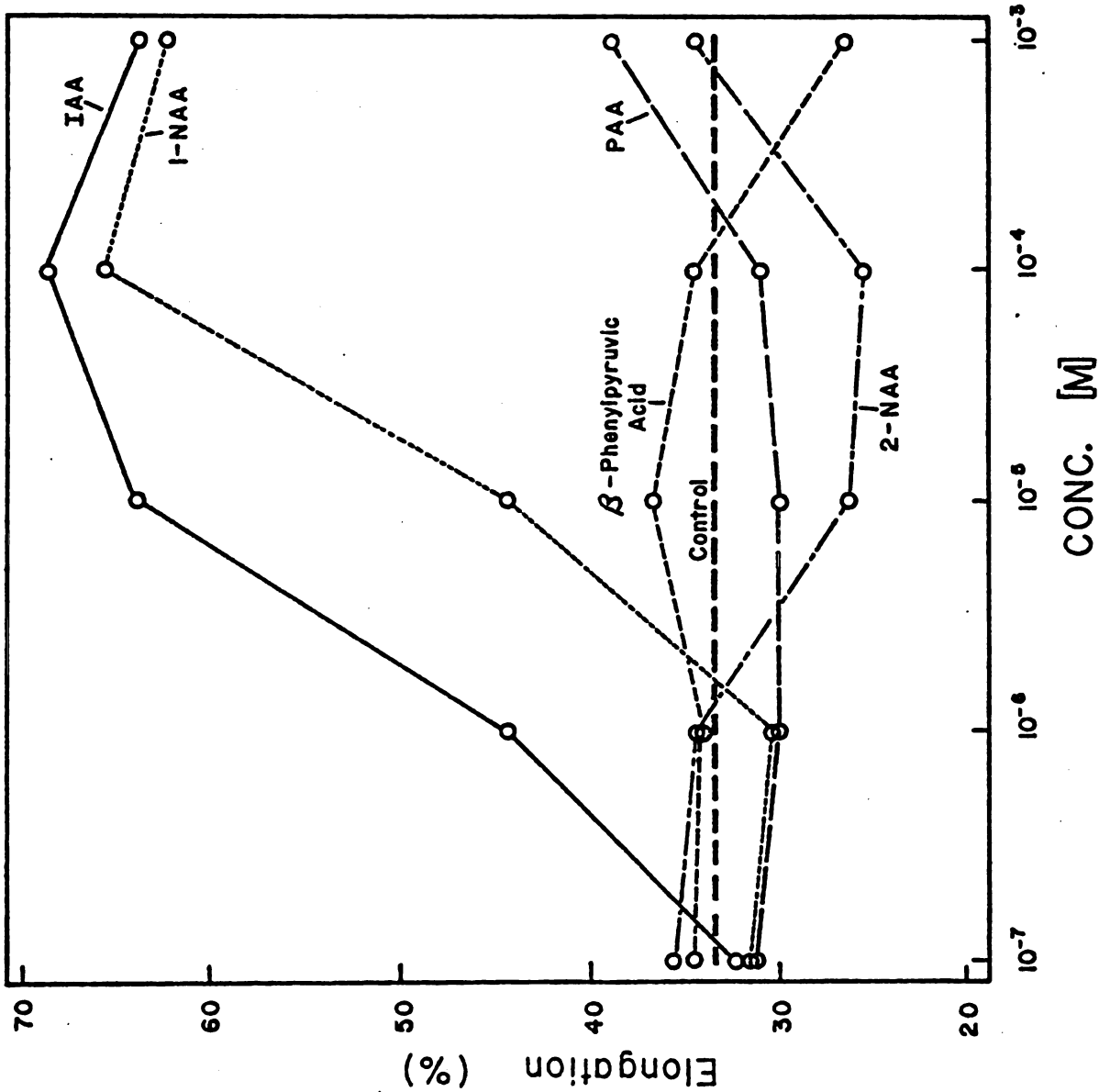
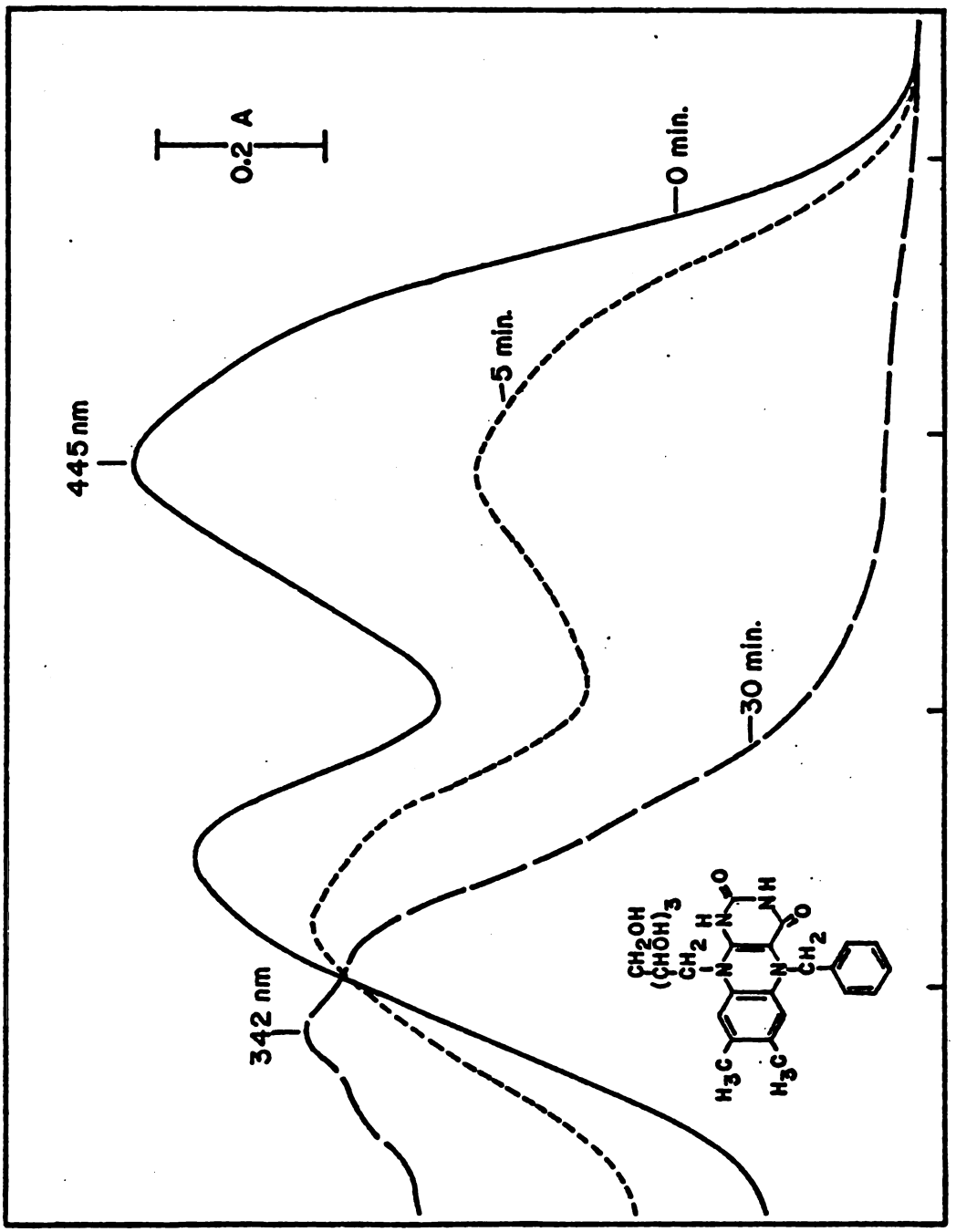

IAA
I-NAA
PAA
β-Phenylpyruvic Acid
Control
2-NAA
Elongation (%)
70
60
50
40
30
20
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
CONC. [M]

Figure 3.2 Aerobic photoreduction of riboflavin by PAA. Absorption spectrum of 0.7 mM riboflavin and 5 mM PAA solution (pH 7.0) irradiated for various time intervals with 5 mW cm^{-2} blue light. The photoproduct absorption maximum at 342 nm indicates that attachment of the benzyl residue to position N-5 of riboflavin (Hemmerich, et al., 1967).


0.2 A
0 min.
5 min.
30 min.
445 nm
342 nm
350
400
450
500
Wavelength (nm)

the five compounds tested, 1-NAA, 2-NAA, PAA and β-phenylpyruvic acid form stable photoadducts, although with different initial velocities (Table 3.1). Such photoadduct formation can be distinguished from riboflavin photodecomposition by differences in the reaction rate as well as the absorption maxima of the products. IAA did photoreact with riboflavin causing an absorbance change as a result of the modification of the indole ring, but the adduct was unstable. During the course of the reaction with IAA, riboflavin became slightly reduced, but the absorbance at 445 nm was fully regenerated in the dark under aerobic conditions.

3.3.3 Effect on corn seedling tropic responses. Several compounds were tested for their ability to inhibit phototropism and geotropism. KCN and SHAM, inhibitors of CN-sensitive and CN-resistant respiration respectively (Solomos, 1977) affected both tropic responses equally at all concentrations tested (Figure 3.3 A,B). Seedlings exposed to 10 mM KCN exhibited no tropic bending while 10 mM SHAM decreased both responses by 10%. Mannitol (Figure 3.3C) also displayed no specificity but, when used at concentrations above 20 mM, bending was significantly reduced.

When the five carboxylic acids were tested for their effects on tropism, the results were divided into three classes, showing: 1) an equally strong inhibition of both photo and geotropism--IAA and 1-NAA. [The concentrations needed to induce 50% inhibition (C_{50}) were 3 mM and 1 mM respectively (Figure 3.3D,E)]; 2) Little effect on either response except at high concentrations--β-phenylpyruvic

Table 3.1 Rates of aerobic photoreduction of riboflavin by various carboxylic acids.

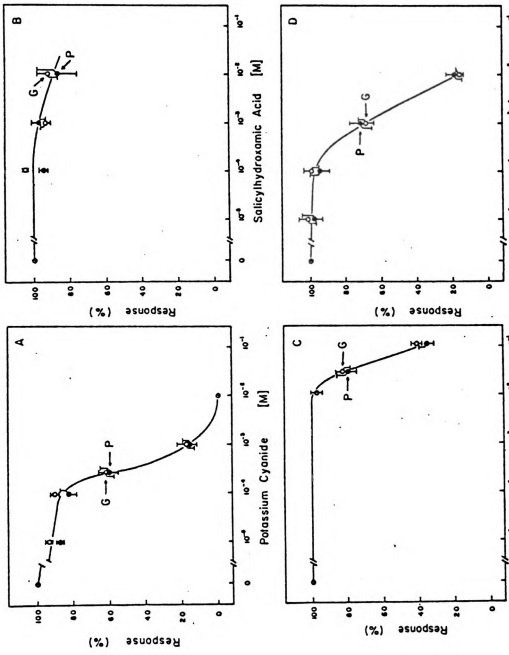
Solutions consisting of 0.7 mM riboflavin, 10 mM phosphate buffer (pH 7.0) and 5 mM of each compound were irradiated with 5 mW cm^{-2} blue light. At various time intervals, an absorption spectrum of each solution was recorded and the initial reaction rate was determined from plots of absorbance at 445 nm versus time. Absorbance maxima of the products were obtained from fully photoreduced riboflavin solutions.

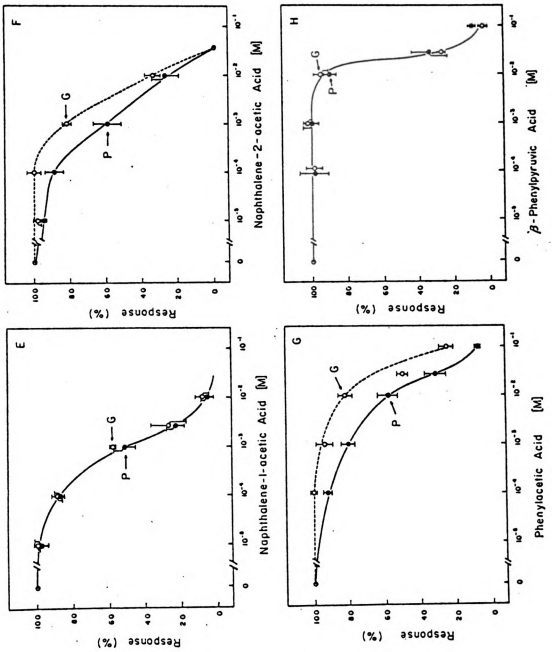
	A_{max} Photoproduct (nm)	Initial Rate of Riboflavin Photoreduction [M]/Min ($\times 10^7$)[a]
PAA	342	104
1-NAA	313	108
2-NAA	347	38
IAA	Unstable	-
β-phenylpyruvic acid	313	55
Buffer	355	6.4[b]

[a]Average of 3 determinations.

[b]Rate of riboflavin photooxidation.

Figure 3.3 Phototropic and geotropic bending in the presence of various potential inhibitors. Phototropic bending (●) relative to control seedlings in distilled H_2O was measured following 4 hour illumination by 2 $\mu W\ cm^{-2}$ blue light (450 nm). Geotropic bending (o) relative to control seedlings in distilled H_2O was measured after 4 hours geotropic stimulus in darkness. Potential inhibitors were: A) KCN, B) SHAM, C) Mannitol, D) IAA, E) 1-NAA, F) 2-NAA, G) PAA, and H) β-phenylpyruvic acid. The vertical bars represent $\pm$ one standard error. Each point represents 4 to 8 independent experiments each comparing 10 test seedlings to 10 control seedlings.


A
B
C
D
Response (%)
Potassium Cyanide [M]
Salicylhydroxamic Acid [M]
Mannitol [M]/2
Indoleacetic Acid [M]
G
P


E
F
G
H
G
P
Response (%)
Naphthalene-1-acetic Acid [M]
Naphthalene-2-acetic Acid [M]
Phenylacetic Acid [M]
β-Phenylpyruvic Acid [M]
0
20
40
60
80
100
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
10⁻¹

acid (Figure 3.3H) (C_{50} = 20 mM); and 3) A significant inhibition of phototropism when compared with geotropism--PAA and 2-NAA (Figure 3.3 F,G) (The C_{50} for phototropism was 15 mM and 2 mM for PAA and 2-NAA respectively, and for geotropism, 40 mM and 5 mM respectively). It should be noted that the percentage geotropic response was the same for the potential inhibitors, PAA and 2-NAA, whether the seedlings were illuminated with 2 μW cm^{-2} blue light (450 nm) from the top and bottom during the geotropic stimulus or kept in darkness.

3.3.4 Uptake of PAA into corn coleoptiles. By incubating the roots with PAA solutions spiked with radioactivity labelled PAA, the *in vivo* PAA concentration that could cause specific inhibition of phototropism was measured (Table 3.2). For example, the concentration of externally applied PAA in the coleoptile was 100 μM after a six hour exposure of the roots to 10 mM PAA (if the photoreceptor pigments were located on the exterior of the plasma membrane, the concentration of PAA in the vicinity of the pigments could be higher). The amount of PAA in the coleoptile increases with time to a plateau after four hours (Figure 3.4).

Table 3.2 Transport of PAA into corn coleoptiles from bathing media.

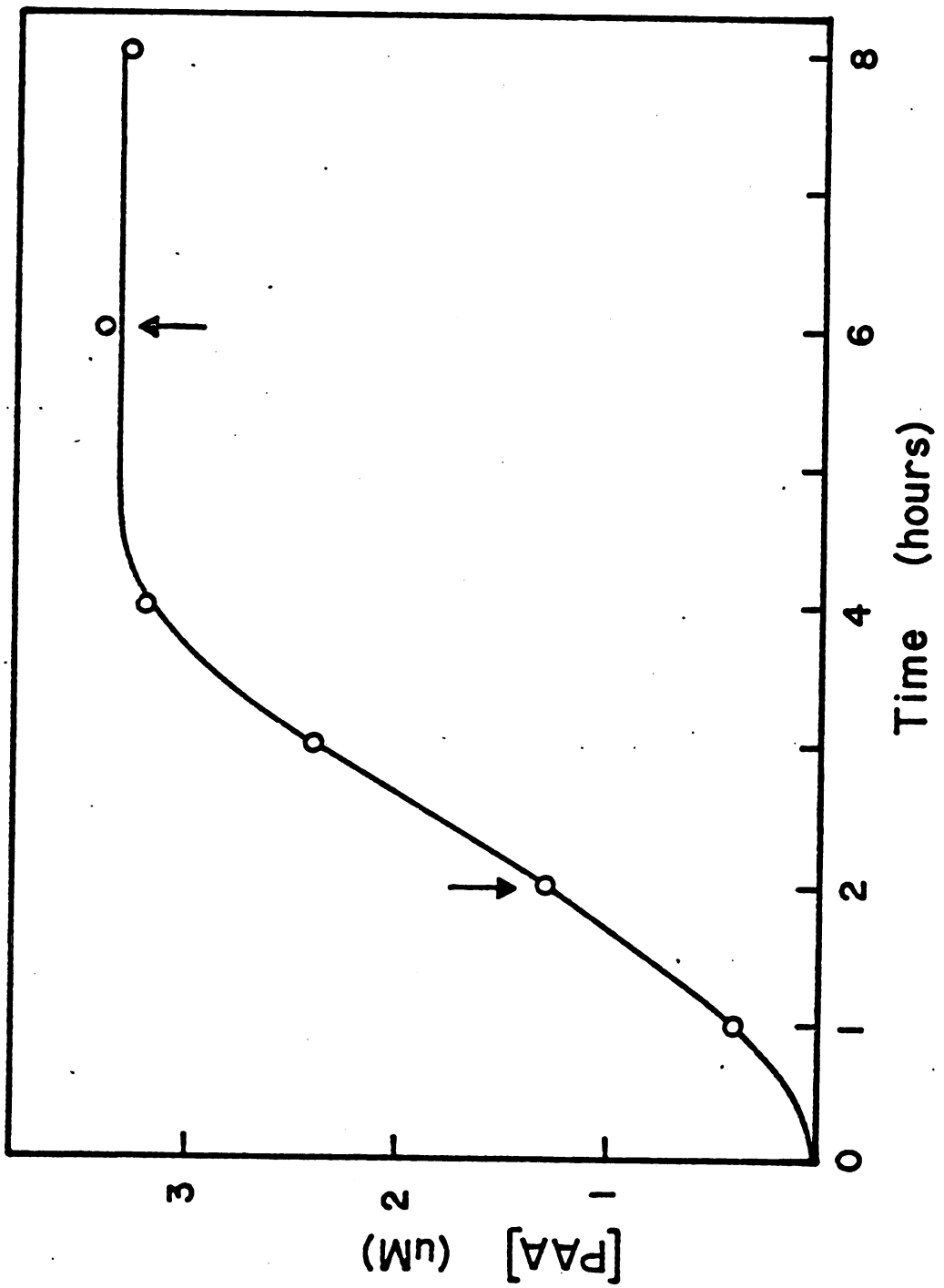
Corn seedlings' roots were incubated in various concentrations of PAA spiked with [^{3}H]PAA. After six hours, the amount of radioactivity in the coleoptile was measured and the concentration of PAA determined. (Coleoptile volumes were approximated from the difference between fresh and dry weights of the tissue.)

Concentration of PAA in bathing solution [M]	Concentration of externally applied PAA in coleoptile [M][a,b]
10^{-4}	1.8×10^{-7}
10^{-3}	4.2×10^{-6}
10^{-2}	9.5×10^{-5}

[a]Average of 2 to 3 determinations.

[b]The endogenous level of PAA, which has been detected in corn (Wightman, 1977), has not been taken into account.

Figure 3.4 Time course of PAA transport into corn coleoptiles. Corn seedlings' roots were immersed into 1.0 mM PAA, (pH 7.0) spiked with [^{3}H]PAA. At various time intervals, the coleoptiles were harvested and the radioactivity measured. Each point represents the average concentration of PAA in 10 coleoptiles. Arrows indicate the beginning and end of the tropic stimilus presentation used in Figure 3.3.


[PAA] (uM)
0
1
2
3
0
1
2
4
6
8
Time (hours)

3.4 DISCUSSION

These results support the conclusion of Schmidt, et al. (1977) that geotropism and phototropism follow similar metabolic pathways after input from the primary sensing mechanism and that specific inhibition of phototropism by PAA results from its photoreactivity with flavins. It is likely that compounds similar to PAA can also react with other photodynamic dyes (Ferri, 1951). However, the facts that PAA will react with flavins (Hemmerich, et al., 1967), that a flavin is implicated in phototropism (Dennison, 1979), and that monochromatic blue light was used in the tropism experiments (thereby eliminating the photoreaction of PAA with non blue-light-absorbing pigments) provide a rationale for the conclusion that PAA is photoreacting with a flavin involved in phototropism.

Inhibitor (KCN, SHAM, and mannitol) that affect functions related to the cell's general metabolism (e.g., respiration) show equal inhibition of both tropic responses, providing justification for the use of geotropism as a control for phototropism. The results using SHAM are noteworthy because of its reputed ability to block light-induced b-type cytochrome reductions in corn coleoptile plasma membrane-enriched fractions (Caubergs, et al., 1978). Such a b-type cytochrome reduction is hypothesized to be involved in the

photoreception of blue light (Britz, et al., 1979). If the flavin/b-cytochrome complex is actually accessible to SHAM when fed through the roots, then the insensitivity of phototropism to the inhibitor implies that the photoreduction of this b-type cytochrome may not be involved in corn phototropism.

A comparison of the auxin activity and flavin photoreactivity of the five carboxylic acids with the inhibition of tropic responses by these acids indicates that auxin activity is related to a compound's effect on tropism in general but is not a prerequisite for the specific inhibition of phototropism. IAA and 1-NAA, which are both effective in stimulating coleoptile growth, substantially inhibit both tropic responses at concentrations greater than 100 μM but do not specifically inhibit phototropism as would be expected if phototropism were more sensitive to auxins. The sensitivity of phototropism and geotropism to IAA would indicate that auxins are equally important to both responses. Addition of high concentrations of IAA would inhibit bending by stimulating growth on all sides of the coleoptile, thus interfering with any growth rate differences set up by light or gravity between two sides.

Of the compounds that do react with flavins, only PAA and 2-NAA demonstrate an ability to inhibit phototropism specifically. This suggests that a weak auxin activity may be required for compounds that photoreact with flavins to specifically inhibit phototropism. A strong auxin (1-NAA) may interfere with phototropism at concentrations too low to

bleach enough chromophores to significantly reduce phototropism while a lack of auxin activity (β-phenylpyruvic acid) might prevent adequate transport of the potential inhibitor into the coleoptile. We suggest that for such compounds to demonstrate specificity, they should weakly mimic IAA to allow uptake and transport, and should also photoreact aerobically with flavins.

Compounds such as PAA and IAA can affect also the excited states of flavins (e.g. fluorescence) by stacking (static quenching) in addition to their photoreaction with flavins (dynamic quenching). This phenomenon is unlikely to be the explanation of the observed inhibition of phototropism because the high concentrations required for static quenching [>30 mM for IAA (Song, et al., 1980)] are 100 to 1000 times greater than that required for specific inhibition in vivo (Table 3.2). In addition, IAA has no specific effect on phototropism but will affect flavins by static quenching.

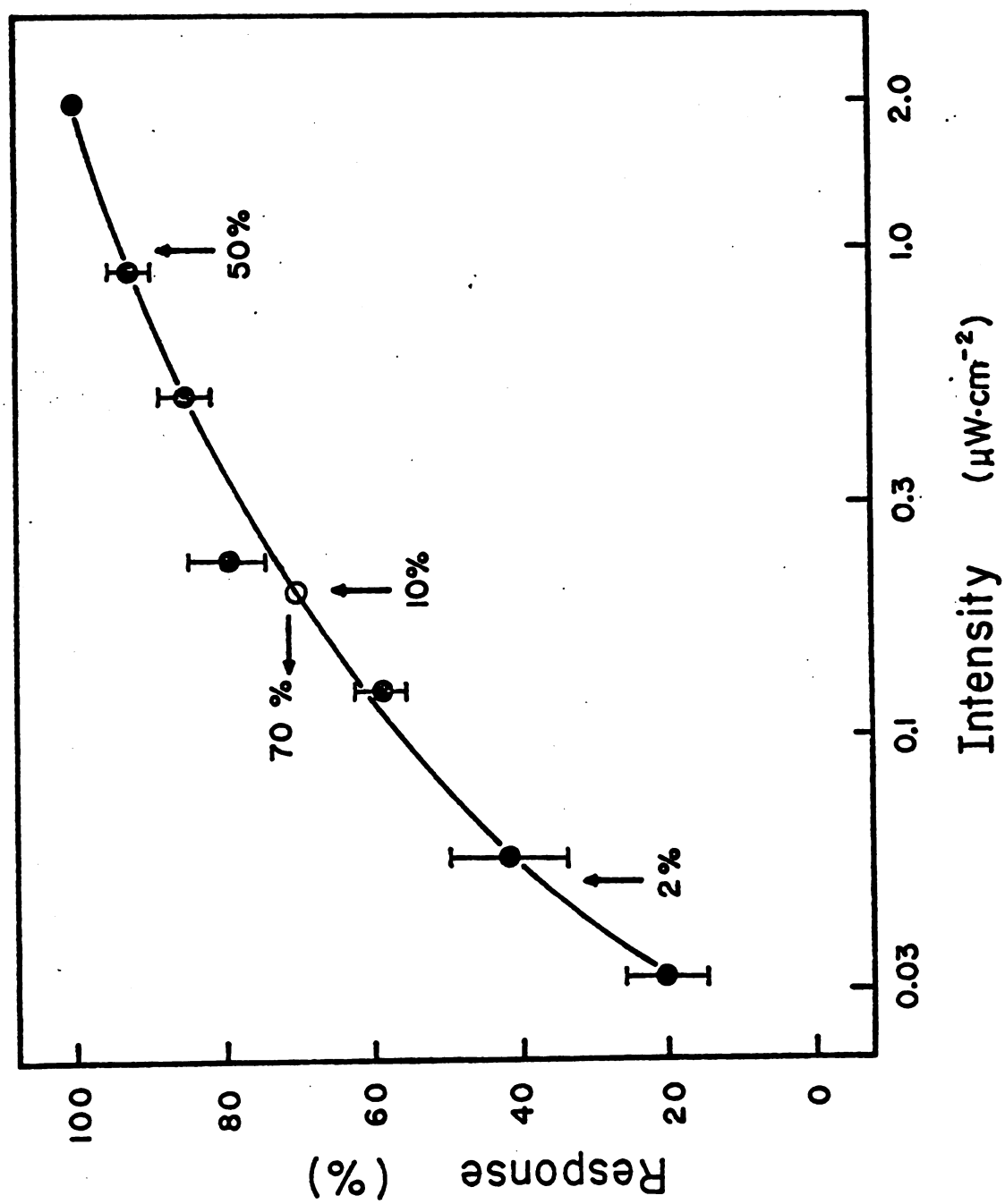
Additional evidence that PAA's ability to inhibit phototropism specifically is not related primarily to its hormonal activity is provided by measurements of PAA transport by corn seedlings. For example, when 10 mM PAA is supplied to the roots (concentrations sufficient to induce significant specific inhibition), the concentration of PAA taken up into the coleoptile was 100 μM (Table 3.2). This concentration is 10 times lower than the concentration of PAA required to stimulate coleoptile growth (Figure 3.1) (Wightman, 1977). Therefore, PAA is exerting an effect at a concentration below which it has appreciable auxin activity. However,

corn does contain significant amounts of endogenous PAA--as much as 1 μM in 2 week old corn seedlings (Wightman, 1977). Although the concentration of PAA in etiolated coleoptiles is not known, the endogenous supply of PAA could photoreact with the flavin chromophore, reducing the control's phototropic response and thus lessen the effects of exogenously applied PAA.

Based on the assumption that the bleaching of a given amount of photoreceptor pigment is equivalent to exposing the coleoptile to a decreased light intensity, an attempt was made to estimate the percentage of photoreceptor pigment bleached by PAA from a fluence response curve (Figure 3.5). [For example, a 30% reduction in curvature (70% response) would result from a 90% drop in light intensity (10 fold) or presumably by inactivating 90% of the photoreceptor pigment in the illuminated half.] In the case of the specific inhibition by PAA, 10 mM PAA inhibited phototropism by 23% relative to geotropism. If the above assumptions are valid, this would correspond to an inactivation of 40% of the total photoreceptor pigment (80% inactivated on the illuminated half). This reasoning suggests that a substantial percentage (>10%) of chromophores must be bleached by PAA to induce specific inhibition.

From studies of compounds capable of photoreacting with flavins, it is noteworthy that most potent auxins [stimulating coleoptile growth at concentrations below 100 μM (Ray, et al., 1977)] appear capable of photoreacting with flavins. Such compounds tested here include IAA, 1-NAA and

Figure 3.5 Fluence response curve for corn seedling phototropism in response to a 4 hour irradiation with blue light (450 nm). Phototropic response for each light intensity was compared on a percentage basis to that elicited by 2 $\mu W\ cm^{-2}$. Each point represents 4 to 5 independent experiments consisting of 10 seedlings irradiated with a given intensity compared with 10 seedlings irradiated with 2 $\mu W\ cm^{-2}$. The vertical bars represent $\pm$ one standard error.


Response (%)
100
80
60
40
20
0
0.03
0.1
0.3
1.0
2.0
Intensity (μW·cm⁻²)
50%
10%
70 %
2%

2,4-dichlorophenoxyacetic acid. Other compounds are not tested, but displaying structural similarities to compounds that are photoreactive, include naphthalene-2-oxyacetic acid, indole-3-n-butyric acid, and the dichloro isomers of phenoxyacetic acid and phenoxy-2-propionic acid. Galston and Baker (1949) first formulated a hypothesis that phototropism was caused by flavins photoreacting with IAA, thereby lowering its concentration on the illuminated side and reducing growth. However, experiments to determine whether light could reduce the IAA concentration in coleoptiles did not support this hypothesis (Briggs, 1963). It may be additionally important that auxin/amino acid conjugates can also react with flavins when irradiated with blue light. Both amide conjugated auxins, indoleacetylglycine and 1-naphthyleneacetylglycine (courtesy of Dr. Roger Hangarter) photoreact with riboflavin resulting, in the case of IAA conjugate, in the destruction of the indole ring. Therefore, auxin conjugation to amino acids will not protect IAA from photodestruction in light-grown plants (Bandurski, et al., 1977).

Attempts to find additional compounds, similar to PAA, capable of inducing specific inhibition of phototropism have thus far been unsuccessful. β-phenylpyruvic acid and 2-phenypropionic acid, which form aerobically stable photoadducts with flavins, showed no specific inhibition of phototropism of corn seedlings. Neither compound inhibited either tropic response except at concentrations greater than 10 mM.

The results reported here indicate that the preferential inhibition of phototropism by PAA is related to the ability

of the inhibitor to photoreact with flavins and not to its auxin activity. Since PAA is effective in inactivating a substantial percentage of photoreceptor pigment at low concentrations, the pigment must be accessible to, and very photoreactive with PAA. Thus, PAA may offer a reasonable approach to the localization and isolation of the flavoprotein phototreceptor pigment for phototropism through photoaffinity labelling of corn coleoptiles.

Chapter 4

Role of Carotenoids in the Phototropic Response of Corn Seedlings

4.1 INTRODUCTION

Plants exhibit a wide variety of physiological and morphological responses which are induced by blue light (Dennison, 1979). For many years, a carotenoid was believed to be the chromophore responsible for these responses supported primarily from action spectra which displayed maxima at 450 nm and 480 nm--characteristic of β-carotene--and evidence that photoresponsive organisms generally contained large amounts of carotenoids (Wald and DuBuy, 1936). A flavin is now thought to be chromphore based on a wide range of experimental evidence, most notably that "carotenoid-less" mutants of Phycomyces (Presti, et al., 1977), Neurospora (Sargent and Briggs, 1967) and Euglena (Checcucci, et al., 1976) display normal light sensitivity, and yet may have less than 0.004% (in the case of Phycomyces) of the wild type carotenoid content.

Obtaining evidence for the role of carotenoids in the blue light response of higher plants (e.g., phototropism of cereal coleoptiles) has been hampered by an inability to obtain mutants lacking carotenoids and by a lack of efficient carotenoid synthesis inhibitors. Bandurski and Galston (1951) studied an albino mutant of maize and found that the coleoptiles displayed 50 to 80% of the normal phototropic response to white light with only 0.1% of the normal

carotenoid content. Using inhibitors, Bara and Galston (1968) reduced carotenoid content of Avena coleoptiles to 20% of the normal concentration and demonstrated that the coleoptiles still retain their normal phototropic response. A new herbicide, SAN 9789 [(4 chloro-5-methylamino)-2-(α,α,α,trifluoro-m-tolyl)-2(2H)pyridazinone] permits the effective inhibition of carotenoid synthesis in vivo (Bartels and McCullough, 1972). This herbicide interferes with the desaturation of cis-phytoene, thus blocking the accumulation of colored carotenoids. Of equal importance is the fact that this herbicide does not appear to significantly alter general metabolism. For example, corn seedlings retain normal light sensitivity for the photomorphogenic responses stimulated by phytochrome (Jabben and Dietzer, 1979).

In this chapter, I report the effects SAN 9789 has on the carotenoid content and the phototropic response of corn seedlings to determine the role, if any, which carotenoids play in such blue light responses.

4.2 MATERIALS AND METHODS

4.2.1. Plant Material. Corn seeds (Zea mays hybrid MS WFg x Bear 38 from National Starch and Chemical Co., Decatur, Ill.) were allowed to imbibe overnight in distilled H_2O and sown, embryo up, on Kimpak germinating-paper dampened with distilled H_2O in trays covered with cellophane. If SAN 9789 treated corn seedlings were required, the seeds were allowed to imbibe overnight in unbuffered solutions, between 10^{-6} and 10^{-4} M, of SAN 9789 (trade name, Norflurazon, obtained at an 80% wettable powder from Sandoz Wander, Inc., Homestead, Fla.) and sown on Kimpak dampened with the same SAN 9789 solution. Trays were kept at 22° ± 1° C for four days in complete darkness and exposed to one hour of 50 μW cm^{-2} red light (630 nm, 30 nm half-band width) on the third and fourth nights to inhibit mesocotyl growth (Blaauw, et al., 1968). Straight seedlings between 4 and 5 cm long were selected for use.

4.2.2 Tropic Response Tests. Under a dim green safe-light, seedlings, with and without the SAN 9789 treatment, were placed in 50 ml test tubes with 2 cm of the coleoptile projecting through a hole in a cork stopper inserted in the opening of the tube and the roots immersed in distilled H_2O. The seedlings were placed in a humid plexiglas chamber for one hour prior to and during the 3 hour test stimulus (light

or gravity). Each experiment consisted of 40 seedlings, 10 germinated with distilled H_2O and 10 each, germinated with three different concentrations of SAN 9789.

To test for phototropism, the seedlings were exposed unilaterally for 3 hours to a quantum flux density of 3.8 x 10^{-12} E cm^{-2} sec^{-1} from either 1.0 μW cm^{-2} 450 nm light (8.5 nm half-band width) or 1.2 μW cm^{-2} 380 nm light (10.4 nm half-band width) from a slide projector in conjunction with a Baird Atomic interference filter. Fluence response curves for 380 or 450 nm light were determined by varing the light intensity with neutral density filters, holding the presentation time constant. This dose corresponds to that which would elicit the second positive curvature (Briggs, 1960). Geotropism was induced by holding the seedlings horizontal for 3 hours.

4.2.3 Measurements of Carotenoid Content and Absorption of Seedlings. Carotenoid concentrations were calculated from absorption spectra of corn seedlings (± SAN 9789). 0.5 grams (fresh weight) of corn coleoptiles, including the primary leaves, were homogenized in 0.5 ml H_2O and the absorption spectrum recorded using a vertical cuvette (Butler, 1972) in conjunction with a single beam spectrophotometer similar to that described by Davis, et al. (1973) on line with a Hewlett Packard 21MX mini-computer. The absorbance at 481 nm was measured for the seedlings germinated with various concentrations of SAN 9789 and related on a percentage basis to the absorbance at 481 nm of seedlings germinated with distilled H_2O.

Localization of the various pigments in corn seedlings was determined by dissection of the corn seedlings. Coleoptiles (0.73 grams) and primary leaves (0.37 grams) were separated from 1.1 grams of corn seedlings and homogenized in 0.5 ml H_2O. The absorption spectra of the various fractions were obtained as described above and then added together to determine the total absorption spectrum.

Absorbance measurements orthogonal to the long axis of single intact corn seedling at 450 nm and 380 nm were made using the single beam spectrophotometer with the seedling enclosed in a small chamber with aligned entrance and exit slits (1 mm width). Each seedling section, 1.2 cm long, was obtained beginning 3 mm from the seedling tip and placed in the chamber parallel to the exit and entrance slits. The slits were pressed firmly onto the seedling to prevent light leaks around the sides. The photomultiplier tube current at 450 nm and 380 nm was recorded with the seedlings in place and the absorbance determined from calibration with neutral density filters.

Total flavin content was determined by the lumiflavin fluorescence method as described by Jesaitis, et al. (1977), using FMN as a standard (See Section 5.2.3.6).

Light intensity measurements were made using a Kettering (model 65) radiometer.

4.3 RESULTS

4.3.1 Effect of SAN 9789 on Carotenoid Content. Corn seedlings germinated in the presence of increasing concentrations of SAN 9789 showed a substantial reduction in the accumulation of colored carotenoids (Figure 4.1). The difference spectrum of corn seedlings germinated in distilled H_2O-minus-seedlings treated with 100 µM SAN 9789 (Figure 4.1) exhibits maxima characteristic of carotenoid absorption spectra [i.e., maxima at 481 and 450 nm with prominent shoulders at 423 and 398 nm (Davis, 1976)]. A K_I of 15 µM was determined from a plot of carotenoid content as a function of SAN 9789 concentration (Figure 4.2, lower). SAN 9789 at 100 µM inhibited 98-99% of the carotenoid accumulation (with respect to that of the control) but did not alter flavin content of the seedlings. (Flavin concentration was approximately 4 µM on a gram fresh weight basis for both control and herbicide treated tissue).

The absorbance of single intact seedlings was measured (Table 4.1) to determine the effect of SAN 9789 on the total absorbance of the seedlings (both absorption and scattering). For seedlings germinated with 100 µM SAN 9789 the absorbance at 380 nm was not significantly altered (~9% reduction) but was reduced by ~25% at 450 nm (2.44 versus 1.81 A). When expressed on a percent transmission basis, SAN 9789 treatment

Figure 4.1 Absorption spectra of corn seedling germinated with H_2O or SAN 9789. The sample consisted of 0.5 grams of seedling tips (including the primary leaf) homogenized in 0.5 ml distilled H_2O. Absorption maxima were determined from the fourth derivative of the difference spectrum.

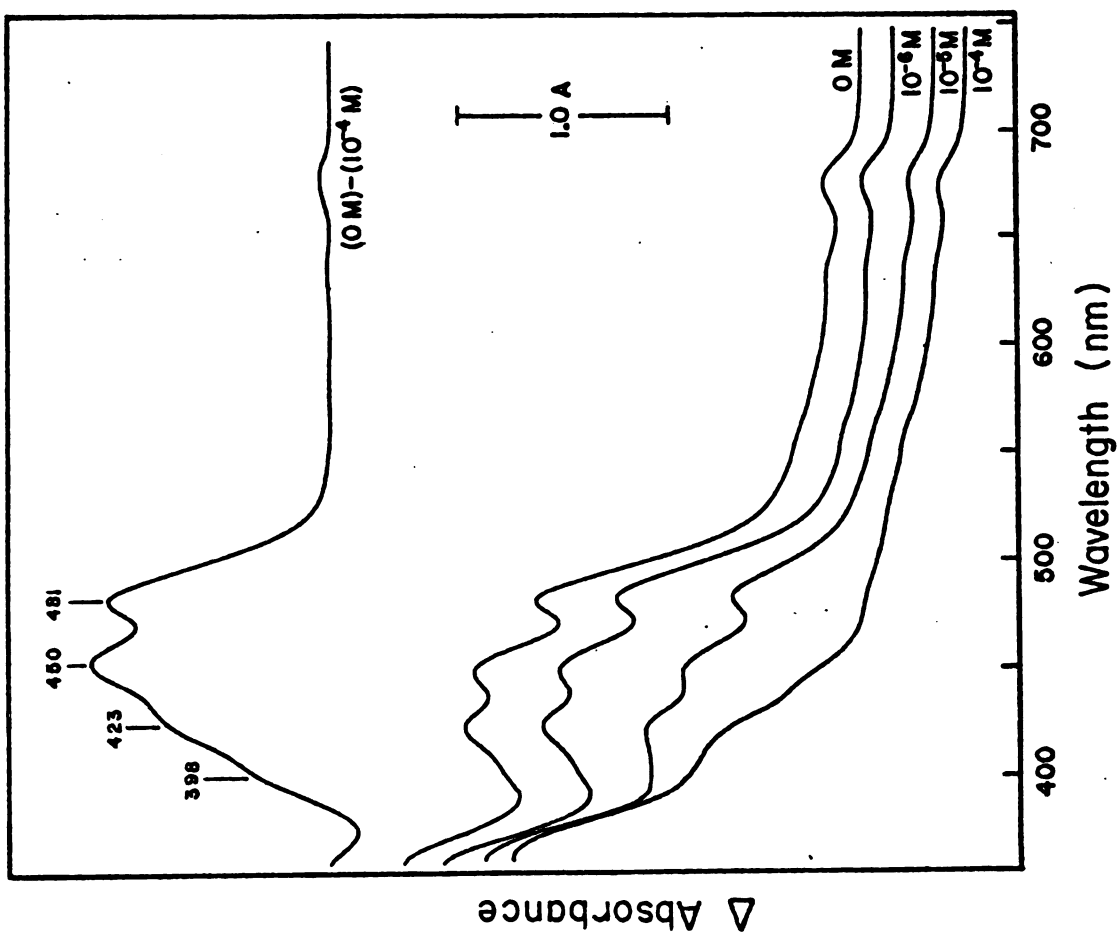

(0 M)-(10⁻⁴ M)
1.0 A
0 M
10⁻⁶M
10⁻⁵M
10⁻⁴M
481
460
423
398
400
500
600
700
Wavelength (nm)
Δ Absorbance

Table 4.1 Absorbance and percentage transmission of single intact corn seedlings germinated with or without 100 μM SAN 9789 at 380 nm and 450 nm.

Absorbance was measured using monochromatic light directed through a 1 mm entrance slit, the intact seedling, a 1 mm exit slit, and then to a photomultiplier tube calibrated using neutral density filters.

ABSORBANCE AND PERCENT TRANSMISSION OF CORN SEEDLINGS WITH AND WITHOUT 100 μM SAN 9789[a]

	Control	100 μM SAN 9789
380 nm	2.69 ± .09 (0.20%)	2.48 ± .19 (0.33%)
450 nm	2.44 ± .07 (0.36%)	1.81 ± .08 (1.54%)

[a]average of 5 seedlings ± one standard deviation

increased light transmission by 4.3 and 1.7 times for 450 and 380 nm light respectively.

4.3.2 Tropic responses of SAN 9789 treated seedlings. The tropic responses of corn seedlings were measured as a function of SAN 9789 concentration (Figure 4.2, upper). Geotropism was reduced 10% by SAN 9789 at all of the concentrations tested. This effect by 1 μM SAN 9789 on geotropism would indicate that the herbicide does effect other processes in addition to carotenoid synthesis in this variety of corn. The inhibition by SAN 9789 of the phototropic response to 380 nm light was comparable to the inhibition of geotropism at all concentrations tested even though carotenoid content was severely reduced at herbicide concentrations greater than 1 μM. In contrast, the inhibition by SAN 9789 of the phototropic response to 450 nm light was significantly greater than the inhibition of geotropism. A 20% reduction in phototropic response to 450 nm light was observed as compared to geotropism when 100 μM SAN 9789 was used.

Phototropic fluence response curves were determined for both control and SAN 9789 (100 μM) treated tissue toward 380 nm of 450 nm light. Fluence was varied by altering the light intensity of a constant 3 hour presentation time. Both control and SAN 9789 treated tissue require the same threshold light intensities (approximately 9×10^{-5} μW cm^{-2} at 450 nm and 2×10^{-4} μW cm^{-2} at 380 nm) for phototropic curvature (Figure 4.3). At saturating light intensities, however, the SAN 9789 treated seedlings developed less of a response than control seedlings and, as might be predicted from Fig. 2,

Figure 4.2 (upper) The effect of SAN 9789 on phototropic and geotropic bending of corn seedlings. Geotropic bending (▲) relative to control seedlings germinated in distilled H_2O was measured after 3 hours geotropic stimulus. Phototropic bending to 380 nm (0) and 450 nm (●) light relative to control seedlings germinated in distilled H_2O was measured after a 3 hour phototropic stimulus. The vertical bars represent ± one standard error. Each point represents 4 to 6 independent experiements comparing 10 seedlings treated with SAN 9789 to 10 control seedlings.

(lower) The effect of SAN 9789 on carotenoid accumulation in corn seedlings. Carotenoid content of SAN 9789 treated seedlings was determined from the absorbance at 481 nm of 0.5 grams of homogenized seedlings tip and compared to that of control seedlings. Error bars represent ± one standard deviation.

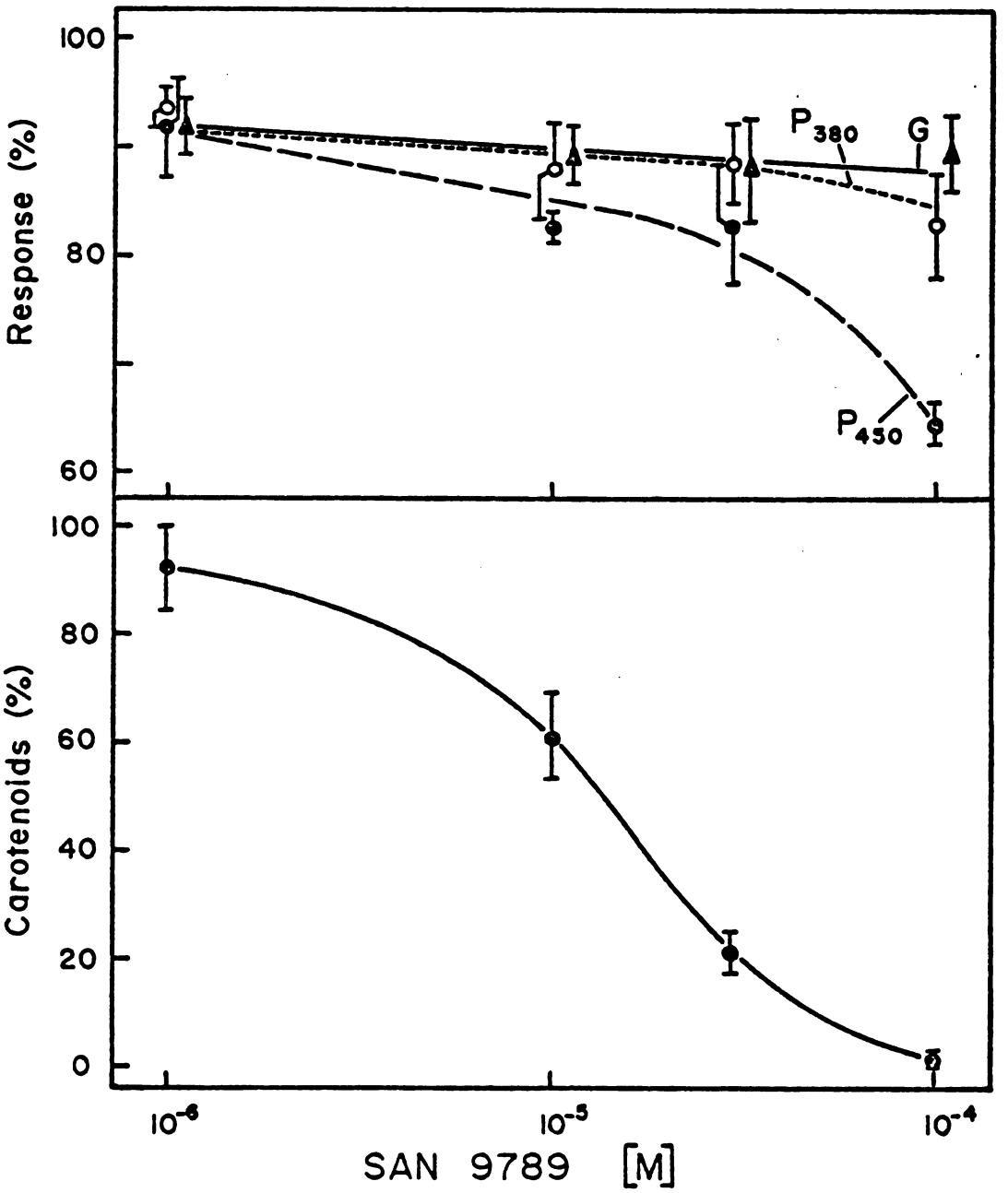
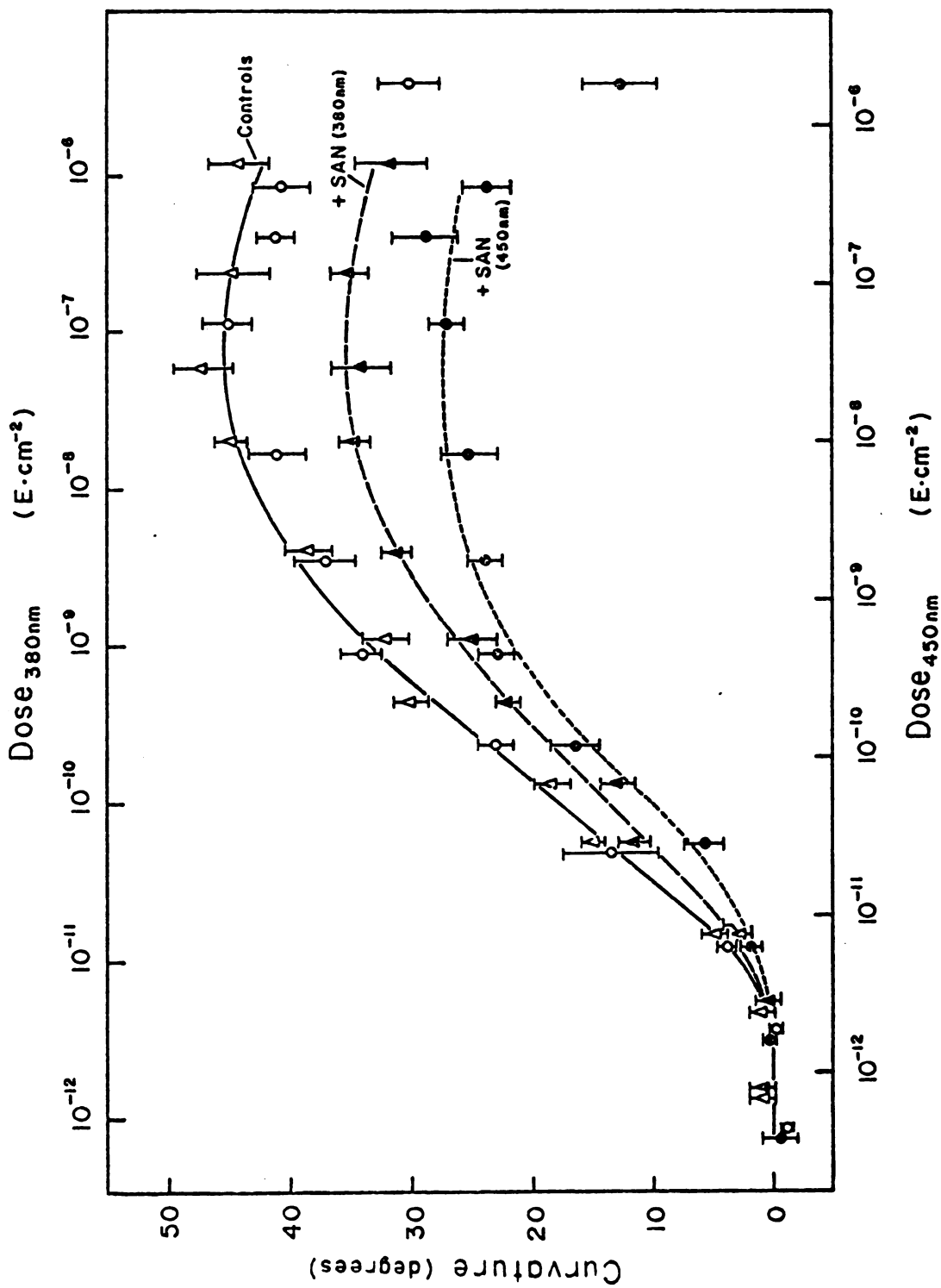

100
80
60
Response (%)
P380
G
P450
100
80
60
40
20
0
Carotenoids (%)
10-6
10-5
10-4
SAN 9789 [M]

Figure 4.3 Fluence response curves for the phototropic response of corn seedlings. Solid figures, 100 µM SAN 9789; open figures, distilled H_2O; triangles, 380 nm light; circles, 450 nm light. Phototropic bending was measured after a 3 hour stimulation. Error bars represent ± one standard error. Each point represents 4 to 6 independent experiments consisting of 10 coleoptiles each. The dose of 380 nm light has been shifted with respect with the dose at 450 nm in order to overlap the fluence response curves of 380 nm light with 450 nm light for seedlings germinated with distilled H_2O.


Curvature (degrees)
0
10
20
30
40
50
Dose380nm (E·cm-2)
10-12
10-11
10-10
10-9
10-8
10-7
10-6
Dose450nm (E·cm-2)
10-12
10-11
10-10
10-9
10-8
10-7
10-6
Controls
+ SAN (380nm)
+ SAN (450nm)

bent more toward 380 nm light than toward 450 nm light.

4.4 DISCUSSION

From these results, I conclude that "bulk" carotenoids are not the photoreceptor pigment responsible for phototropism in corn seedlings. The percentage response for phototropism toward 380 nm light was indistinguishable from that for geotropism for seedlings treated with increasing concentrations of SAN 9789 even though the accumulation of carotenoids was drastically reduced at SAN 9789 concentrations above 1 µM.

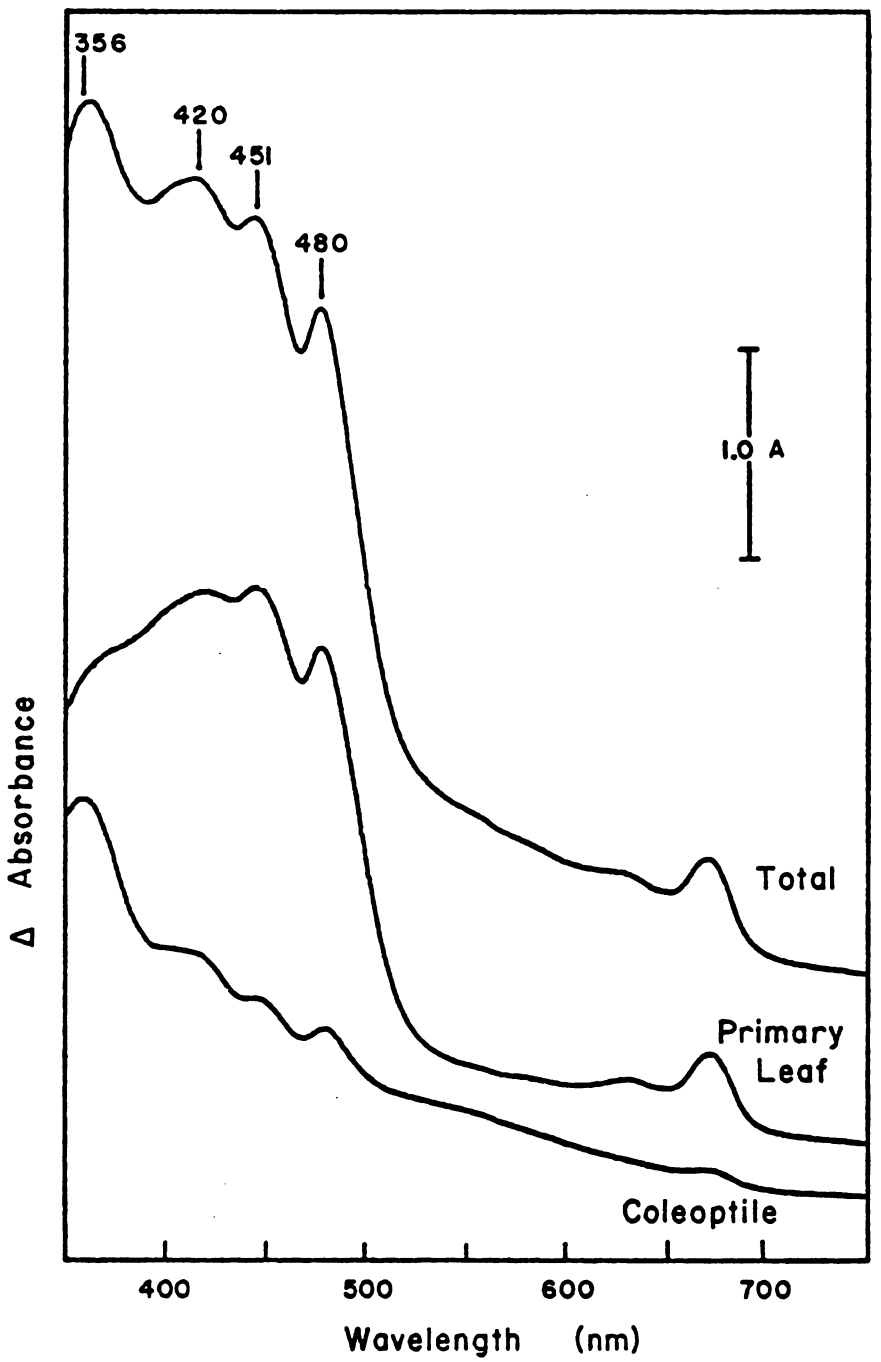
In addition, fluence response curves for seedlings treated with 100 µM SAN 9789 extrapolate to the same threshold intensity indicating that the amount of photoreceptor was not affected by the herbicide. Assuming that a prerequisite number of photons must be absorbed by the photoreceptor pigment, and that the amount of excited photoreceptor pigment is the rate-limiting factor, a significant decrease in photoreceptor pigment content would have resulted in an increase in the threshold intensity. Thus, a reduction in bulk carotenoid by 99% should have increased the threshold by 100 times.

Because the coleoptiles do contain carotenoids. even at the highest concentrations of SAN 9789 tested (~1 x 10^{-8} M in coleoptiles germinated with 100 µM SAN 9789), it is not possible to rule out all carotenoids completely since the photoreceptor pigment may be required at only very low

concentrations [theoretical minimum concentration is 3×10^{-7} M for Phycomyces (Bergman, et al., 1969) and 1×10^{-9} M for Avena (Briggs, 1964)]. Schmidt, et al. (1977) have shown that compounds affecting flavins will specifically inhibit phototropism. The data from Schmidt, et al. (1979) and Chapter 3, in addition to these results, are consistent with the conclusion that a flavin and not a carotenoid is the photoreceptor chromophore for phototropism.

However, although I conclude that a carotenoid is not the photoreceptor chromophore, carotenoids appear to be involved in photoreception, acting as an internal light filter. For phototropism to occur, the seedling must detect a difference in light absorption between the front and back of the coleoptile. Approximately 90% of the carotenoids of the shoot are located in the center of the coleoptile in the primary leaves (Figure 4.4). Because the absorbance of carotenoids and flavins overlap at wavelengths between 400 and 500 nm, carotenoids must enhance the light gradient perceived by the seedlings when irradiated with 450 nm light. Any reduction in carotenoid content would allow more light to be transmitted to the backside, collapsing the light gradient and reducing phototropic curvature. SAN 9789 treated seedlings exhibited little reduction of absorbance at 380 nm and hence, little inhibition of their phototropic response toward 380 nm light while there was a sizable drop in absorbance at 450 nm light. When expressed on a percent transmission basis, germination with 100 μM SAN 9789 increased the coleoptile's transmission by 430% thus allowing approximately 4 times more

Figure 4.4 Absorption spectra of the primary leaves and coleoptiles from dissected corn seedlings. 0.73 grams of coleoptiles and 0.37 grams of primary leaves were separated from 1.1 grams of seedlings, homogenized in 0.5 ml distilled H_2O and absorption spectrum taken in a vertical cuvette. The absorption spectrum of the total seedling was obtained by computer addition of the individual spectra of the coleoptile and the primary leaf.


356
420
451
480
1.0 A
Total
Primary
Leaf
Coleoptile
Δ Absorbance
400
500
600
700
Wavelength (nm)

light to reach the coleoptile's shaded side. It is interesting to note that the difference between phototropism toward 450 nm and 380 nm light and geotropism of seedlings exposed to 100 μM SAN 9789--25% and 6% respectively--correlate well with the loss of absorbance by such seedlings due to the SAN 9789 treatment--26% at 450 nm and 9% at 380 nm. The fact that such SAN 9789 treated coleoptiles can still respond phototropically, even with a severe reduction in absorption by pigments in the tissue indicates that scattering is also a major component in the development of a light gradient in corn.

The hypothesis that coleoptiles respond phototropically by detecting a light gradient induced by scattering and internal filters, especially carotenoids, was formulated by Reinert (1953) studying in vitro reactions of flavins in the presence of β-carotene. Evidence that the "filter" theory was valid in vivo was obtained from experiments with coleoptiles whose phototropic sensitivity could be significantly enhanced by the addition of artificial internal light filters (Brauner, 1955; Bunning, 1953). Using a derivation of the Beer-Lambert law ($I=I_o e^{-a}$), Thimann and Curry (1960) developed a model for photoresponses that involve detection of a light gradient. They suggested that if a screen (carotenoids, etc.) were present in excess of a photoreceptor pigment, the difference in light absorbed by the photoreceptor pigment between the illuminated and shaded sides (ΔI) could be approximated, neglecting the effects of scattering, by the equation:

$$\Delta I = I_o a(1-e^{-m})$$

where: I_o = incident light intensity

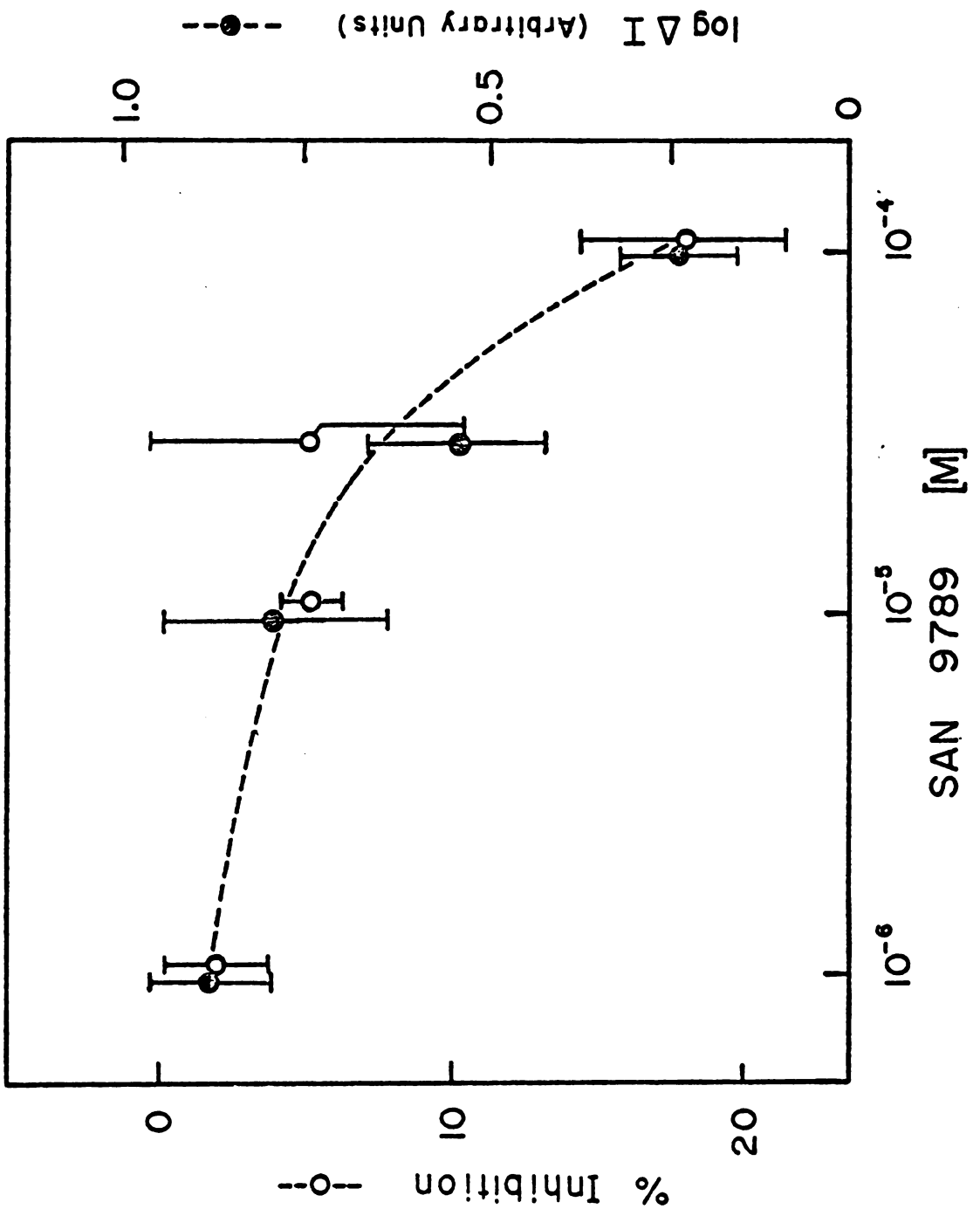
a = absorbance of the photoreceptor pigment

m = absorbance of the screen

SAN 9789 treatment will not significantly affect the light intensity on the illuminated side of the coleoptile but will increase the light intensity on the shaded side by an increase in light transmission. I have assumed that the shaded side of the coleoptile responds linearly to log I (see Figure 4.3), and therefore, have used the logarithm of ΔI to relate the model to the actual response of the seedling upon SAN 9789 treatment. Thus, because of the logarithmic nature of the equation, a significant decrease in the phototropic response would occur only at substantially reduced carotenoid levels and therefore, at high SAN 9789 concentrations. Using this equation, I find that there is good agreement between the predicted response and the percentage inhibition of phototropism using 450 nm light (Figure 4.5).

Since perception of light direction in corn seedlings involves other pigments in addition to the photoreceptor chromophore, any action spectrum for phototropism will be a function not only of the absorption spectrum of the photoreceptor pigment, but also of the shading pigments. In Phycomyces, for example, which uses focusing to detect light direction , negative phototropism in response to UV light has been attributed to screening of the photoreceptor pigment by gallic acid (Delbrück and Shropshire, 1960). In corn seedlings, carotenoids also will have some effect on the action spectrum. Fluence response curves for untreated

Figure 4.5 Comparison of the percentage inhibition of phototropism toward 450 nm light (0) (relative to geotropism) with the predicted decrease phototropic response (●) (log ΔI) as a function of SAN 9789 concentration. Percentage inhibition of phototropism toward 450 nm light was obtained from Fig. 2 (upper). Error bars for the percentage inhibition of phototropism represent $\pm$ standard error. The predicted decrease in phototropic bending was calculated from the equation; $\log \Delta I = \log(\text{constant})(1-e^{-m})$ using for m, the absorbance at 450 nm of seedlings treated with increasing concentrations of SAN 9789. Error bars for log ΔI represent $\pm$ one standard deviation of $\log \Delta I$ (calculated from 5 independent determinations of m).


log Δ I (Arbitrary Units)
1.0
0.5
0
% Inhibition
0
10
20
10^{-4}
10^{-5}
10^{-6}
SAN 9789 [M]

coleoptiles gave a 450 nm/380 ratio of 0,5 for the fluence required to elicit a 20° curvature [a similar ratio is reported in Avena (Evert and Thimann, 1968; Thimann and Curry, 1960)]. For seedlings treated with 100 μM SAN 9789, this ratio was 0.75 which is in better agreement with the 0.85--370 nm/445 nm absorbance ratio for riboflavin.

In summary, we have presented evidence that "bulk" carotenoids are not the photoreceptor pigment in corn seedling phototropism. These results, in connection with other studies involving flavin inhibitors, provide further evidence favoring a flavin as the chromophore. Carotenoids, however, appear to be involved secondarily in phototropism, acting as an internal light screen important in establishing the light gradient required for the perception of light direction. Because of this involvement, the action spectra for phototropic responses of cereal coleoptiles will be a function of carotenoids in addition to the photoreceptor pigment and thus may differ significantly from the absorption spectrum of the photoreceptor pigment.

Chapter 5

Localization of Light-Induced Phenylacetic Acid Binding in Corn Coleoptiles -- Possible Association of the Blue Light Photoreceptor Pigment with the Plasma Membrane

5.1 INTRODUCTION

It has been shown in the preceding chapters that a wide range of experimental data now favors a flavin rather than a carotenoid as the chromphore responsible for much of the blue light-sensitive photophysiology. Many questions still remain concerning the nature of the flavoprotein photoreceptor pigment(s) and the components of the sensory transduction chain between the photoreceptor pigment and the observed response. Attempts to localize and isolate the photoreceptor pigment will prove difficult because of the many flavoproteins in a cell and the lack of an assay specific for the photoreceptor pigment. Work involving the flavin(s) involved in light-induced b-type cytochrome reduction could be fruitful but a direct causal link between this photoreduction and observed photoresponse has not been accomplished, making the conclusions ambiguous.

In this chapter, the results of Schmidt, et al. (1977), and Chapter 3 will be extended in an effort to develop an approach to the localization and isolation of the photoreceptor pigment by exploiting several characteristics of PAA. First, PAA will photoreact with flavins producing a stable benzyl-derivatized flavin (Hemmerich, et al., 1979); second, PAA will specifically inhibit phototropism because of its reactivity toward flavins and not its auxin activity (Schmidt,

et al., 1977; Chapter 3); and third, although PAA will photoreact with most flavoproteins, it must be accessible to and very photoreactive with the photoreceptor pigment when considering the low concentrations of PAA required to photoreduce a significant percentage of the flavoprotein in question (Chapter 3). Therefore, PAA can be used as a somewhat specific photoaffinity label for the photoreceptor pigment. It is likely that other flavoproteins will also be derivatized in addition to the photoreceptor pigment, but the technique should allow us to eliminate a majority of those flavoproteins that are non-photoreactive. Using [^{3}H] PAA, an attempt was made to localize coleoptile membranes that are photoreactive with PAA in vivo. The in vivo photoaffinity labelling was then compared to in vitro PAA binding. In addition, the photoreactivity of PAA with several flavins and purified flavoproteins was measured to determine the effects apoproteins have on light-induced PAA binding.

I have concerned myself only with membrane fractions from corn coleoptiles because the "active" photoreceptor pigment is believed to be membrane bound based on theoretical arguments, evidence from other blue-light sensitive responses and implications from the nature of early biochemical events after blue light reception in coleoptiles (See Haupt, 1980). Evidence from other photoreceptor pigments supports the conclusion that an association of the photoreceptor pigment with a membrane is an absolute requirement, e.g., bacteriorhodopsin (Stoeckenius, 1976; Hildebrand and Dencher, 1977), rhodopsin (Hagins, 1972), chlorophyll and accessory photosynthetic

pigments (Sane, 1977; Nultch, 1980) and phytochrome (Haupt, 1972; Satter and Galston, 1973).

In addition to other systems, results from blue light responsive organisms also indicate that a membrane bound photoreceptor pigment is involved. For example, an action dichroism exists for phototropism in Phycomyces (Jesaitis, 1974), phototaxis in Euglena (Bound and Tollin, 1967) and polarotropism in Spherocarpos and Dryopteris (Steiner, 1967), suggestive of a highly oriented photoreceptor pigment, hence an association with a stable cell structure, i.e., membranes. Microbeam experiments with blue light-induced chloroplast movements and cytoplasmic streaming indicate that the pigment is not freely mobile in the alga Vaucheria (Fischer-Arnold, 1963; Blatt and Briggs, 1980). Electrical potential changes and ion fluxes, almost certainly regulated by membranes, are controled by blue light in the guard cells of onion (Moody and Zeiger, 1978) and Vicia (Hsiao, et al., 1973), pulvini of Albizzia (Satter and Galston, 1973) and the zygotes of Fucus (Weisenseel, 1979). Indirectly, the location of light-induced b-type cytochrome reduction on membranes from Neurospora and corn (Brain, et al., 1977; Britz, et al., 1979) could infer a membrane bound photoreceptor pigment. Additionally, detailed blue light action spectra (See Figure 1.1) exhibit striking similarities to the absorption spectra of flavins dissolved in hydrophobic solvents or held in a rigid matrix (Song and Moore, 1976).

Phototropism of corn coleoptiles is believed to be mediated by blue light control of polar auxin transport (Dennison,

1979). Such transport is membrane regulated (Goldsmith, 1977) [possible by the plasmalemma and/or tonoplast (Lembi, et al., 1971)] and can be affected by blue light irradiation (Briggs, 1963; Shen-Miller, et al., 1969).

Based on these results, the blue light photoreceptor pigment is belived to be associated with membranes. This provides the rationale for our attempts to localize light-induced PAA binding on corn coleoptile membrane fractions as a first approximation for the location of the blue light photoreceptor pigment.

5.2 MATERIALS AND METHODS

5.2.1 Plant Material. Corn seeds (Zea mays hybrid MS WFg x Bear 39 from National Starch and Chemical Co., Decatur, Ill.) were allowed to imbibe overnight in distilled H_2O and sown on Kimpak germinating-paper dampened with distilled H_2O in trays covered with cellophane. Trays were kept at 22° ± 1° C for 4 to 5 days in complete darkness and exposed to one hour of 50 μW cm^{-2} red light (630 nm, 30 nm half-band width) on the third and fourth nights to inhibit mesocotyl growth (Blaauw, et al., 1968). Under dim green light, the apical 1 to 2 cm of the coleoptile was harvested, separated from the primary leaves and put on ice.

5.2.2 Homogenization and Fractionation. Coleoptiles were chopped with a razor blade and then grounded in a chilled mortar with extraction buffer (0.5 ml/gram of coleoptiles). Extraction buffer contained 0.25 M sucrose, 50 mM MES, 50 mM HEPES (pH adjusted to 8.0 with KOH and HCl), 1 mM Na_2EDTA and 0.1 mM $MgCl_2$ (Jesaitis, et al., 1977). The homogenate was squeezed through a 100 μm nylon mesh and the remaining cake was reground in a second, equal volume of extraction buffer and again, squeezed through the nylon mesh. The combined filtrates (pH approximately 7) were centrifuged at 500 x g for 10 min (1,800 rpm, Sorvall HB-4 rotor) and the pellet discarded.

For differential centrifugation of the homogenate, the 500 x g supernatant (10 ml from 6 g fresh wt of coleoptiles) was layered onto a 3 ml linear 15 to 45% sucrose gradient topped with 1 ml of 15% sucrose. Gradient media consisted of either 15 or 45% sucrose (w/w), 10 mM MES, 10 mM HEPES (pH adjusted to 7.0 with KOH and HCl), 1 mM Na_2EDTA, 1 mM KCl and 0.1 mM $MgCl_2$. The tube was centrifuged for 1 hour at 198,000 x g (40,000 rpm, SW-40 rotor in a Beckman L2-65B ultracentrifuge). The region between 15 and 45% sucrose was collected, diluted with 20 ml of 15% sucorse and the resuspended membranes sequentially centrifuged for 6,800 x g (15 min), 21,000 X g (15 min) and 95,000 X g (20 min) using a Sorval HB-4 and Beckman SW-27 rotors. The pellets termed 6.8 KP, 21 KP and 95 KP were then resuspended in 0.5 ml/g fresh weight of 15% sucrose.

For sucrose gradient centrifugation, the 500 X g supernatant was layered on a linear 15 to 45% sucrose (w/w) gradient. Linear gradients (15-45%, 25 ml) were constructed over 2 ml of 45% sucrose in the bottom of a 40 ml cellulose nitrate tube. A layer of 15% sucrose was applied to the top, the volume of which (between 4 and 7 ml) depended on the volume of the crude homogenate to be applied (total gradient volume was 39 ml). The 500 X g supernatant was pipetted over the 15% sucrose layer and the tube centrifuged at 95,000 X g for various time periods (27,000 rpm, SW-27 rotor). For isopycnic gradients, centrifugation for 3 hours was found to be sufficient (Ray, 1977). After centrifugation, the gradient was fractionated from the top by injecting 55% sucrose

into the bottom of the tube with automatic collection of 1 to 2 ml fractions. Turbidity (absorbance at 435 nm) was monitored using an ISCO (Model UA-5) absorbance monitor. Sucrose concentration (w/w) was determined using a hand-held refractometer (Bausch and Lomb).

5.2.3 Assays

5.2.3.1 Protein. Total protein was assayed using a procedure developed by Bradford (1972). The sample (50 to 100 µl) was added to 5 ml of 100 mg/l Coomassie Brilliant Blue G (Sigma) in 8.5% phosphoric acid. Protein content was determined from the absorbance at 595 nm using BSA as the standard.

5.2.3.2 Cytochrome-c Oxidase. Cytochrome-c oxidase activity was determined using the technique described by Appelmans, et al., (1955). Horse heart cytochrome-c (Sigma) (0.53 mg/ml) in 50 mM tris-acetate buffer (pH 7.4) containing 0.05% Triton-X 100 was reduced with 1 µl/ml of a 1 M dithionite solution (sufficient to reduce 80 to 90% of the cytochrome-c) prepared immediately before use with distilled H_2O. The sample to be assayed (10 µl) was added to 1 ml of this solution in a 1.3 ml cuvette. After mixing, the rate of cytochrome-c oxidation was monitored by the loss of absorbance at 550 nm using a Gilford recording spectrophotometer. Activity was determined from the linear portion of the reaction rate curve.

5.2.3.3 NADH-Dependent Cytochrome-c Reductase. NADH dependent cytochrome-c reduction was measured as described by Jesaitis, et al. (1977). The sample (5 to 10 µl) and 20

µl of 50 mM NADH (Sigma) was added to a 1 ml solution of horse heart cytochrome-c (0.53 mg/ml) containing 50 mM tris-acetate (pH 7.4) and 1 mM KCN. The reaction was followed by the increase in absorbance at 550 nm. There was little reduction of cytochrome-c in the absence of NADH, thus verifying that the reaction was dependent on NADH.

5.2.3.4 Naphthalphthalamic Acid Binding. Naphthylphthalamic acid (NPA) binding was measured as described by Ray (1977). [2,3,4,5 -^{3}H] (sp. act. 16.5 Ci/mol) was obtained courtesy of Dr. Gary Gardner and unlabelled NPA (trade name ALANAP) from U.S. Rubber Co. Sample solutions (0.6 ml) were added to 1.0 ml of NPA binding medium containing 0.5 nM [^{3}H] NPA (7000 cpm/ml) 4.5 µM unlabelled NPA, 0.25 M sucrose, 10 mM Na-citrate and 5 mM $MgSO_4$ (pH 5.3) at 4° C. The samples were then centrifuged at 88,500 x g for 20 min (30,000 rpm, SW 56 rotor) in cellulose nitrate tubes. The pellets were collected by cutting off the bottom of the tubes and radioactivity measured by scintillation counting after dissolving the pellets in 10 ml of Bray scintillation fluid (60 g naphthalene, 4 g PPO, 0.2 g POPOP, 100 ml absolute methanol, made to 1 liter with dioxane). The difference in counts per minute between the pellets exposed to [^{3}H] NPA and [^{3}H] NPA plus an excess of unlabelled NPA was used as specific NPA binding for each fraction.

5.2.3.5 Glucan Synthetases I and II. Glucan synthetases I and II were measured according to Ray (1977). 200 µl of sample solution was added to 80 µl of reaction medium containing 50 mM tris (pH 8.0) and 1.5 nM UDP [^{14}C] glucose (sp.

act. 303 Ci/mol, from ICN). For glucan synthetase I, the reaction medium also contained 200 mM $MgCl_2$ and for glucan synthetase II; 2 mM unlabelled UDPG (Sigma). Samples were incubated for 20 min at 26° C and the reaction terminated by boiling for 2 min after the addition of 2 ml of 70% ethanol, 100 µl BSA (50 mg/ml) and, to the glucan synthetase II assay only, 50 µl of 50 mM $MgCl_2$. The mixture was chilled to -10° C overnight and the precipitate pelleted at 2000 X g and washed 4 times with 70% ethanol. The ethanol-insoluble material was then suspended in scintillation fluid and the radioactivity measured.

5.2.3.6 Flavins. Total flavin content was determined as described by Jesaitis, et al. (1977) using a modification of the lumiflavin fluorescence method of Yagi (1962). One ml of 1 M NaOH was added to 0.5 ml of the sample and the solution irradiated for 1 hour with a bank of fluorescent lights. Following irradiation, the solution was neutralized with 1 ml 20% acetic acid and the lumiflavin extracted with 3 ml of chloroform. The fluorescence excitation spectrum (530 mm emission) of the chloroform layer was recorded using an Aminco Bowman (model H-8202) spectrophotofluorometer and the excitation peak height at 460 nm determined. The assay was calibrated using FMN as a standard and was linear for FMN concentrations between 10 nM and 100 µM (the assay could easily detect as little as 10 pmol FMN). The addition of PAA in the dark before NaOH did not interfere with the lumiflavin assay, but if the FMN solution was irradiated in the presence of PAA before the assay, the resulting photoadduct was stable

under the assay procedures and the fluorescence causally reduced.

5.2.3.7 Carotenoids. Carotenoid content was determined from the absorbance at 480 nm (Davies, 1976) of the sample which was measured with a Cary 15 spectrophotometer.

5.2.3.8 Cytochromes. Cytochrome content of each sample was determined from low temperature (77K) reduced-minus-oxidized absorption spectroscopy. Absorption spectra were measured using a cylindrical vertical cuvette in conjunction with a single beam spectrophotometer on line with a Hewlett Packard 21 MX computer (Butler, 1972; Davis, et al., 1973). The vertical cuvette was inserted in a liquid nitrogen-filled Dewar with an optical window on the bottom. Spectra of oxidized conditions were obtained from 0.5 ml of sample and spectra of reduced conditions obtained from the same sample with the addition of several grains of dithionite after thawing. Reduced-minus-oxidized absorption spectra were calculated by computer subtraction and absorption maxima determined from fourth derivative analysis (Butler, 1972).

5.2.4 Electron Microscopy. Gradient fractions were diluted with 15% sucrose buffer and pelleted at 88,500 X g for 20 min (30,000 rpm SW-56 rotor). The pellet was resuspended and fixed overnight at 2° C with 1 ml of a 2% glutaraldehyde solution (0.1 M Na_2PO_4, pH 7.2). The suspension was repelleted and washed 3 times with 1 ml buffer alone and then treated witha 1% OsO_4 (0.1 M Na_2PO_4, pH 7.2) solution for 1 hour. After rinsing 3 times with distilled H_2O, the pellets were dehydrated with a graded acetone series and embedded in

Epon-Araldite resin (Lembi, et al., 1971). Sections were observed and photographed using a Phillips Model 300 Electron Microscope.

5.2.5 In vivo Light-Induced PAA Binding. 6.0 grams fresh weight of 1 to 2 cm corn coleoptile tips were harvested and chopped into approximately 3 mm sections. The sections were added to a 4 ml solution of 2.7 nM (200 μCi) [ring-4-^{3}H] PAA (sp. act. 18.5 Ci/mmol, IRE, Belgium). The coleoptiles were kept on ice either in darkness or while irradiated from top and bottom with blue light (5 or 0.1 mW cm^{-2}) from a projector in combination 3 cm aqueous 10% $CuSO_4$ and a Balzers DT Blau filter (370 to 470 nm transmission). All steps before and after the light treatments were performed under dim green safelights (530 nm emission maximum). The sections were stirred at 10 minute intervals and, after 1 hour, were washed three times with 25 ml of 50 mM unlabelled PAA (pH 7.0 adjusted with KOH) for 20 min. The sections were blotted dry with a paper towel and homogenized and fractionated according to the aforementioned procedures with the addition of 50 mM PAA and 0.1 mM IAA to the extraction and 10 mM PAA and 0.1 mM IAA to the gradient media (pH adjusted with KOH to 8.0 and 7.0 respectively). Approximately 10% of the radioactivity applied was taken up into the coleoptiles and survived washing with unlabelled PAA (50 mM).

After differential centrifugation, the radioactivity in the 6.8, 21 and 95 KP fractions was measured by scintillation counting of the pellets dissolved in 15 ml of ACS scintillation fluid (Amersham). After sucrose gradient centrifugation,

the radioactivity in each gradient fraction was measured either by scintillation counting after burning 0.5 ml of sample in a Packard Sample Oxidizer with 300 mg of Kimwipe and 200 µl of Combustaid (Packard) or by adding the sample (100 µl) directly to 15 ml of Biosol (New England Nuclear) scintillation fluid.

Light-induced PAA binding was calculated from the difference in counts per minute between identical membrane fractions from differential or sucrose gradient centrifugation of coleoptile sections incubated in the light or kept in darkness. Membrane fractions from "light" and "dark" sucrose gradients were matched according to their % sucrose as well as the gradient's absorbance profile at 435 nm.

5.2.6 In vitro Photoreaction of Membrane Bound Flavins with PAA. 11 grams of corn coleoptiles were homogenized and fractionated according to section 5.2.2. Isopycnic sucrose gradient fractions (400 µl) were added to either 100 µl of distilled H_2O and kept in darkness or 100 µl of 250 mM PAA (± Triton-X 100) (pH 7.0) and irradiated for 1, 5 or 30 minutes with 1 mW cm^{-2} blue light from a projector in combination with two DT Blau filters. Afterwards, flavin content was assayed by the luminflavin method as described in Section 5.2.3.6.

5.2.7. Photoreactivity of Purified Flavins and Flavoproteins. Flavins; riboflavin, FMN, and FAD (Sigma) and flavoproteins, glucose oxidase, diaphorase, and D-amino acid oxidase (Worthington) were dissolved in 10 mM K_2HPO_4 (pH 7.0) (A_{445nm} 0.8). Using a vertical cuvette, a solution

consisting of 0.75 ml of a flavin or flavoprotein and 0.25 ml of 20 mM PAA or distilled H_2O, was irradiated with blue light (5 mW cm^{-2}) from a projector in combination with 3 cm aqueous 10% $CuSO_4$ and a DT Blau filter. At various time intervals, absorption spectra were recorded using a single beam spectrophotometer (See Section 5.2.3.7). From plots of the absorbance maximum (450 nm) of the flavin or flavoprotein versus time, the initial rate of photoreduction in the presence of PAA or photobleaching in the presence of distilled H_2O could be measured. Flavin fluorescence intensity was determined from the fluorescence emissions of flavins in 10 mM KH_2PO_4 (pH 7.0) at 525 nm using an Aminco Bowman spectrophotofluorimeter (Model H-8202).

5.3 RESULTS

5.3.1 Localization using Differential Centrifugation. Localization of in vivo light-induced PAA binding on corn coleoptile membranes involved the use of differential centrifugation of the 500 X g supernatant. Table 5.1 shows the distribution of various enzyme markers, expressed on a per mg protein basis, used for localization of distinct membrane fractions (See Quail, 1979). The mitochondrial marker, cytochrome-c oxidase (Appelmans, et al., 1955) was enriched in the 6800 X g pellet (6.8 KP), while the endoplasmic reticulum marker, NADH-dependent cytochrome-c reductase (Lord, et al., 1973) was found in all three fractions (6.8, 21 and 95 KP) with a peak of activity in the 95 KP. Approximately 60% of the mitochondria were recoverable in the 6.8 KP and 40% of the endoplasmic reticulum in the 95 KP. Two putative plasma membrane markers, 1-N-naphthylphthalamic acid (NPA) binding (Lembi, et al., 1971) and glucan synthesis II [high UDPG, Mg^{+2} independent (Ray, 1977)], were present in all three pellets with the 6.8 and 95 KP exhibiting the most activity. Marker enzyme distributions are similar to those reported by Jesaitis, et al. (1979) and Dohrmann, et al. (1978) with the exceptions that, in this case, the plasma membrane is not enriched in the 21 KP. This difference most

Table 5.1 Distribution of protein, membrane markers, flavins and light-induced PAA binding following differential centrifugation. All activities are express on a per mg protein basis. Numbers in parentheses are percent activity as compared to the most active fraction.

Fraction	Protein	Cyt-c Oxidase	NADH Cyt-c Reductase	NPA Binding	Glucan Synthetase II	Flavins	Light-Induced PAA Binding	Specific Light-Induced PAA Binding
	mg/ml	rel. act.		cpm	cpm x 10^{-3}	mol x 10^{7}	cpm x 10^{-2}	cpm/mol flavin (x 10^{9})
6.8 KP	0.57	205(100)	68(78)	805(84)	37(100)	11.8	43.6(100)	3.7
21 KP	0.48	109(53)	67(76)	604(63)	24(65)	11.5	17.2(40)	1.5
95 KP	0,68	34(17)	88(100)	957(100)	28(85)	8.5	24.4(56)	2.9

likely results from the initial sucrose gradient centrifugation step used here (see below). The flavin content of each fraction was approximately 1 µmol/mg protein and appeared to be tightly bound as suggested by the fact that it remained pelletable after the intervening gradient centrifugation step.

To determine in vivo light-induced PAA binding to corn membrane fractions, chopped coleoptiles were incubated in 2.7 nM solutions of [^{3}H] PAA in light or darkness. A dark control was required due to the large excess of auxin-related (Ray, et al., 1977) and non-specific (Murphy, 1979) binding of PAA to corn membranes. The crude homogenate was first centrifuged into a small 15 to 45% linear sucrose gradient before pelleting to separate the particulate material from the non-bound radioactivity. This step removed 98% of the radioactively labeled PAA from the particulate material. There was still a significant quantity of non-specifically bound radioactivity despite this extra centrifugation step and extensive washings with 50 mM PAA and 0.1 mM IAA. Therefore, the in vivo light-induced PAA binding was determined by subtracting the membrane-associated radioactivity of coleoptiles irradiated with blue light from that of coleoptiles kept in the dark.

Using this procedure, the three pelletable fractions were assayed for in vivo light-induced PAA binding (Table 5.1). Light treatment increased the pelletable radioactivity from 6 g of coleoptiles by 15,000 cpm, a 30% increase over dark controls. The 6.8, 21 and 95 KP fractions exhibited increases in radioactivity of 60, 43 and 17% respectively after

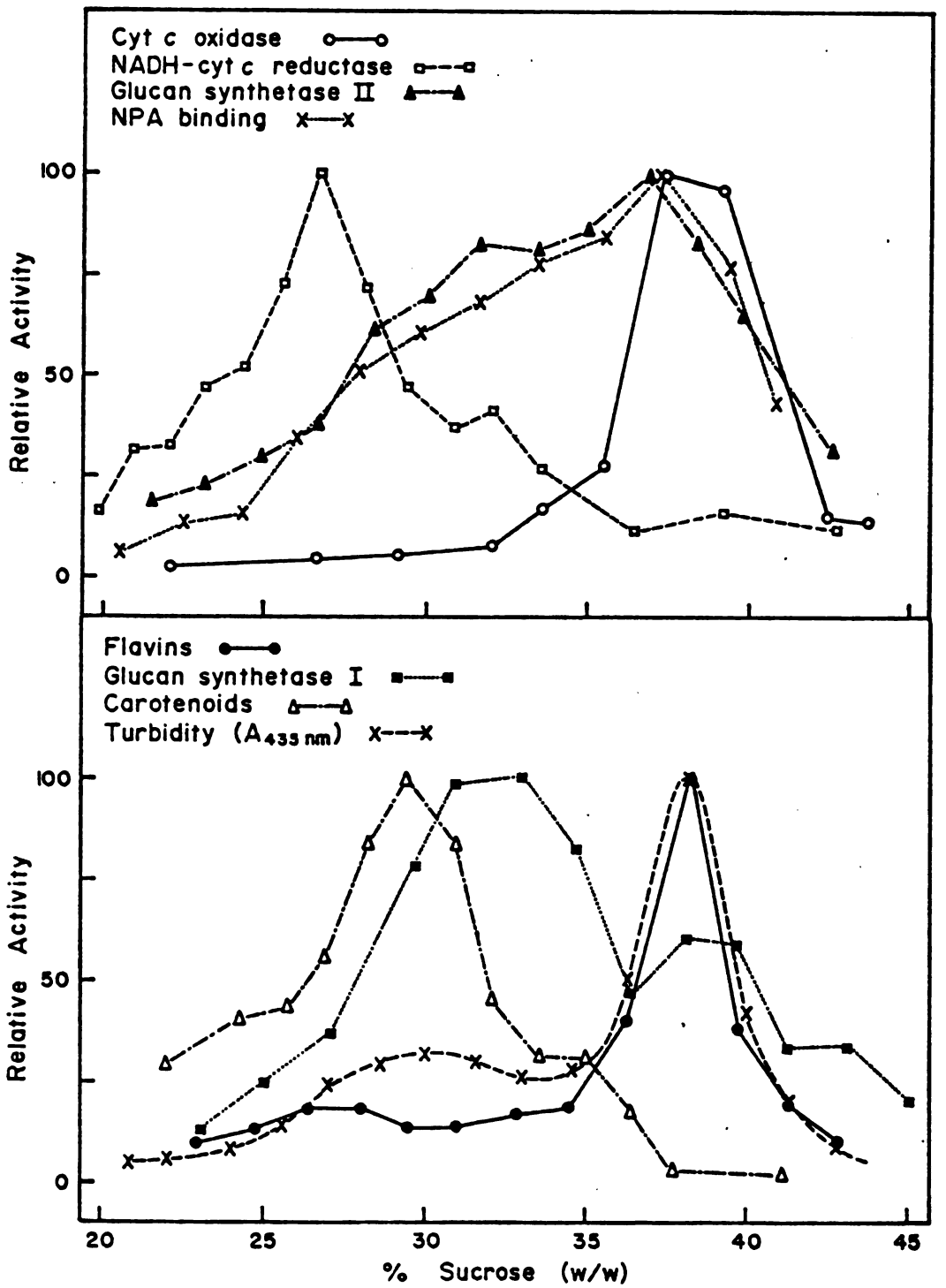
irradiation with blue light. When expressed on a per mg protein basis, the 6.8 KP was the most active fraction containing 50% of the total light-induced pelletable radioactivity. Correlation with enzymatic activities indicated a possible association with the plasma membrane and mitochondria. More importantly, significant heterogeneity exists between fractions in the photoreactivity of the membrane associated flavins with PAA when expressed on a per flavin-basis. For example, flavins of the 6.8 KP were 2.5 times more photoreactive than those of the 21 KP. Such differences in specific light-induced binding suggested to me that membrane fractions do exist that contain flavins that are more photoreactive with PAA than flavins of other fractions.

5.3.2 Localization Using Sucrose Gradient Centrifugation.

5.3.2.1 Localization of Distinct Membrane Fractions on Isopycnic Gradients. To achieve better resolution of the various membrane fractions, isopycnic centrifugation was employed using linear 15 to 45% sucrose gradients. Relative activities of marker enzymes specific for the various membrane fractions are in Figure 5.1. Cytochrome-c oxidase and NADH-dependent cytochrome-c reductase have peaks of activity centered at 38% sucrose (w/w) (density 1.17 g cm^{-3}) and 27% sucrose (1.11 g cm^{-3}) respectively. These two enzymes located the equilibrium sedimentation behavior of mitochondria and endoplasmic reticulum.

The distribution of mitochondria on the isopycnic sucrose gradients was further substantiated by reduced-minus-oxidized absorption spectroscopy of the gradient fractions

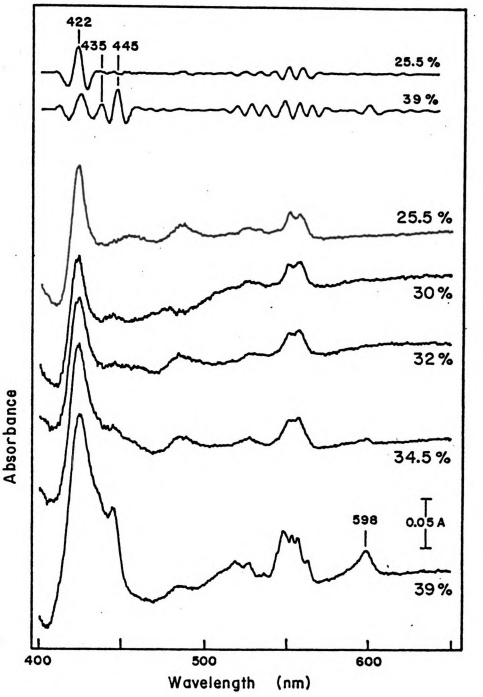
Figure 5.1 Distribution of marker enzyme activities after isopycnic sucrose gradient centrifugation. The 500 X g supernatant was layered on a 15 to 45% linear sucrose gradient and centrifuged for 3 hours at 95,000 X g. Afterwards, the gradient was fractionated and marker enzyme activities determined and relative activity expressed as a function of sucrose concentration.


Cyt c oxidase
NADH-cyt c reductase
Glucan synthetase II
NPA binding
Relative Activity
100
50
0
Flavins
Glucan synthetase I
Carotenoids
Turbidity (A435 nm)
Relative Activity
100
50
0
20
25
30
35
40
45
% Sucrose (w/w)

at 77 K (Figure 5.2 a,b). The appearance of cytochrome-oxidase (A_{max} of the Soret and α bands at 445 and 598 nm respectively) and cytochrome-c (A_{max} 548 of the α band), in such spectra is diagnostic for intact mitochondria [both cytochrome oxidase and cytochrome-c are components of the mitochondrial electron transport chain (Davis, et al., 1973)]. The distribution of cytochrome oxidase absorption at 598 nm correlated well with the presence of cytochrome-c oxidase activity in similar gradients. For example, fractions from 34.5% sucrose contain less than 20% of the cytochrome oxidase and cytochrome-c oxidase activity of fractions from 39% sucrose. In addition, an absorption spectrum of regions enriched in endoplasmic reticulum (25.5% sucrose) exhibited b-type cytochromes similar to that found by Jesaitis, et al. (1977) (i.e., α band maxima at 550 and 557 nm).

The plasma membrane--as assayed by the markers NPA binding the glucan synthetase II--sedimented over a broad range of sucrose concentrations with a maximum centered at 35 to 36% sucrose. Carotenoid content, indicative of proplastids reached a maximum at 29% sucrose and glucan synthetase I [low UDPG, Mg^{+2} dependent (Ray, 1977)], characteristic of golgi, peaked at 32%. Flavins, as determined by the lumiflavin asay, were associated primarily with mitochondria (~60% of the total) with a small peak in the region enriched for the endoplasmic reticulum. These flavins were considered to be tightly bound as judged by their inability to be lost after extensive (24 hrs) dialysis. The sedimentation behavior of these enzyme markers match closely those reported

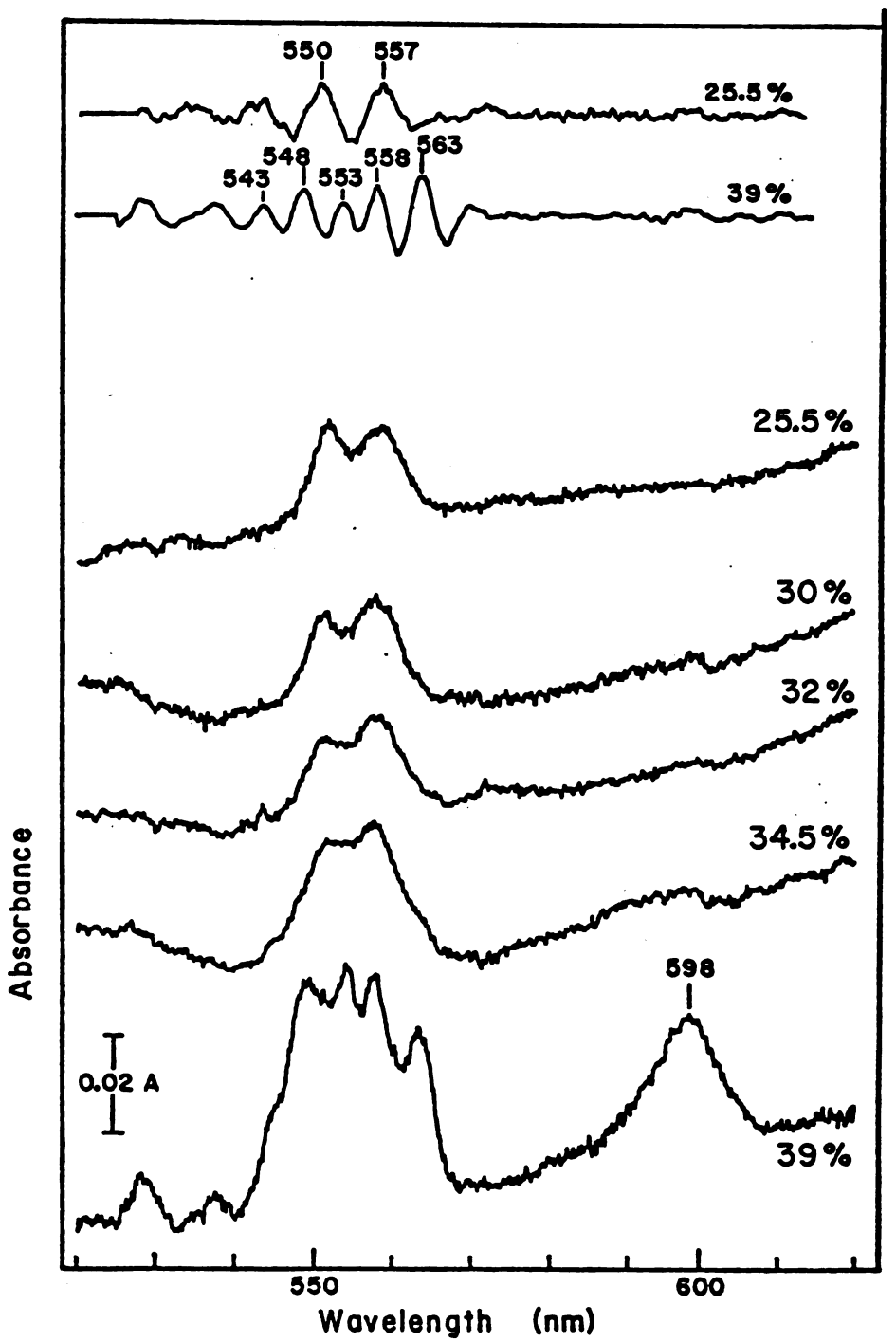
Figure 5.2 a,b Low temperature reduced-minus-oxidized difference spectra of various sucrose gradient fractions from corn coleoptile membranes. Absorption spectra of oxidized samples were obtained from 0.5 ml of sample frozen to 77K and spectra of reduced samples obtained similarly with the addition of dithionite after thawing. Absorbance maxima were determined by fourth derivative analysis (top). a) Absorption spectra between 400 and 650 nm and b) from 520 to 620 nm. Percentages indicate the percentage sucrose of each fraction.

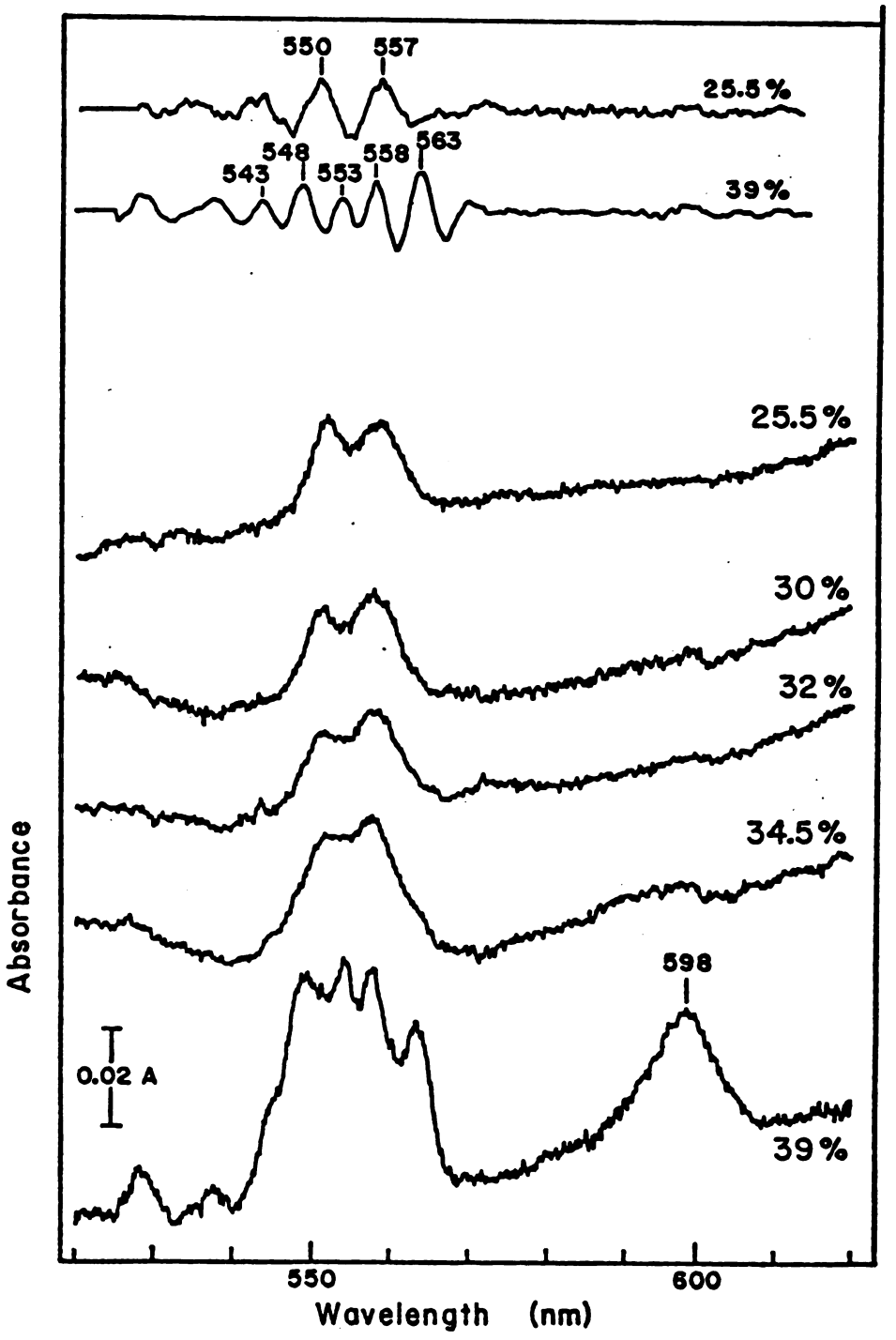

422
435
445
25.5 %
39 %
25.5 %
30 %
32 %
34.5 %
598
0.05 A
39 %
Absorbance
400
500
600
Wavelength (nm)

elsewhere (Jesaitis, et al., 1977; Ray, 1977; Dohrmann, et al., 1978).

Electron microscopy of particulate material from the various sucrose gradient fractions provide further evidence as to the position of recognizable membranes (Figure 5.3, a, b,c,d). Fractions at 39% sucrose contain almost exclusively mitochondria with a few large membrane vesicles, whereas those from 35% sucrose show much less mitochondria with a heterogeneous collection of smooth vesicles. Prolamella bodies derived from proplastids and golgi are evident at 29% sucrose and the 26% sucrose fraction contains non-distinct small vesicles suggestive of smooth endoplasmic reticulum (rought endoplasmic reticulum were stripped of their ribosomes with the use of a low Mg^{+2} homogenization buffer).

5.3.2.2 Localization of Light-Induced PAA Binding on Isopycnic Gradients. Using similar centrifugation procedures, as in Section 5.3.2.1, attempts were made to identify coleoptile membranes that were able to specifically bind PAA after blue light irradiation. After incubation in radioactively labelled PAA, the coleoptile sections were extensively washed with unlabelled PAA and IAA to remove non-bound radioactivity. PAA binding to coeloptile membranes kept in darkness occurred over the whole gradient [Figure 5.4 (lower)] with small peaks at 37 and 26% sucrose. This non-specific binding correlated loosely with protein distribution but correlated very little with trubidity (Figure 1), which is indicative of vesicle content and hence, particle surface area. The peak at 26% may reflect an auxin binding site described by Ray (1977) to


550
557
25.5%
548
543
553
558
563
39%
25.5%
30%
32%
34.5%
598
0.02 A
39%
Absorbance
550
600
Wavelength (nm)


550
557
25.5%
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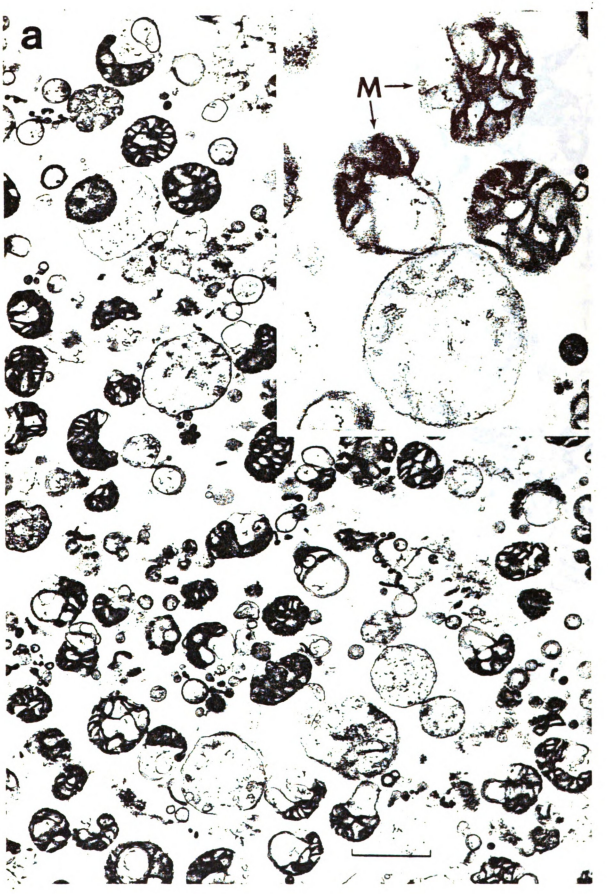
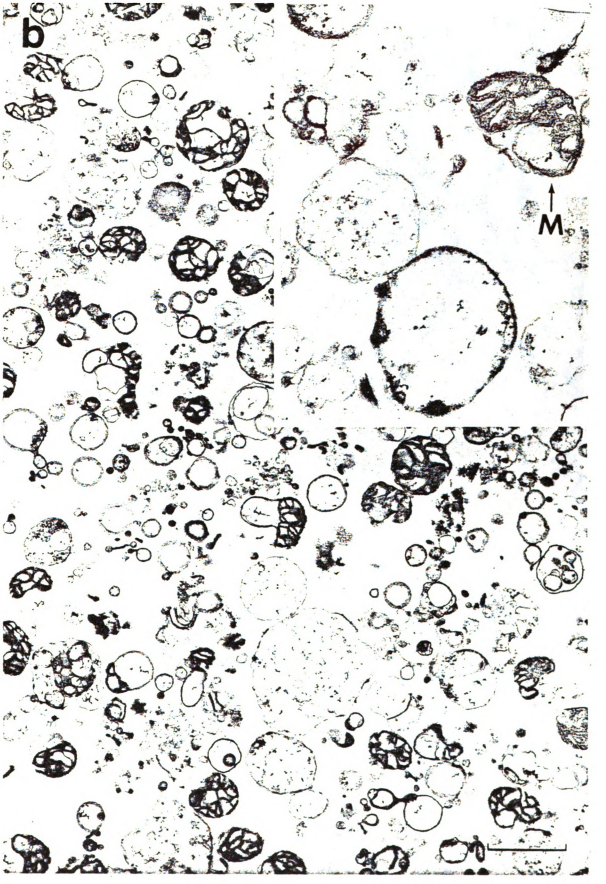
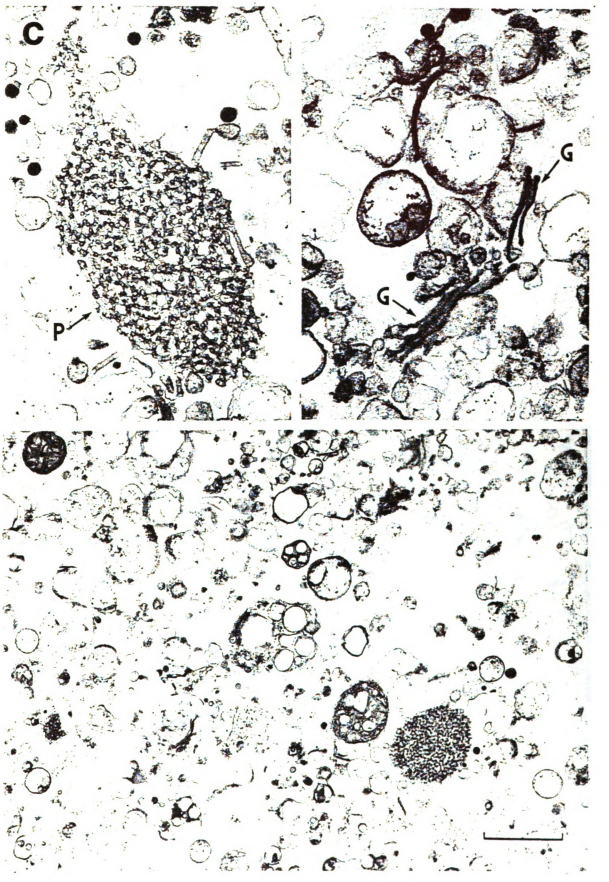
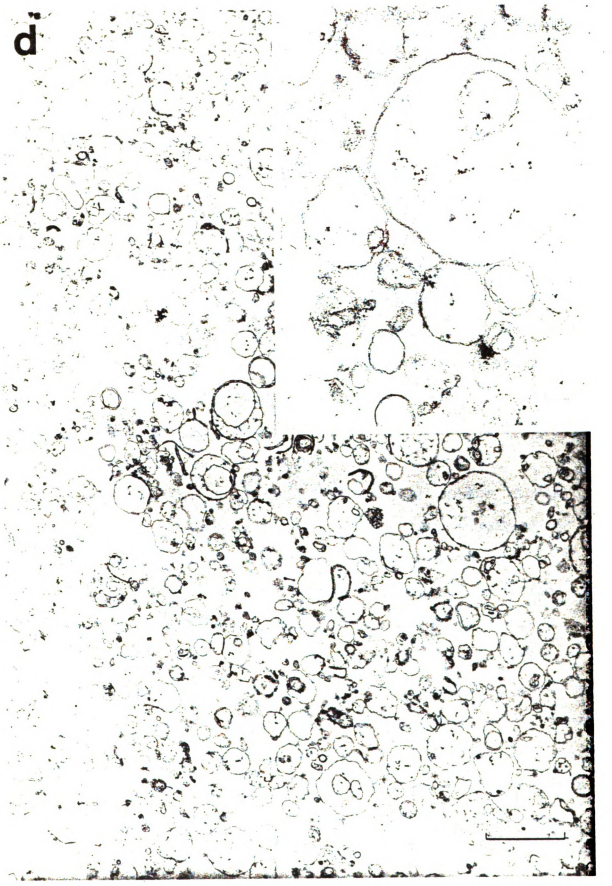
548
553
558
563
39%
25.5%
30%
32%
34.5%
598
0.02 A
39%
Absorbance
550
600
Wavelength (nm)

Figure 5.3 a,b,c,d Electron micrographs of various particulate fractions after isopycnic sucrose density gradient centrifugation. a) 39% sucrose fraction containing the peak of cytochrome-c oxidase activity (See Figure 5.1), b) 35% sucrose, peak of NPA binding and glucan synthetase II activity, c) 29% sucrose containing the peak for carotenoids and glucan synthetase I activity, and d) 26% sucrose fraction containing maximum activity of NADH dependent cytochrome-c reductase. Magnification is 21,000 times with the bar representing 1 μm. Inserts are 3 times enlargements (final magnification of 63,000 times) of membranes characteristic of each fraction, M: mitochondria; G: golgi; and P: prolamella bodies derived from proplastids.


a
M


b
M


C
P
G
G


d

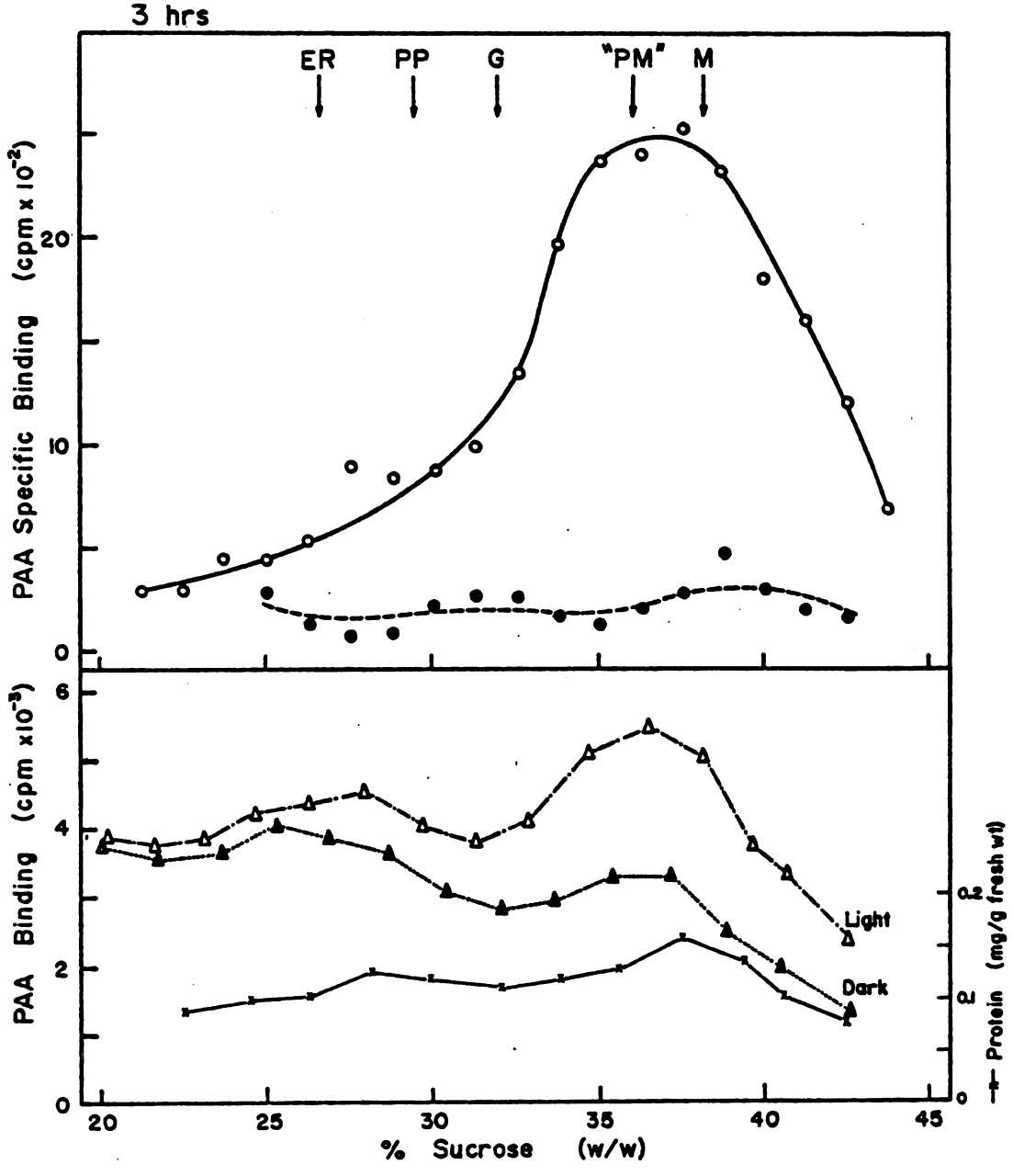
exist on the endoplamic reticulum.

Irradiation of the coleoptiles with 5 mW cm^{-2} blue light for 1 hour stimulated a significant increase in radioactivity associated with membraneous material (~65% increase at 36% sucrose). When expressed as the difference between radioactivity from light-irradiated and dark tissue [Figure 5.4 (upper)], a peak in light-induced PAA binding was observed at 36% sucrose with significant activity from 28 to 43% sucrose. This peak, based on the location of marker enzymes, was not associated primarily with either endoplasmic reticulum, proplastids, or golgi, but did sediment at sucrose concentrations characteristic of the plasma membrane and mitochondria. More importantly, as in the differential centrifugation studies, this peak did not follow the flavin profile for similar gradients (Figure 5.1).

In an effort to demonstrate that the increase in PAA binding is light dependent, similar experiments were were performed using 1/50 the light intensity (0.1 mW cm^{-2}). A reduction in light intensity by 98% theoretically should reduce the light-induced binding by a similar percentage. Using the lower light intensity, the difference in radioactivity between membranes from light irradiated coleoptile and those in darkenss was not significant [Figure 5.4 (upper)]. This indicated that light-induced PAA binding does require blue light irradiation, suggesting a photochemical reaction.

5.3.3 Localization using Rate-Zonal Centrifugation. Based on the difference in sedimentation behavior between the plasma membrane and mitochondria, the two membranes were

Figure 5.4 Distribution of light-induced PAA binding following isopycnic sucrose gradient centrifugation (3 hours). (lower) Profile of radioactivity associated with membrane fractions from 6 grams of corn coleoptiles incubated with [^{3}H] PAA and irradiated with 5 mW cm^{-2} blue light (Δ) or kept in darkness (▲) and protein content of similar isopycnic gradients (X) plotted as a function of sucrose concentration. (upper) Distribution of light-induced PAA binding measured as the difference in radioactivity between light and dark treatments. Open and closed circles represent differences in radioactivity between corn coleoptiles irradiated with 5 mW cm^{-2} or 0.1 mW cm^{-2} respectively and those kept in darkness. Specific binding using 5 mW cm^{-2} represents the average of 4 separate experiments involving a comparison between 4 light and 4 dark treatments. Arrows indicate peaks of activity for membrane markers from Figure 5.1 (ER: endoplasmic reticulum; PP: proplastids; G: golgi; "PM": plasma membrane, and M: mitochondria).


3 hrs
ER
PP
G
"PM"
M
PAA Specific Binding (cpm x 10⁻²)
0
10
20
PAA Binding (cpm x10⁻³)
0
2
4
6
Light
Dark
0
0.1
0.2
Protein (mg/g fresh wt)
20
25
30
35
40
45
% Sucrose (w/w)

separated using a modification of the rate-zonal centrifugation technique. Because of their density and compact size, mitochondria will sediment more quickly in sucrose gradients then will the large vesicles and sheets of the plasma membrane. Therefore, by using shorter periods of centrifugation, it was possible to have significant quantities of mitochondria present in the gradient without the plasma membrane.

Centrifugation of the 500 X g supernatant at 95,000 X g for 30 min (Figure 5.5) was sufficient for the mitochondria to reach their equilibrium density (38% sucrose) while only 70% of the activity associated with the plasma membrane had reached equilibrium with the remaining activity smeared toward lower sucrose concentrations. The endoplasmic reticulum, as measured by NADH dependent cytochrome-c reductase activity had just begun to enter the gradient after this centrifugation period. When this procedure was used to examine the distribution of *in vivo* light-induced PAA binding, the peak of radioactivity at 37% sucrose was reduced with a concomitant increase in radioactivity at sucrose concentrations less than 30% when compared to the distribution after a 3 hour centrifugation. This modification was coincident with the shift in the plasma membrane marker, glucan synthetase II.

After an even shorter centrifugation time period, 15 minutes (Figure 5.6), the mitochondria were close to their final equilibrium sedimentation density (36% sucrose). Alternatively, the enzyme activity associated with the plasma membrane had just begun to enter the gradient while the endoplasmic reticulum still remained in the supernatant. The

Figure 5.5 Distribution of light-induced PAA binding and enzyme activities following a 30 minute centrifugation. The 500 X g supernatant from 6 grams of corn coleoptiles was layered onto 15 to 45% linear sucrose gradient identical to that used in Figure 5.1, but centrifuged at 95,000 X g for 30 minutes. (Upper) Profile of light-induced PAA binding measured as the difference in radioactivity of membrane fractions from coleoptiles irradiated with 5 mW cm^{-2} blue light and those kept in darkness, plotted as a function of sucrose concentration. Specific binding represents the average of 2 separate experiments involving a comparison between two light and 2 dark treatments. (Lower) Relative activity of enzymatic membrane markers after 30 minutes centrifugation step. Activities are expressed as a percentage of those attainable from membrane fractions from the same amount of corn coleoptiles centrifuged for 3 hours (M: mitochondria).

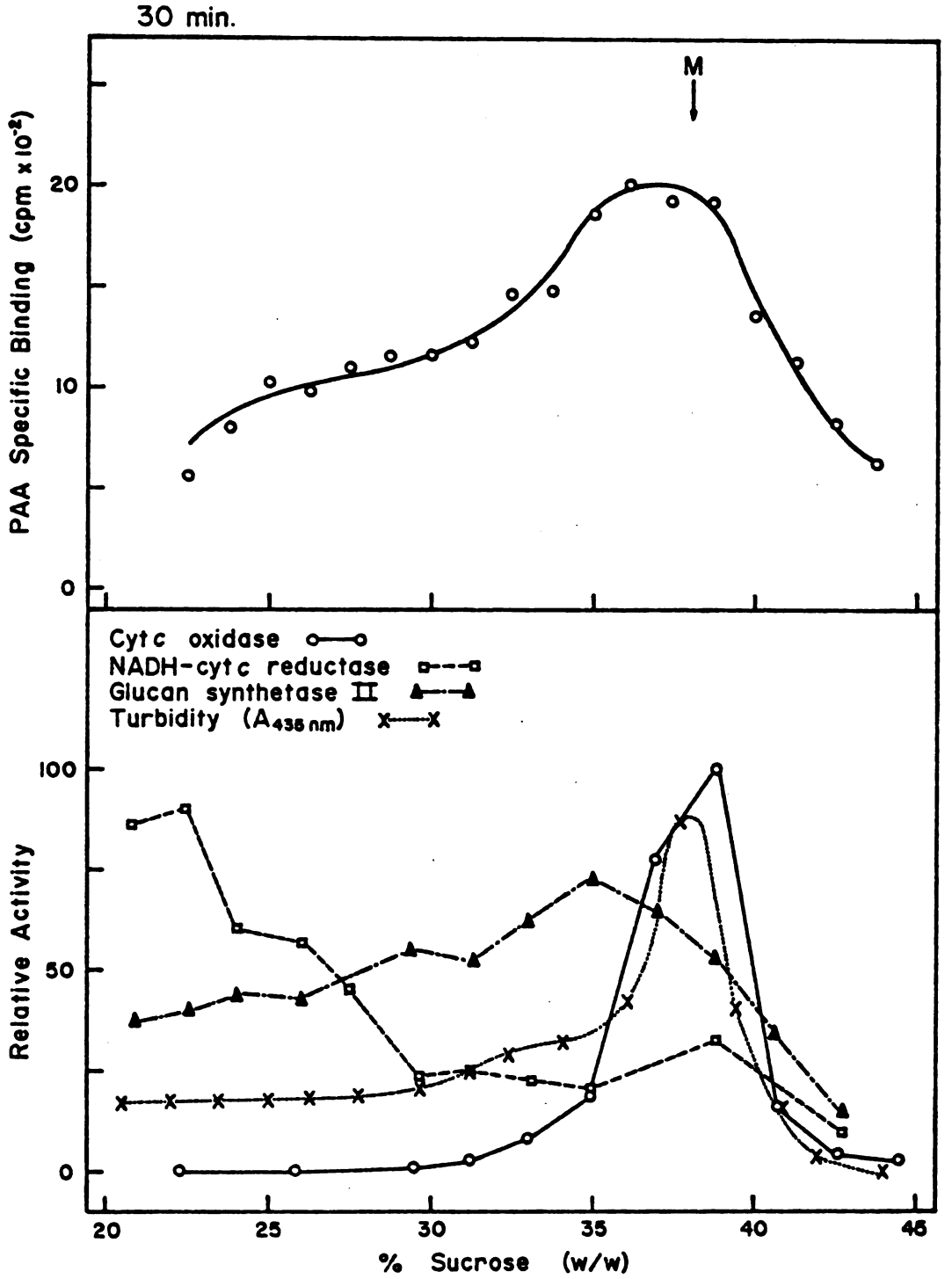
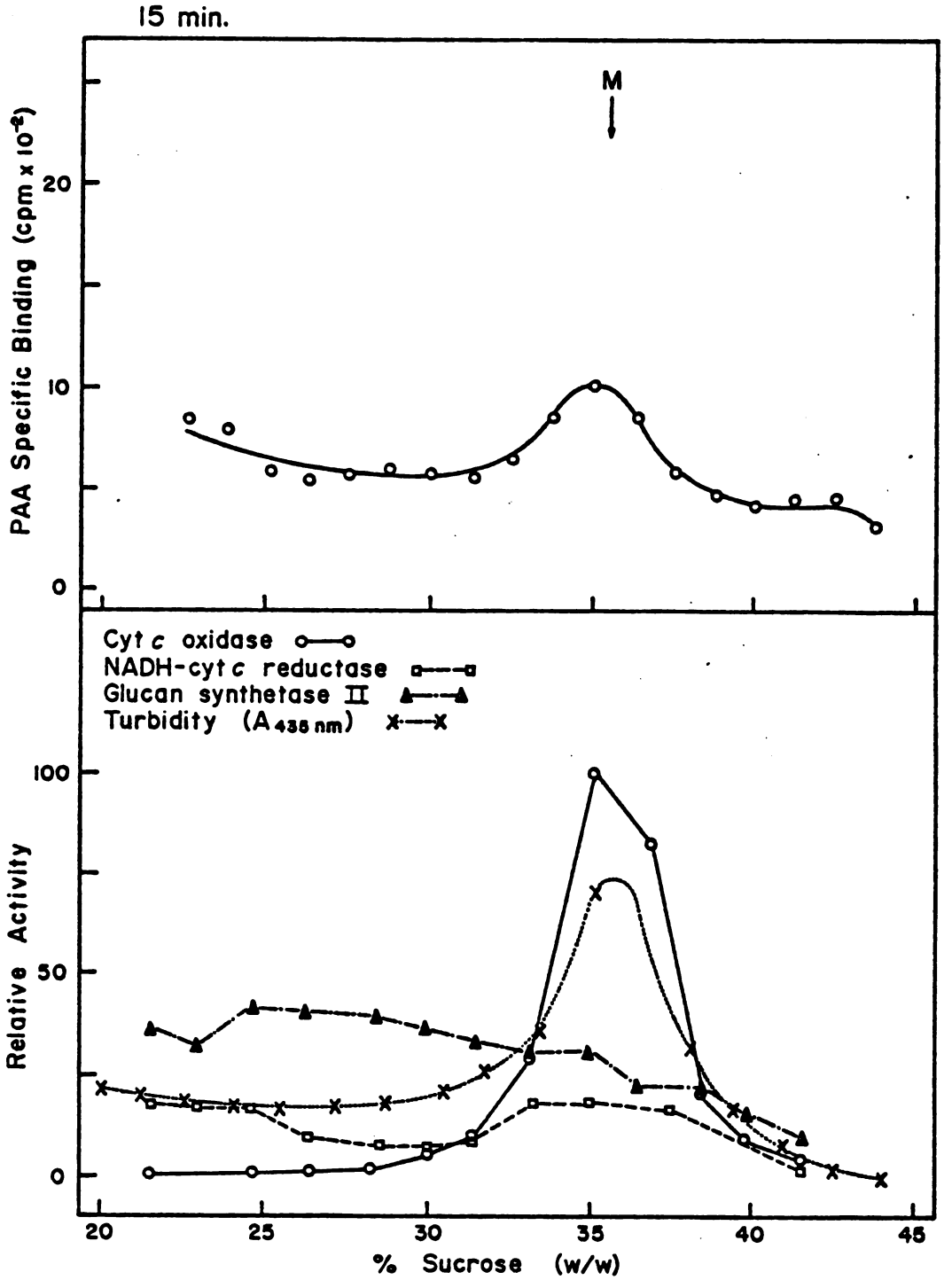

30 min.
M
PAA Specific Binding (cpm x 10⁻²)
20
10
0
Cyt c oxidase
NADH-cyt c reductase
Glucan synthetase II
Turbidity (A436 nm)
Relative Activity
100
50
0
20
25
30
35
40
45
% Sucrose (w/w)

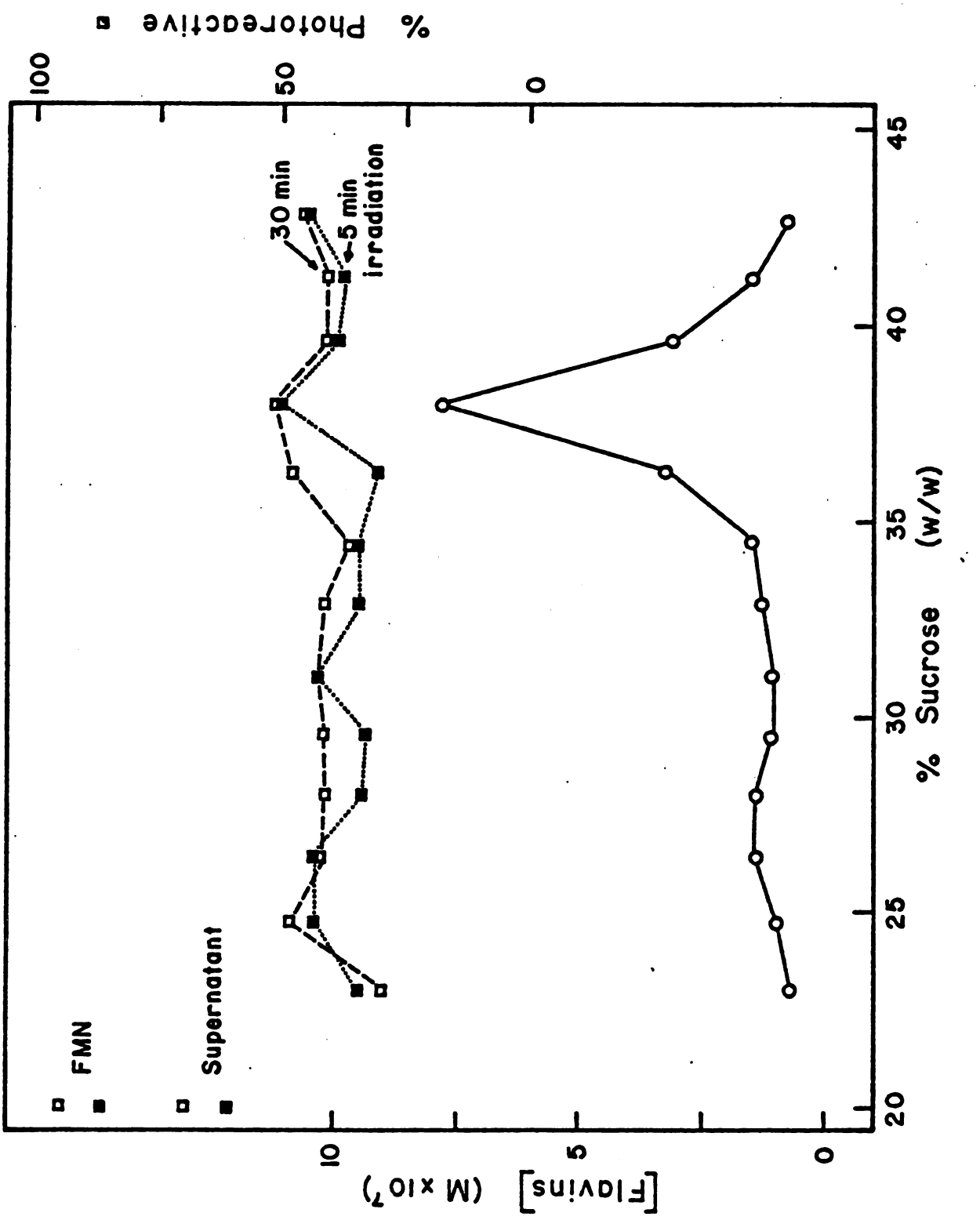
Figure 5.6 Distribution of light-induced PAA binding and enzymatic activities following a 15 minute centrifugation. The 500 X g supernatant from 6 grams of corn coleoptiles was layered onto a 15 to 45% linear sucrose gradient identical to that used in Figure 5.1, but centrifuged at 95,000 X g for 15 minutes. (Upper) Profile of light-induced PAA binding measured as the difference in radioactivity of membrane fractions from coleoptiles irradiated with 5 mW cm^{-2} blue light and those kept in darkness, plotted as a function of sucrose concentration. Specific binding represents the average of 3 separate experiments involving the comparison between 3 dark and 3 light treatments. (Lower) Relative activity of enzymatic markers after a 15 minute centrifugation step. Activities are expressed as a percentageof those attainable from membrane fractions from the same amount of corn coleoptiles centrifuged for 3 hours (M: mitochrondria).


15 min.
M
PAA Specific Binding (cpm x 10⁻²)
0
10
20
Cyt c oxidase
NADH-cyt c reductase
Glucan synthetase II
Turbidity (A 435 nm)
Relative Activity
0
50
100
20
25
30
35
40
45
% Sucrose (w/w)

profile of *in vivo* light-induced PAA binding after a 15 minute centrifugation period exhibited a substantial loss of specifically-bound radioactivity (~70%) at 35% sucrose as compared to the distribution after a 3 hour centrifugation even though the size of the mitochondrial peak was unaffected. It is evident that the absence of plasma membrane particles from the gradient will affect the amount and distribution of light-induced radioactivity associated with corn coleoptile membranes. From these results, it appears that approximately 80% of the *in vivo* light-induced [^{3}H] PAA binding is associated with the plasma membrane with the remaining 20% associated with the mitochondria and/or the distribution of membrane-bound flavins.

5.3.4 *Photoreactivity of membrane bound flavins with PAA in vitro*. Corn coleoptile membrane fractions obtained from isopycnic sucrose gradient centrifugation were then assayed for their ability to photoreact with PAA *in vitro*. PAA (final concentration of 50 mM) was added to aliquots of each membrane fraction, irradiated with blue light, and the flavin content, as assayed by the lumiflavin method, compared to that of non-irradiated samples. The flavin distribution on coleoptile membranes is in Figure 5.7 (flavin concentrations were between 0.1 and 0.7 μM). When such fractions were irradiated with blue light (1 mW cm^{-2}), a significant percentage of flavins in each fraction became photoreduced by PAA (17% after 1 minute, 38% by 15 minutes, and 43% by 30 minutes). This rate is slower than that of a free flavin, FMN (0.3 μM) which was 80, 90 and 95% photoreduced after 1, 5 and 30

Figure 5.7 In vitro photoreactivity of PAA with membrane bound flavins obtained from isopycnic sucrose gradient centrifugation. The 500 X g supernatant from 10 grams of corn coleoptiles was layered onto a linear 15 to 45% sucrose gradient and centrifuged for 3 hours at 95,000 X g. An aliquot from each membrane fraction was assayed for the ability of its flavins to photoreact with PAA. (Circles) Profile of flavin content before irradiation. Percentage photoreactive (squares) represents the amount of flavins undetected by the lumiflavin assay after irradiation in the presence of PAA for 5 minutes (closed) and 30 minutes (open). The photoreactivity of a 0.3 μM solution of FMN and aliquot from the supernatant (5.4 μM flavin) was used for comparison.


% Photoreactive
100
50
0
30 min
5 min
irradiation
FMN
Supernatant
[Flavins] (M x 10⁷)
10
5
0
20
25
30
35
40
45
% Sucrose (w/w)

minutes, respectively. The photoreactivity of soluble flavins and flavoproteins from the supernatant (flavin concentration of 5.4 μM) was between that of free and membrane bound flavins indicative of a mixture containing both free flavins and soluble flavoproteins.

The inability of many membrane-bound flavoproteins to photoreact with PAA after 30 minutes (~60% of the total), suggests that these remaining flavins are weakly or non-photoreactive. The addition of 0.1% Triton X-100 to such membrane fractions did not increase the percentage of flavins that photoreacted with PAA. This treatment would have eliminated the problems of the accessibility of PAA to flavins enclosed in membrane vesicles or embedded in the membrane lipid bilayer.

It should be noted that the percentage of *in vitro* photoreactive flavins does not change as a function of sucrose concentration and hence, membrane type. Therefore, *in vitro* light-induced PAA binding (photoreactivity) does reflect the flavin distribution on such gradients. This is in contrast to the *in vivo* light-induced PAA binding which does not correlate with flavin content but with specific enzymatic membrane markers. It should be noted that this procedure is less sensitive than those involving [^{3}H] PAA because of the high concentrations of PAA (50 mM) used. This assay should detect all flavins that are at least weakly photoreactive, masking subtle differences that may exist between different flavoproteins on different membranes.

5.3.5 Photoreactivity of purified flavins and flavoproteins. To compare the effects that the binding of a flavin to the apoprotein would have on the photoreaction rate, purified flavins and flavoproteins were tested for their photoreactivity with PAA. Flavoproteins generally have reduced photoreaction rates when compared to free flavins due to quenching of the flavin excited states by the apoprotein (Oster, et al., 1962; McCormick, 1977; Song, et al., 1980) and reduced accessibility of the flavin to compounds such as PAA (Swoboda, 1969). For example, complexing of free flavins with proteins generally quenches the flavin fluorescence.

Of the free flavins tested, riboflavin and FMN exhibited similar photochemical activities (rates of photoreduction by PAA, rates of photobleaching, and fluorescence) while FAD was significantly lower (Table 5.2). [This reduction is due to the interaction of the nucleotide portion of FAD with the flavin nucleus thus quenching the excited states (Oster, et al., 1962)]. When the rate of photoreduction by PAA of glucose oxidase, D-amino acid oxidase, and diaphorase were compared to their free flavin cofactors, glucose oxidase (40% of the rate of free FAD) and diaphorase (63% of FMN) had reduced initial photoreaction rates, as expected, while D-amino acid oxidase surprisingly showed an increase in reaction rate (140% of FAD). This would indicate that binding of the flavin to the apoprotein can significantly affect the flavins' excited states allowing the flavin to become more or less photoreactive than the free flavin.

Table 5.2 Photoreactivity of flavins and flavoproteins. Rate of photoreduction by PAA was determined from solutions containing 5 mM PAA, 10 mM K_2HPO_4 (pH 7.0) and the flavin or flavoprotein (A_{445} 0.45) irradiated with 450 nm blue light (5 mW cm^{-2}). Rate of photobleaching was determined similarly without the addition of PAA. Percent fluorescence intensity was calculated as a percentage of the fluorescence emission at 525 nm of that observed by riboflavin.

	Riboflavin	FMN	FAD	Glucose Oxidase FAD(2)[a]	D-Amino Acid Oxidase FAD(2)	Diaphorase FMN(1)
Photoreduction by PAA (M/min x 10^7)	98	71	13	5.1(40%)[b]	19 (140%)	45(63%)
Photobleaching (M/min x 10^7)	6.1	10	0.7	-	-	-
Fluorescence Intensity (%)	100	96	18	-	-	-

[a] number and type of flavin associated with the protein.

[b] percentage of the reaction rate as compared to the free flavin.

5.4 DISCUSSION

It is evident that blue light irradiation does increase the binding of radioactively labeled PAA to corn coleoptile membranes. Because benzyl-derivatized flavins have not been isolated from such membranes after light treatment, I can not conclusively demonstrate the light-induced PAA binding is *via* covalent attachment to flavoproteins. However, the response does require high intensities of blue light [well above that needed to stimulate for phototropism (Figure 4.3) and affect auxin transport (Elliott and Shen-Miller, 1976)] suggestive that a photochemical and not a photobiological reaction is involved. If a photobiological response (such as the active transport of PAA as an auxin analogue) using the blue light photoreceptor were involved, one would expect it to saturate at much lower light intensities.

It should be noted that the light dosage needed to detect light-induced PAA binding (5 mW hr cm^{-2}) was substantially greater than the light dosage used to demonstrate specific inhibition of phototropism [8 μW hr cm^{-2} (Chapter 3)]. However, the reaction velocity of flavin photoreduction by PAA is determined by the product of the number of excited flavin molecules, and hence light intensity, and the concentration of PAA (second order reaction). Based on this assumption, a 10 fold reduction in the concentration of PAA

would require a 10 fold increase in light intensity to maintain the same initial reaction rate. For example, the in vivo concentration of PAA required to specifically inhibit phototropism was 4×10^{-6} M (Table 3.2), while the in vivo [^{3}H] PAA concentration used to for light-induced PAA binding was $\sim 2 \times 10^{-10}$ M. This corresponded with a reduction of 5×10^5 times in the concentration of PAA which correlates with only a 625 times increase in light dosage between the light dosage used to detect light-induced PAA binding that used to stimulate for phototropism. Thus, one could expect less light-induced binding of PAA to the photoreceptor pigment using radioactively labelled PAA than would occur in experiments involving the specific inhibition of phototropism.

Evidence from differential and sucrose gradient centrifugation studies indicated that a majority of the in vivo light-induced PAA binding was associated with the plasma membrane as determined by correlation with enzymatic markers. Moreover, this activity did not correlate with markers for endoplasmic reticulum, golgi, or plastids. Approximately 80% of the activity was associated with the plasma membrane with the remaining, 20%, coincident with either mitochondria and/or the distribution of bound flavins. Because the mitochondria contain most of the pelletable flavins, it was not possible to distinguish between the two possibilities.

The majority of the light-induced PAA binding was determined not to be associated with mitochondria even though this activity and the distribution of mitochondria overlapped after isopycnic gradient centrifugation. Isopycnic sucrose

gradient fractions low in mitochondria (~35% sucrose), determined by enzymatic markers, absorption spectroscopy and electron microscopy, still had substantial amounts of light-induced radioactivity. Additionally, fractions from rate-zonal centrifugation that contained mitochondria without the plasma membrane showed little light-induced PAA binding.

The plasma membrane was located using the enzymatic markers, NPA binding and glucan synthetase II. Because the association of these two markers and the plasma membrane involved the use of the controversial phosphotungstate-chromate stain [PTA-CrO_3 (Roland, et al., 1972)], the plasma membrane cannot be located unambiguously (See Quail, 1979). The distribution of the putative plasma membrane markers, however, does not coincide with the markers for either endoplasmic reticulum, golgi, proplastids, or mitochondria. The remaining distinct membrane, the tonoplast, which has no specific markers in corn, is an unlikely alternative because it is not stained by the PTA-CrO_3 procedure used to identifty possible plasma membrane markers. Thus, NPA binding and glucan synthetase II activity can be indirectly linked with the plasma membrane by process of elimination.

It is logical that such markers be located on the plasma membrane when considering the type of activities they represent. For example, NPA inhibits auxin transport (Lembi, et al., 1972) presumed to be regulated by the plasma membrane (Goldsmith, 1977). Glucan synthetase II activity involves the production of wound callose [β,1-3 linked glucan (Anderson and Ray, 1978)] and would also likely be associated

with the plasma membrane. Although not conclusive, it is probable that the markers, NPA binding and glucan synthetase II, can be used to determine the location of the plasma membrane in corn coleoptile membrane preparations.

The profile for *in vitro* photoreactivity of membrane bound flavins with PAA was different from the profile obtained *in vivo* using [^{3}H] PAA. When PAA was added to previously fractionated corn coleoptile membranes, the membrane bound flavins from all fractions were roughly equal in their photoreactivity with PAA. As a consequence, the distribution of *in vitro* light-induced PAA binding would correlate with the distribution of flavins. When light-induced PAA binding was assayed *in vivo*, the majority of this distribution was not coincident with that for flavins, but with the plasma membrane.

These results demonstrate that PAA is more accessible to certain membranes from intact corn seedlings supporting the hypothesis of Schmidt, *et al*. (1977). It is likely that PAA would be more accessible to and thus would more extensively photoaffinity label flavoproteins bound to the plasma membrane. The plasma membrane would be the first barrier PAA would encounter when entering an intact cell. In contrast, for PAA to enter the mitochondria, containing most of the flavins, it would have to cross at least three membrane barriers (the plasma membrane and the double membrane of the mitochondria).

Because the procedures to detect *in vivo* light-induced

PAA binding and to measure specific inhibition of phototropism involve intact corn coleoptile cells, one would expect the results from the *in vivo* light-induced PAA binding to stimulate that which would occur during the experiments to detect the specific inhibition of phototropism in intact seedlings (Chapter 3). Based on the assumption that the blue light photoreceptor is membrane bound and estimates that a substantial fraction of the photoreceptor pigment is affected by PAA, the photoreceptor pigment would be expected to be associated with membranes containing substantial light-induced PAA binding activity. Therefore, one could postulate that because the plasma membrane bound flavins are highly labeled with [^{3}H] PAA *in vivo*, the blue light photoreceptor pigment is affected by PAA because of its location on the plasma membrane.

The location of the blue light photoreceptor on the plasma membrane is also suggested by other results in addition to those presented here. Action dichroism in the phototropic responses of *Phycomyces* (Jesaitis, 1974) and polarotropism in *Dryopteris* and *Spherocarpos* (Steiner, 1967) which indicate membrane binding of the photoreceptor pigment, implicate the plasma membrane more specifically. Metabolic processes that are affected by blue light such as membrane potential changes and ion fluxes are primarily located on the plasma membrane and/or tonoplast. The location of blue light-induced b-type cytochrome reduction (possibly involved in blue light physiology), has been correlated with the plasma membrane in corn and *Neurospora* (Brain, *et al.*, 1977) [Of

interest is that this b-type cytochrome photoreduction is inhibited by PAA (Caubergs, et al., 1979)]. Because blue light affects the growth of phototropically responding organs [e.g., coleoptiles (Elliott and Shen-Miller, 1976)], it is likely that the photoreceptor pigment would be located near the cell wall, i.e., the plasma membrane.

From results reported here, a majority of the membrane bound flavoproteins in corn coleoptiles can be tentatively eliminated as possible candidates for the blue light photoreceptor pigment. Because the photoreceptor pigment is expected to be highly photoreactive with PAA, flavins bound to the endoplasmic reticulum, golgi, proplastids and possibly mitochondria, may be ruled out by the inability of these membranes to demonstrate significant light-induced PAA binding. Additionally, of the flavins that are membrane bound, only ~40% will photoreact with PAA evenafter detergent treatment. This suggests to us that the remaining 60% of the flavins bound to the plasma membrane are not photoreactive and thus, not likely candidates. From this reasoning, the photoreceptor pigment is most likely to be among the 40% of the flavins photoreactive with PAA that are bound to the plasma membrane.

The blue light photoreceptor would be expected to be highly photoreactive [a decrease in photoreactivity should concomitantly reduce the chromophore's efficiency (McCormick, 1977)]. As can be seen from studies involving purified flavoproteins, the apoprotein can significantly affect a flavin's photoreactivity with PAA. Most flavoproteins should

be less photoreactive that the free flavin, due to quenching by the apoproteins' amino acids and reduced accessibility of the flavin to the outside environment (e.g., glucose oxidase and diaphorase). However, the apoprotein can also make the flavin more photoreactive (e.g., D-amino acid oxidase), as one would theoretically expect from the apoprotein of the blue light photoreceptor pigment [it should be noted that D-amino acid oxidase is also more efficient in photoreducing a b-type cytochrome isolated from Dictyostelium than either glucose oxidase, diaphorase, or FAD (Manabe and Poff, 1978)].

In conclusion, photoaffinity labelling of membrane bound flavins from intact corn coleoptiles using [^{3}H] PAA indicates that the majority of this label is associated with the plasma membrane. Comparison of the in vivo light-induced [^{3}H] PAA binding with the in vitro photoreaction of membrane bound flavoproteins with PAA suggests that the distribution of the in vivo light-induced PAA binding is mainly because plasma membrane bound flavins are more accessible to PAA than other membrane bound flavins and not because they are more photoreactive with PAA. Because PAA can specifically inhibit phototropism by photoreacting with the blue light photoreceptor pigment, and because plasma membrane bound flavins are highly accessible to PAA, I postulate that the blue light photoreceptor pigment is located on the plasma membrane. Identification of PAA photoaffinity-labeled flavins from corn coleoptile plasma membranes should provide a promising method

for isolating possible candidates for the blue light photoreceptor pigment responsible for the phototropic response in corn seedlings.

Chapter 6

General Discussion and Conclusions

Using the assay developed by Schmidt, et al. (1977), the nature and tentative location of the blue light photoreceptor pigment was examined in corn seedlings. Compounds known to affect either flavins or carotenoids were tested for their ability to specifically inhibit phototropism using geotropism as a control.

Xenon, a specific quencher of the triplet excited state of flavins was found not to affect corn seedling phototropism. This result, in addition to the non-specific quenching of flavin excited states by high concentrations of iodide, suggests, in contrast to the conclusions of other authors, that the flavin singlet state may be a more likely reactive species than the flavin triplet state in corn phototropism.

Using analogues of PAA with varying degrees of auxin activity and photoreactivity with flavins, the mechanism for the specific inhibition of phototropism by PAA was explored. Specific inhibition by PAA was determined to be related primarily to its photoreactivity with flavins and not its auxin activity. Estimates of the percentage of photoreceptor pigment inactivated by PAA, indicated that a substantial fraction (~80% of the pigment molecules on the illuminated side) were affected. This further substantiates the hypothesis that the photoreceptor pigment is highly accessible to and/or very photoreactive with PAA.

The role of carotenoids in corn seedling phototropism was examined with the use of the carotenoid synthesis inhibitor, SAN 9789. A reduction in carotenoid content by 99% with the addition of 0.1 mM SAN 9789 did not specifically inhibit phototropism toward 380 nm light or significantly shift the threshold intensities for phototropism toward 380 nm or 450 nm light. This indicated that bulk carotenoids are not the photoreceptor pigment. Results using 450 nm suggest, however, that carotenoids are involved in phototropism acting as an internal light filter, thus enhancing the light gradient use by corn seedlings to detect light direction. As a result, action spectra for phototropism in coleoptiles is a function of the absorption spectra of screening pigments, such as carotenoids, as well as the absorption spectrum of the photoreceptor pigment.

Because PAA will photoreact with flavins, leaving the benzyl-residue covalently attached to the flavin and because the photoreceptor pigment photoreacts with PAA, radioactively labeled PAA was used as a photoaffinity label in an effort to locate the photoreceptor pigment. *In vivo* light-induced [^{3}H] PAA binding was found to occur almost exclusively on the plasma membrane from corn coleptile membrane preparations. Comparison of the *in vitro* photoreactivity of PAA with membrane bound flavins with the *in vivo* light-induced [^{3}H] PAA binding indicate that the plasma membrane bound flavins are more accessible to PAA than other membrane bound flavins. These results provide a tentative link between the photoreceptor pigment and the plasma membrane,

consistent with the existing hypothesis concerning the photoreceptor pigment location.

This thesis provides the first step toward the identification of the blue light photoreceptor pigment. [^{3}H] PAA labeled flavoprotein should be purified from plasma membranes enriched fractions to identify possible candidates. As a first step, polyacrylamide gel electrophoresis could be employed. Initial studies with SDS polyacrylamide gels indicate that corn coleoptile membranes do contain a wide variety of proteins (at least 30 are found in large quantities in membrane fractions from isopycnic sucrose gradients) and their presence can be correlated with specific membranes (e.g., endoplasmic reticulum or mitochondria). Because denaturation of the flavoprotein would release the flavin and radioactive label, techniques involving non-denaturating gels would have to be employed. It should be possible to detect such flavoproteins by the binding of radioactively labelled PAA and by flavin fluorescence (lumiflavin fluorescence assays are sensitive well below a picomole of free flavin).

Once candidates have been isolated, several criteria could be used to estimate whether they could be involved in photoreception.

1. Do they fluoresce? To be an efficient photoreceptor pigment, the chromophore should be minimally quenched by the apoprotein. Preliminary results by Song, et al. (1980) have detected the presence of highly fluorescent flavoproteins in plasma membrane enriched fractions from corn

(these flavoproteins also have exceedingly short fluorescent lifetimes indicating a highly photoreactive nature).

2. Are they very photoreactive with PAA? From these results, it can be concluded that the photoreceptor pigment must be highly photoreactive with PAA. It is possible that plasma membrane bound flavoproteins similar to D-amino acid oxidase will exist where the flavoprotein is more photoreactive with PAA than the free flavin.

3. Do such flavoproteins efficiently photoreduce b or c-type cytochromes? There is a tentative link between such light-induced b-type cytochrome reductions and blue light photoresponses. Recently, a flavin-mediated photoreducible b-type cytochrome has been partially purified from corn coleoptile plasma membrane enriched fractions (Briggs, personal communication). This membrane bound b-type cytochrome can be washed or solubilized without loss of photoactivity indicating an association of a specific flavin to the b-type cytochrome. Is this flavin/b-type cytochrome complex photoaffinity labelled with [^{3}H] PAA?

Although these techniques will isolate possible candidates, assuming more than one flavoprotein on the plasma membrane is labelled by [^{3}H] PAA, it cannot definitely prove that a particular candidate is involved. This would require mutant analysis, correlating a blind phenotype with a deficiency in a particular flavoprotein.

Once one blue light photoreceptor has been identified, many questions would still remain. How does it work in corn? Does it interact with auxin transport or directly affect

growth? Is the photoreceptor pigment similar in other organisms with phototropic responses, e.g., Phycomyces? Is this pigment and its sensory transduction pathway responsible for other blue light responses found in the same organism? Evidence from mutant analysis in Phycomyces indicates that its blue light responses (phototropism, carotenogenesis and sporangiophore formation) are all mediated by the same photoreceptor pigment (Russo, 1979) while results in Neuorspora for entrainment of the circadian rhythm and carotenogenesis suggest the involvment of different pigments (Brain, et al., 1977). In closing, the results from this thesis provide the first step in isolating one of the most intriguing and elusive photoreceptor pigments in the plant kingdom.

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