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The Effect of Visible Light on Carotenogenesis of Arthrosporulating Trichophyton Mentagrophytes

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THE EFFECT OF VISIBLE LIGHT ON CAROTENOGENESIS OF ARTHROSPORULATING *TRICHOPHYTON MENTAGROPHYTES*

by

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DEDICATION

TO DR. T. HASHIMOTO, FOR HIS PATIENCE, INSPIRATION, AND GUIDANCE THROUGHOUT THIS STUDY

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LIFE

Ruth Carol Mock was born to George and Rose Styskal in Oak Park, Illinois, on June 29, 1945.

She graduated from J. Sterling Morton High School East in June of 1963, and received an Associate of Arts degree from Morton Jr. College in June of 1965. At Morton College, Mrs. Mock was elected president of the national junior college honor society, Phi Theta Kappa. She further obtained a Bachelor of Science degree from the Department of Biology at the University of Illinois (Chicago Circle Campus) in March of 1968 and received a Master of Science degree from the Department of Biological Sciences at the same university in March of 1972. While attending the University of Illinois, she was elected to the national honor society, Phi Kappa Phi.

After working for several years as a research assistant in Professor G. B. Bouck's laboratory and as a biology instructor at Central Y.M.C.A. Community College, Mrs. Mock matriculated in the Department of Microbiology at Stritch School of Medicine, Loyola University of Chicago, in October, 1976. While pursuing her Ph. D. degree, she was granted Basic Sciences Fellowships in 1977, 1978, and 1980; she was a recipient of the Loyola University of Chicago Dissertation Fellowship in 1979. She also served as an instructor in the Microbiology Department of the Loyola University Dental School in 1978, 1979, and 1983.

Mrs. Mock is currently a member of the American Society for Microbiology and the American Association for the Advancement of Science. She is also an associate member of the Society of Sigma Xi.

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LIST OF ABBREVIATIONS AND SYMBOLS

A	absorbance
approx	approximately
avg	average
β	beta
°C	degrees Celsius
cm	centimeter
d	dry weight
diam	diameter
e	base of natural logarithm
$E^{1\%}_{1cm}$	extinction coefficient
EDTA	ethylenediamine tetra-acetic acid
Fig	figure
γ	gamma
GE	General Electric
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
in	inch
kV	kilovolt
KU	Klett unit
λ_{max}	maximum wavelength
μg	microgram
μm	micrometer
M	molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
N	normal
nm	nanometer
%	per cent
p	probability
P_2O_5	phosphorous pentoxide
PI	pigmentation index
psi	pounds per square inch
r	coefficient of correlation
R_f	rate of flow
rpm	revolutions per minute
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
sec	second
SE	standard error of the mean
TEM	transmission electron microscopy

UV	ultraviolet
v	volume
v/v	per cent by volume
W	watts
W/m^2	watts per square meter
w/v	weight-volume per cent
wt	weight
X	times
χ^2	Chi-square

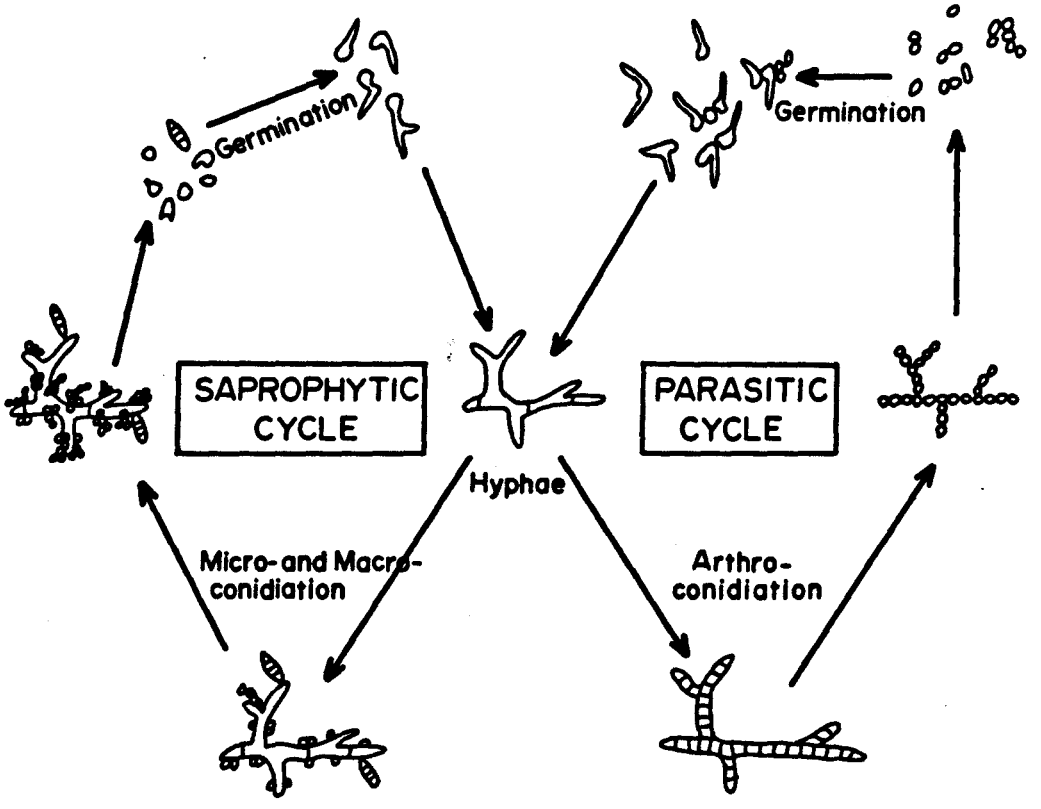
CHAPTER I

INTRODUCTION AND REVIEW OF RELATED LITERATURE

Dermatophytes are a group of fungi which infect keratinized tissue of the epidermis, nails, hair, etc. in both man and animals. This fungal group is composed of three genera: Epidermophyton, Microsporum, and Trichophyton. Trichophyton mentagrophytes is one of the dermatophytes most frequently involved in human dermatophytoses (Rippon, 1982). This fungus produces three types of asexual spores (Fig. 1). Microconidia and macroconidia are formed when hyphae are grown saprophytically. Arthrospores (arthroconidia), on the other hand, are produced by parasitically growing hyphae. These arthrospores are suspected to play important roles in the spread, transmission, and recrudescence of infection (Rippon, 1982).

Until recently, it was generally believed that the main pigments produced by dermatophytes were naphthoquinones or their derivatives (Walker and Milovanovic, 1970). These pigments, which are synthesized primarily during hyphal growth, can diffuse into a surrounding culture medium and serve as diagnostic criteria for dermatophyte identification (Ito et al., 1965). The biological functions of dermatophytic naphthoquinones are unknown. A few studies have examined the effects of xanthomegnin, a yellow 1,4-naphthoquinone, which has been isolated in relatively pure form from several dermatophytes. Tests indicate that xanthomegnin may act as a strong uncoupler of oxidative phos-

Fig. 1. Asexual life cycles of T. mentagrophytes (diagram slightly modified from R. Emyanitoff, Ph. D. dissertation, Loyola University of Chicago, 1978). Under saprophytic conditions, clusters of single-celled microconidia and occasional multicellular macroconidia are produced. When grown parasitically, chains of arthrospores develop from pre-existing hyphae.


Germination
Germination
SAPROPHYTIC CYCLE
PARASITIC CYCLE
Hyphae
Micro- and Macro-conidiation
Arthro-conidiation

phorylation in isolated rat liver mitochondria (Ito et al., 1973) or may serve as an electron transport bypass to the mitochondrial respiratory chain (Kawai and Nozawa, 1978).

The development of *in vitro* methods for producing large amounts of arthrospores led to the identification of a second major class of pigments in the dermatophyte *Trichophyton mentagrophytes* (Hashimoto et al., 1978). Mature arthrospores were found to contain a mixture of carotenoids which included phytoene, phytofluene, β-carotene, neurosporene, lycopene, and γ-carotene. In addition, these pigments appeared to accumulate within intracellular lipid granules of arthrospores. Carotenoids were not detected in hyphae. While examining factors which influence carotenogenesis in arthrosporulating *T. mentagrophytes*, we noted that arthrospores exposed to moderate intensities of white fluorescent light contained lower levels of carotenoids than their dark counterparts (R. Mock and T. Hashimoto, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I 26, p. 88). This observation appeared of special interest because visible light usually induces or stimulates fungal carotenogenesis (Ruddat and Garber, 1983).

The carotenoids are among the most widespread and important class of pigments in nature (Weedon, 1971). They are found throughout the plant and animal kingdoms as well as in microorganisms. Although frequently masked by other pigments, such as chlorophyll, carotenoids are present in all photosynthetic tissue and photosynthetic bacteria. They also occur in certain vertebrates, invertebrates, nonphotosynthetic bacteria, and fungi.

The ability to produce carotenoids appears to have developed at an early stage in evolution, for plants, fungi, and bacteria synthesize carotenoids de novo. In contrast, animals, especially higher orders, are dependent on dietary carotenoids which may subsequently undergo enzymatic alteration (Singh and Cama, 1975).

The wide distribution of carotenoids has led to an examination of their fundamental biological role. On the basis of the array of effects, carotenoid functions have been subdivided into several areas which include protection of cells from the harmful effects of light, extension of the spectral sensitivity of photosynthesis, membrane stabilization, and the influence of secondary metabolites, such as vitamin A and trisporic acids (Krinsky, 1971). To date, a universal function for these pigments has not been demonstrated.

The primary purpose of this dissertation is to study in detail the effect of visible light on carotenogenesis in T. mentagrophytes. Whether visible light can induce or stimulate carotenogenesis in this dermatophytic fungus will be examined. Conditions under which light-mediated suppression of carotenoid accumulation occurs in arthrospores will be characterized. Besides carotenogenesis, additional parameters such as growth and arthrosporulation will be tested for sensitivity toward light. The mechanism of light-mediated suppression of carotenogenesis in arthrosporulating T. mentagrophytes will be investigated. In addition, the process of the development of carotenoid-containing granules during arthrosporulation will be studied by light and electron microscopy. Isolated pigment granules from arthro-

spores will be structurally characterized and chemically analyzed with particular emphasis on carotenoid content.

As stated above, the major aim of this study is to examine the effect of visible light on carotenogenesis in T. mentagrophytes. It is germane in this regard to review the effects of visible light on carotenogenesis, the focus primarily being on studies associated with fungi and nonphotosynthetic bacteria. These effects have recently been summarized (Harding and Shropshire, 1980; Rau, 1980).

Visible Light Effect - General Aspects

Visible light is known to exert several different types of effects on carotenogenesis in nonphotosynthetic microorganisms. In some bacteria and fungi, carotenogenesis is photostimulated. The fungus Phycomyces blakesleeanus, for example, produces substantial amounts of carotenoids in the dark, and white or blue light causes an increase in the level of pigment produced (Jayaram et al., 1979). In a number of other bacteria and fungi, irradiation with visible light is obligatory for the accumulation of appreciable amounts of colored carotenoids. Since relatively small amounts of pigment are present in the dark, biosynthesis of carotenoids in these organisms is thought to be strictly "induced." Among bacteria, photoinduction of carotenogenesis has been detected and thoroughly studied in Flavobacterium dehydrogenans (Weeks et al., 1973), in Myxococcus xanthus (Burchard and Hendricks, 1969) and several species of Mycobacterium (Batra, 1971; Goodwin, 1980). Fungi in which strict photocontrol of carotenogenesis has been well characterized include: Fusarium aquaeductuum, Neuro-

spora crassa, and Verticillium agaricinum. A more extensive list of fungi demonstrating photoinduced carotenogenesis has recently been compiled by Goodwin (1980). No attempt has previously been made to examine photoinduction of carotenogenesis in dermatophytes.

There are few and infrequent reports which indicate that visible light suppresses carotenogenesis in nonphotosynthetic microorganisms. It has been observed that a mutant of the aquatic fungus Blastocladiella emersonii grown under continuous white fluorescent light (approx 660-880 lux) until fully developed had a 3- to 5-fold reduction in the amount of carotenoid pigment (γ-carotene) compared with corresponding dark-grown cells (Cantino and Horenstein, 1956). It is unknown whether this reduction of carotenoid accumulation under light is due to inhibition of carotenoid biosynthesis, photodestruction of pigment, or a combination of these two processes.

Alternate periods (12 h) of white fluorescent light (100 lux) and darkness at 25°C have been shown to cause mated cultures of the phycomycete Choanephora cucurbitarum to contain approx 40% less carotenoids (measured as β-carotene) than dark-grown cultures at the end of two weeks (Chu and Lilly, 1960). Carotenogenesis in this fungus was only slightly reduced when the cultures were shifted to white light (10,000 lux) at stationary growth (7 days). In these studies, it could not be ascertained whether visible light inhibits carotenoid biosynthesis or catalyzes the destruction of these pigments.

Mated or individual cultures of Blakeslea trispora grown under continuous "Cool White" fluorescent light (4.13 W/m^2) for approx 5

days accumulated 40% of the β-carotene of cultures grown in darkness (Sutter, 1970). Although the exact mechanism of this reduction was not determined, it was concluded that an increased destruction of β-carotene probably occurred in either type of culture.

Constant illumination under white incandescent light at 28°C for 6 days has been found to inhibit carotenogenesis in the yeasts Rhodotorula glutinis, Sporobolomyces pararoseus, and S. roseus (Bobkova, 1965). In comparison with corresponding dark controls, total carotenoid levels decreased approx 40% for R. glutinis at 1500 lux, 30% for S. pararoseus at 1500 lux, and 50% for S. roseus at 600 lux. These were highest intensities of light where growth was not inhibited. Dark-grown cultures contained more β-carotene than torularhodin (an acidic carotenoid). In light, this ratio became reversed, and the extent of this reversal was dependent upon light intensity. In addition, low intensities of light proved stimulatory to carotenoid accumulation in S. pararoseus. Based on the relative amounts of β-carotene and torularhodin in light and darkness, it was concluded that moderate intensities (600-1500 lux) of white light inhibit carotenoid biosynthesis in these yeasts.

It has been reported that continuous irradiation with "Daylight" fluorescent light (1500 lux) at 25°C for 75-85 h caused a quantitative reduction (approx 25%) of the four major carotenoids in the mycelium of Epicoccum nigrum compared with dark-grown counterparts (Gribanovski-Sassu and Foppen, 1969). Furthermore, white light decreased the amount of ergosterol. As will be recalled, the biosynthetic pathways

of carotenoids and sterols share a number of common intermediates. When the temperature was raised to 28°C, white light stimulated carotenoid accumulation in hyphae, and photosuppression of pigmentation was not observed. Although the exact mechanism by which white light suppressed pigmentation could not be determined, these authors suggested that light probably inhibits carotenoid biosynthesis since the major carotenoids become proportionally reduced.

From the above studies, it is clear that the mechanism for carotenogenic photosuppression, which occurs after prolonged exposure to fairly moderate intensities of white light, has not been firmly established. Since one of the main functions of carotenoids in fungi may be photoprotection (Krinsky, 1971), this suppressive effect of visible light on carotenoid accumulation is noteworthy. It should be noted that fungi which exhibit this type of photoeffect synthesize fairly large amounts of carotenoids in the dark. Furthermore, positive photoregulation of carotenogenesis was not demonstrated under the experimental conditions used for photosuppression. Interestingly, P. blakesleeanus mutants, which are totally incapable of photostimulated carotenogenesis, have decreased levels of carotenoids when continuously irradiated with moderate intensities (1 W/m^2) of blue light (López-Díaz and Cerdá-Olmedo, 1980).

In a recent report, Valadon et al. (1979) found that far-red light reverses the near-red light induction of carotenoid biosynthesis in Verticillium agaricinum. These authors suggested that this response is mediated by phytochrome, a well-known plant photoreceptor,

which regulates chloroplast development as well as carotenogenesis. This is the only report which implicates phytochrome in the regulation of fungal carotenogenesis.

Primary Reaction and Action Spectra

Fungi and bacteria which demonstrate strict photoinduction of carotenogenesis have been mainly used to investigate the series of reactions that intervene between absorption of light and pigment production. Harding and Shropshire (1980) have proposed that photo-induced carotenogenesis of nonphotosynthetic microorganisms is divided into three phases which include a light reaction, a period of protein synthesis, and carotenoid accumulation.

The initial photoreaction is independent of temperature (Harding, 1974; Mathews, 1963; Rau, 1962; Rilling, 1962; Seviour and Codner, 1973; Valadon and Mummery, 1971; Weeks et al., 1973; Zalokar, 1955). It has therefore been concluded that this reaction is a purely photo-chemical process.

Oxygen is also essential during the primary photochemical reaction for maximal photoinduction of carotenogenesis (Howes et al., 1969; Rau, 1969; Rilling, 1962, 1964; Seviour and Codner, 1973; Valadon and Mummery, 1971). There are currently two opposing view-points concerning the role of oxygen in this event. Rau (1969) has concluded that oxygen functions as an electron acceptor which keeps the photoreceptor in a proper state of oxidation. In contrast, Rilling (1964) and Batra (1971) have proposed that O_2 participates directly in the photochemical reaction.

The energy requirement for the initial photoreaction varies with the specific organism and the wavelength of light (Batra, 1971). Light saturation of photoinduced carotenogenesis has been reported for Mycobacterium sp. (Howes and Batra, 1970), Mycobacterium marinum (Batra, 1971), Flavobacterium dehydrogenans (Weeks et al., 1973), and Neurospora crassa (Zalokar, 1955; DeFabo et al., 1976). In Fusarium aquaeductuum, saturation could not be achieved (Rau, 1976a). Photoinduction of carotenogenesis in these microorganisms also follows the Roscoe-Bunsen reciprocity law (constant light fluence yields constant response). This implies that the photochemical act is probably the main rate-limiting step during photoinduction.

An important step in elucidating the basis of a biological photoresponse is to establish the chemical nature of the photoreceptor pigment, which is a chromophore that absorbs light effective in bringing about the response. The identity of this pigment is usually deduced from an action spectrum, which is a plot of the relative response of a system to different wavelengths of light. The action spectrum of an effect should approximately correspond to the absorption spectrum of the chromophore involved.

In nonphotosynthetic microorganisms, detailed action spectra of photoinducible carotenogenesis have been measured in Mycobacterium marinum (Batra and Rilling, 1964), Myxococcus xanthus (Burchard and Hendricks, 1969), Flavobacterium dehydrogenans (Weeks et al., 1973), Mycobacterium sp. (Batra and Rilling, 1964; Howes and Batra, 1970; Rilling, 1964), Neurospora crassa (DeFabo et al., 1976; Zalokar, 1955),

and Fusarium aquaeductuum (Rau, 1967a)

The action spectra for carotenogenesis are similar in Mycobacterium marinum and Myxococcus xanthus. Based on their shape (principal peak at 400-410 nm; subsidiary peaks in the 450-650 nm range) and by comparison with absorption spectra of porphyrin-containing fractions of bacterial cell homogenates, it is likely that the photoreceptor pigment is a porphyrin. In Flavobacterium dehydrogenans, the photoreceptor chromophore for light-induced carotenogenesis appears to be a flavin-porphyrin complex.

In the remaining microorganisms mentioned above, the photoreceptor absorbs light in the long wavelength ultraviolet (greater than 330 nm) to the blue range of the spectrum, but not at wavelengths longer than 520 nm. Besides photoinduction of fungal carotenogenesis, this type of action spectrum has been observed for a variety of photobiological responses in both photosynthetic and nonphotosynthetic organisms (Senger, 1980). The pigment or chromophore which mediates these responses has often been termed the "blue light photoreceptor" or crytochrome (Gressel, 1979). In most blue light sensitive systems, the identity of this photoreceptor has aroused considerable controversy. The two most likely candidates are generally believed to be flavins and carotenoids. The relative merits of each of these pigments serving as blue light photoreceptors have been presented (Song and Moore, 1974; Briggs, 1976; Presti and Delbrück, 1978; Tan, 1978). To date, the nature of this photoreceptor is still an open question (DeFabo, 1980).

In Neurospora crassa, a detailed action spectrum of photoinduced carotenogenesis was determined, and β-carotene was proposed to be the photoreceptor (DeFabo et al., 1976). On the other hand, a more recent cell-free study of phytoene biosynthesis in N. crassa contraindicates the involvement of a carotenoid photoreceptor. Spurgeon et al. (1979) found that a blue light treatment of whole cells caused a nine-fold increase in the overall enzymatic activity required for the conversion of isopentenyl pyrophosphate to phytoene. This effect was observed for both the wild type strain and an albino-1 mutant which is blocked in the conversion of phytoene to carotenoids. Since this albino strain contains only trace amounts of carotenoids, it is unlikely that these pigments are serving as photoreceptors for light-mediated phytoene biosynthesis. In addition, the possibility exists that a different photoreceptor may regulate the conversion of phytoene to the remaining carotenoid pigments. Schrott (1980a) has demonstrated that the fluence response of blue light-induced carotenoid biosynthesis in N. crassa is under biphasic regulation. Although further experimental clarification is needed, he suggested that different types of photoreceptors may mediate each phase.

Carotenoids do not appear to function as photoreceptors for photoinduced carotenogenesis in Fusarium aquaeductuum (Lang-Feulner and Rau, 1975). Normally carotenoid production in this fungus is induced by blue light. When photodynamic dyes, such as methylene blue, toluidine blue, and neutral red, were added to mycelial suspensions, red light also became an effective inducer. Since flavins can undergo

photosensitizing oxidations while carotenoids cannot (Song et al., 1972), it is likely that these dyes were acting as artificial photoreceptors which carry out photooxidations in the first step of photoinduction. Flavins were proposed to be the endogenous photoreceptors for this response.

Dose response curves for the induction of carotenoid biosynthesis have been reported to be biphasic in Fusarium aquaeductuum (R. R. Theimer, Ph. D. thesis, Universität München, 1968), F. coeruleum (Schrott, unpublished data), and F. oxysporum (Schrott, 1980b). The second phase, which is the high fluence response, does not show saturation up to an energy fluence of 5×10^6 J/m^2.

As mentioned previously, carotenogenesis in Phycomyces blakesleeanus is stimulated by blue light. On the basis of genetic analysis (Bergman et al., 1973), it is assumed that in Phycomyces, a common photoreceptor pigment is utilized for several blue light response systems including phototropism and photostimulation of carotenogenesis. Pigment-deficient mutants of Phycomyces which are blocked in all six steps of carotenoid biosynthesis from phytoene to β-carotene exhibit phototropic thresholds identical to those of the wild type strain (Presti et al., 1977). Therefore, it is likely that β-carotene, the predominant carotenoid in wild type Phycomyces, is not the blue light photoreceptor.

Jayaram et al. (1979) have recently observed a biphasic dose response curve for blue light-stimulated carotenogenesis in Phycomyces. One component is saturated by relatively low light fluences (20-40

J/m^2), while the second component is responsive to much higher irradiation fluences (saturating at approximately 1,000 J/m^2). Based on the different sensitivities of each response to transcription and translation inhibitors, such as actinomycin D and cycloheximide, the suggestion was made that carotenoids may mediate the low-fluence response, and flavins may serve as photoreceptors under higher fluences. To further test this hypothesis, Whitaker and Shropshire (1981) determined the relative quantum effectiveness of 445- 405-, and 365-nm light for both low- and high-fluence components of light-induced carotenoid synthesis in Phycomyces. By comparing the relative absorbances of the total carotenoid extract (in acetone) with the quantum effectiveness of the three wavelengths for the low-fluence component, they concluded that β-carotene was the blue light photoreceptor for the low-fluence component. Although the high-fluence component had a somewhat different quantum effectiveness, this difference was attributed to screening by β-carotene already produced in the mycelium. Therefore, it is likely that β-carotene, the predominant carotenoid in Phycomyces, serves as the photoreceptor for both responses.

At least two photoreceptor systems have been postulated to operate in photoinduced carotenogenesis of Verticillium agaricinum (Valadon et al., 1982). Carotenoids and flavins, however, are not implicated in either system. An action spectrum for photocarotenogenesis in V. agaricinum contained major peaks of activity in the near-ultraviolet (near-UV) as well as in red regions of the spectrum.

The photoreceptor which absorbs in the near-UV region appears to be a novel pigment of unknown chemical nature (Valadon et al., 1982). Since the red light-induced carotenogenesis in V. agaricinum can be reversed by far-red light and reinduced by red light, Valadon et al. (1979) suggested that phytochrome is also involved. In further studies, Hsiao and Bjorn (1982) have found that red light and far-red light were not effective in inducing carotenoid biosynthesis in V. agaricinum. It should be pointed out, however, that their experimental conditions, including light sources, were different from those reported by Valadon et al. (1979, 1982).

Phytochrome is a photoreversible chromoprotein (probably a biliprotein) which has been shown to control many aspects of plant growth and development (Kendrick and Spruit, 1977). Since phytochrome has not been isolated in relatively pure form from fungi, proof of its involvement must await further studies. Recently the induction of carotenogenesis by red light was extensively examined in several other fungi (Schrott et al., 1982). In contrast to V. agaricinum, phytochrome does not appear to mediate carotenogenesis in Fusarium aquaeductuum and Neurospora crassa.

Level of Regulation

At present, the series of reactions immediately following illumination of the photoreceptor are not well understood. In cases where a porphyrin or flavin apparently acts as the photoreceptor in carotenogenesis, Rau (1969) has suggested that some type of photo-oxidation must occur. Evidence in favor of this type of reaction is

supported by data indicating that oxygen is essential for optimum photoinduction of carotenogenesis. Furthermore, strong reducing substances, such as dithionate (hydrosulfite) or hydroxylamine, inhibit photoinduced carotenoid synthesis completely and specifically when applied before or immediately after illumination (Theimer and Rau, 1970). A model illustrating the events during photoinduction of carotenoid biosynthesis has recently been proposed (Rau, 1980).

The photochemical reaction leads to a series of dark reactions which culminate in carotenoid accumulation. In organisms so far investigated, photoinduction allows for synthesis of a whole set of carotenoids. Variation in the types and amounts of carotenoids may occur, but these changes are usually attributed to cultural conditions (Rau, 1976). Such differences are not related to the mechanism of photoinduction.

With the exception of Flavobacterium dehydrogenans (Weeks et al., 1973), pigment accumulation begins only after a lag period. This period varies from approximately 1 h in Mycobacterium sp. (Rilling, 1962), Neurospora crassa (Rau et al., 1968), and Fusarium aquaeductuum (Rau, 1967a) to about 4 h in Mycobacterium marinum (Batra, 1967).

During the lag period, protein synthesis is necessary for the initiation of photoinduced carotenoid biosynthesis. When added immediately after irradiation, cycloheximide, an inhibitor of eucaryotic protein synthesis, has been shown to block carotenoid accumulation in the fungi Neurospora crassa (Harding, 1974; Harding and Mitchell, 1968; Rau et al., 1968), Fusarium aquaeductuum (Rau, 1967b), and

Verticillium agaricinum (Mummery and Valadon, 1973). Corresponding results using chloramphenicol, an inhibitor of protein synthesis in procaryotes, have been obtained in the bacteria Mycobacterium sp. (Howes and Batra, 1970; Rilling, 1962, 1964), Mycobacterium marinum (Batra, 1967; Batra and Rilling, 1964) and Flavobacterium dehydrogenans (Weeks et al., 1973). From these results, it has been proposed that visible light causes the de novo synthesis of one of more enzymes involved in the carotenoid pathway. Thus, photoregulation appears to occur by a classic induction mechanism.

The recent discovery of biphasic fluence response curves for light-induced fungal carotenogenesis has prompted a renewed interest in the mechanism of enzymatic regulation for pigment synthesis. In Phycomyces blakesleeanus, only the second component of the response appears to require protein synthesis (Jayaram et al., 1979). As mentioned previously, however, carotenogenesis in Phycomyces is not strictly photoregulated. Whether the protein synthesis requirement will be the same in N. crassa remains to be determined.

The hypothesis that irradiation induces de novo production of carotenogenic enzymes has been criticized (Schopfer, 1977). For verification, enzymatic activities must be accurately estimated. Currently, only a few cell-free systems have been established for strictly photoregulated carotenoid biosynthesis. Evidence in favor of the induction hypothesis has been found using cell-free extracts of Mycobacterium sp. (Gregonis and Rilling, 1973; Johnson et al., 1974) and Neurospora crassa (Spurgeon et al., 1979; Mitzka-Schnabel and

Rau, 1981). These studies will be described in more detail below.

Assuming de novo production of carotenogenic enzymes, Batra (1971) has proposed that the light reaction causes the inactivation of a repressor, and the organism subsequently produces carotenogenic enzymes via transcription and translation. On the other hand, Rilling (1964) has suggested that some type of inducer mechanism is involved. Based on either model, it is plausible that control would be at the gene level, and transcription would be regulated by light. Inhibition of photoinduced carotenoid biosynthesis by compounds known as inhibitors of transcription has been reported in a few nonphotosynthetic microorganisms. In Mycobacterium sp. (Batra and Storms, 1968), proflavin inhibited light-induced pigment accumulation, while the effect of proflavin was unspecific in Fusarium (E. Schrott, Ph. D. thesis, Universität München, 1973). Actinomycin D prevented synthesis of carotenoids in Flavobacterium dehydrogenans (Weeks et al., 1973), but in Verticillium agaricinum (Mummery and Valadon, 1973) and N. crassa (Subden and Bobowski, 1973), inhibition was only partial. Distamycin A has been found by Rau (1976) to completely block light-induced carotenogenesis in Fusarium aquaeductuum.

A major argument against the use of transcriptional inhibitors has been their possible unspecific effects. Direct evidence for the involvement of m-RNA has been obtained from further investigations of Fusarium (Schrott and Rau, 1977). Using affinity chromatography of double-labeled polysomal RNA on poly(U)-sepharose, they were able to demonstrate different $^{3}H/^{14}C$ ratios for the adsorbed poly(A)RNA-con-

taining fraction when compared to the unbound fraction. This shift was not observed when both sample and control were kept in darkness.

Photoinduction of carotenogenesis may also be regulated at the translational level. In Verticillium agaricinum, the 80 S ribosomes from light-treated cultures are more active in the polymerization of phenylalanine than ribosomes from dark controls (Valadon et al., 1975). Although this light effect was proposed to be at the level of the ribosome, its relationship to photoinduced carotenogenesis in V. agaricinum remains to be determined.

If photoinduction involves gene derepression, light may inactivate a repressor of carotenogenesis. Therefore, this light effect might be mimicked by adding an inhibitor of the repressor. The compounds p-chloromercuribenzoate (PCMB) or p-hydroxymercuribenzoate (PHMB) are known to inactivate sulfhydryl (-SH) groups and could possibly inactivate the repressor, which is presumably a protein. Addition of either compound to dark-grown Fusarium aquaeductuum has been shown to induce carotenoid synthesis (Rau, 1969; Theimer and Rau, 1972). Other -SH group blocking substances such as iodobenzoate, iodoacetamide, and ethylmaleimide, however, were ineffective. Since the effect of light and PHMB were found to be additive, it is likely that the site of action of PCMB and PHMB is different from that of light. As further proof of independent sites of action, mutants of Fusarium aquaeductuum which have become constitutive for carotenoid biosynthesis also made increased levels of carotenoids in the dark after the addition of either PHMB or PCMB (Theimer and Rau, 1969).

In contrast to their inductive capacity of carotenogenesis in F. aquaeductuum, PCMB and PHMB are ineffective in mycobacteria (Batra, 1971). Antimycin A, however, has been reported to induce carotenoid formation in M. marinum in the absence of light (Batra, 1967). The use of structural analogs of antimycin A has revealed that different functional groups of the molecule are involved in the inhibition of electron transport and for the induction of carotenogenesis (Batra, 1971). It is interesting to note that, at every concentration tested, the inductive effects of this antibiotic in combination with light were additive.

Under the various experimental conditions used, the above mentioned chemoinductions have been shown to be additive to photoinduction. In addition, both chemoinduction and photoinduction are blocked by inhibitors of protein synthesis. Since exactly the same types of carotenoids are synthesized under either type of induction (Batra, 1967), it is likely that the initial target sites may be different, but subsequent events share a common mechanism (Batra, 1971).

Carotenogenesis in mucoraceous fungi may be stimulated approximately twenty-fold during mating. This increase is governed by a set of diffusable, low molecular weight pheromones which belong to the trisporic acid system (Bu'Lock et al., 1976; Ende, 1978; Gooday, 1978). Trisporic acid has been shown to promote sexual differentiation in Blakeslea trispora and Mucor mucedo (Ende et al., 1970). During this process, carotenoids become abundant in zygophores, suspensors, and

gametangia. Trisporic acid, a C_{18} apocarotenoid, is synthesized from β-carotene (Bu'Lock et al., 1970). In Blakeslea trispora, trisporic acid stimulation of carotenogenesis is inhibited by cycloheximide (Thomas et al., 1967). This observation has led to the suggestion that trisporic acid probably derepresses a limiting enzyme involved in the isoprenoid pathway.

Recent studies suggest that trisporic acids also exert their effects through transcriptional regulation of new RNA synthesis, and their action is in part mediated by adenosine cyclic monophosphate (cAMP) (Bu'Lock et al., 1976). Evidence in favor of cAMP involvement includes the finding that in M. mucedo, cAMP levels are 2- and 3-fold higher in areas of mating. Furthermore, the addition of N,N-dibutyryl-cAMP to nonmated cultures causes increased carotenoid pigmentation and marked morphological changes such as hyphal dilation, increased septation, and multiple branching (Jones, 1977). Similar results have been observed in Blakeslea trispora (Dandekar and Modi, 1980).

Cell-free Systems for Carotenogenesis

The overall pathway of carotenoid formation in bacteria, fungi, and higher plants is reasonably well-defined. Various aspects of the pathway have been summarized in many reviews, the more recent of which are cited (Britton, 1976; Davies, 1979; Davies and Taylor, 1976; Goodwin, 1979). The two major approaches which have been used to delineate this pathway include indirect kinetic studies and the more direct use of isotopes (Davies, 1980). The indirect approach relies

on quantitative measurements of carotenoids in organisms at different stages of development, in mutant strains, or under various conditions of biosynthetic inhibition. To prove that these reactions did occur, substrates are labeled with ^{3}H, ^{14}C, and ^{18}O.

In addition to pathway elucidation, these studies can be used to determine which steps in carotenogenesis are photoregulated. A comparison of the levels of intermediates in light- and dark-grown cells can suggest which part of the pathway is photoinduced. More compelling evidence for determining whether an enzyme in the carotenoid biosynthetic pathway is induced by light and consequently synthesized de novo must involve a comparison of enzymatic activities from light- and dark-grown cultures (Harding and Shropshire, 1980; Rau, 1980). This requires the preparation of crude and purified enzyme solutions as well as an in vitro assay system for the accurate estimation of enzymatic activity.

Biosynthetic studies using cell-free extracts have been plagued by a number of problems, mainly because of the nature of carotenoids themselves (Davies, 1976). Besides their instability in dilute solution and on chromatography, carotenoids are in many cases sensitive to heat, light, oxygen, and acids. Their hydrophobic nature has largely precluded their use as substrates in aqueous preparations. Therefore, most studies have relied on the use of radioactive substrates which serve as precursors not only for carotenoids but also for sterols and terpenoids. Incorporation of these precursors into individual carotenoids is usually quite low, and samples may be contam-

inated with other radioactive terpenoids which may have similar chromatographic behavior. Thus, extensive purification of carotenoid products is often required, and as mentioned previously the chances of loss are enhanced. In addition, radioactive intermediates after isopentenyl pyrophosphate are not available, and in many cases have to be synthesized.

Despite these practical difficulties, some progress has been made in the development of cell-free systems capable of synthesizing carotenoids (Britton, 1976). In higher plants, for example, incorporation of radioactive isopentenyl pyrophosphate into phytoene was shown using extracts of tomato plastids (Jungawala and Porter, 1967) and spinach leaves (Subbarayan et al., 1970). Recently an enzyme complex from tomato plastids which catalyzes this series of reactions has been purified to homogeneity and partially characterized (Porter and Spurgeon, 1979). Furthermore, a soluble enzyme system which converts phytoene to lycopene and several other colored carotenoids has been isolated from tomato fruit plastids (Kushwaha et al., 1970). In the fungus Phycomyces blakesleeanus, a cell-free system has been shown to synthesize phytoene from mevalonic acid (Davies, 1973), while crude extracts from several mutants can synthesize colored carotenoids as well (Bramley and Davies, 1976).

In bacteria demonstrating strict photoregulation of carotenogenesis, the only cell-free system examined in detail has been derived from Mycobacterium sp. (Johnson et al., 1974). Initial studies with intact cells demonstrated that little or no phytoene is synthesized in

the dark; however, the phytoene level is greatly increased after irradiation (Rilling, 1974). Since this increase is blocked by the addition of chloramphenicol immediately after irradiation, it is likely that light regulates one or more of the enzymes involved in phytoene biosynthesis. This hypothesis was in part confirmed by studies using cell-free extracts of Mycobacterium sp. (Gregonis and Rilling, 1973; Johnson et al., 1974). With this system, the authors found that the enzyme required for the conversion of geranylgeranyl pyrophosphate to prephytoene pyrophosphate is totally absent in dark-grown cells, while an in vivo light treatment significantly increased its activity. Furthermore, the rather low activity of geranylgeranyl pyrophosphate synthetase, which is normally present in dark-grown cultures, substantially increased after irradiation of intact cells. The activity of isopentenyl pyrophosphate synthetase, however, remained unchanged. Therefore, it was concluded that prephytoene pyrophosphate synthesis is probably the first fully photoinduced step in carotenogenesis of this bacterium. Initial studies suggest that photoregulation of carotenoid biosynthesis in Flavobacterium dehydrogenans also appears to occur at an early stage, since phytoene and other carotenoids have not been detected in dark-grown populations (Weeks and Garner, 1967).

In contrast to Mycobacterium sp., Neurospora crassa accumulates phytoene in dark-grown cultures (Zalokar, 1954). Based on in vivo studies, it was initially thought that light was responsible for the induction of those enzymes in the carotenoid pathway after phytoene

formation (DeFabo et al., 1976; Zalokar, 1955). However, it was later found that an albino mutant had increased phytoene levels after irradiation (Lansbergen et al., 1976). In further studies, Spurgeon et al. (1979) isolated an enzyme system which catalyzed the conversion of isopentenyl pyrophosphate to phytoene. Cell-free extracts from dark-grown cells were shown to exhibit a low level of phytoene synthesizing activity, while a short irradiation of cultures with blue light resulted in an increase of phytoene biosynthesis. Although somewhat different isolation procedures were used, Mitzka-Schnabel and Rau (1981) also obtained a cell-free carotenogenic system from N. crassa. Illumination of mycelia caused a several-fold increase in the conversion of mevalonic acid to phytoene and subsequent colored carotenoids. It should be noted that, in both cell-free systems of Neurospora, the the light-mediated increase in phytoene and other carotenoids was blocked by the addition of cycloheximide to cells during, or shortly after, irradiation.

Regulatory Mutants

Mutants which are blocked at different steps in the carotenoid biosynthetic pathway have been identified mainly in Neurospora crassa and Phycomyces blakesleeanus (reviewed by Cerdá-Olmedo and Torres-Martínez, 1979). To further elucidate the mechanism of photocarotenogenesis, the characterization of regulatory mutants either blocked in the photoinduction of carotenogenesis or capable of synthesizing carotenoids in the absence of light would be useful (Rau, 1980). To date, only a few isolates have been tentatively assigned to this category.

Theimer and Rau (1969) have obtained several mutant strains of Fusarium aquaeductuum which synthesize more pigment in the dark than the fully photoinduced wild type. Recent investigations indicate, however, that small amounts of pigment are photoinduced in these mutants (Rau, 1980).

In Neurospora crassa, several albino mutants which are blocked in the photoinduced synthesis of colored carotenoids have been fairly well characterized (Harding and Shropshire, 1980; Harding and Turner, 1981). Most defects appear to occur in structural genes involved in the pathway leading to carotenoid biosynthesis. Of particular importance is a strain designated wc (white collar) which has albino mycelia but normal pigmentation in conidia. In wild type Neurospora, mycelia and conidia demonstrate a fundamental difference in photocontrol of carotenogenesis. Whereas mycelia require light for carotenoid production, conidia accumulate carotenoid pigments in the dark. Thus, conidia can bypass the light requirement for pigment production. In the mycelial stage of the wc strain, phytoene biosynthesis is unaffected by light; therefore, it is likely that wc is a regulatory mutant. Based on the defects found in albino mutants, Harding and Shropshire (1980) have proposed a model for the regulation of photoinduced carotenogenesis in N. crassa. According to this scheme, a product of the wc+ gene is required early in photoinduction. This model predicts that other wc genes are likely to be found, and at least one group of wc mutants should be defective in the blue light photoreceptor. It should be mentioned that, according to their hypoth-

esis, albinos which have mutations in structural genes should have reduced carotenoid biosynthesis in both conidia and mycelia. The levels of carotenoids in conidia of these mutants have not as yet been determined.

The genetical aspects of carotenogenesis have been most extensively characterized in the fungus Phycomyces blakesleeanus. As mentioned previously, Phycomyces synthesizes significant amounts of carotenoids in the dark. Besides stimulation by light, three other independent regulatory pathways for carotenogenesis have been described (reviewed by Cerdá-Olmedo and Torres-Martínez, 1979). These include repression by a product of the carS gene; stimulation by retinol (vitamin A) or β-ionone; and stimulation by trisporic acids through sexual interaction. A mutant of Phycomyces blakesleeanus, presumed to be regulatory, has been found to synthesize approximately 500 times more β-carotene in the dark than wild type (Murillo et al., 1978). This mutant, however, has more than one defect in the regulatory pathways of carotenogenesis mentioned above. A new group of Phycomyces mutants, designated pic (photoinduced carogenogenesis), are specifically defective in photocarotenogenesis (López-Díaz and Cerdá-Olmedo, 1980). Unfortunately this defect is only partial, and pic mutants synthesize carotenoids, although in reduced amounts, under light. A strain of Phycomyces carrying a double mutation in the carotenoid genes has been found to synthesize a little more β-carotene in the dark than wild type but is altogether unable to photoinduce carotenogenesis (López-Díaz and Cerdá-Olmedo, 1980). Under higher intensities

of light, this mutant has decreased amounts of carotenoid accumulation, probably due to photolysis.

Light regulates many other aspects of the Phycomyces life cycle (Bergman et al., 1969). Some mutants which are defective in phototropism have also been shown to be partially defective in photocarotenogenesis (Bergman et al., 1973). Based on genetic and physiological studies of wild type cells, mutants defective in phototropism, mutants defective in carotene regulation, and pic mutants, it has been proposed that photocarotenogenesis in Phycomyces depends on a single sensory pathway which shares its early steps with that of phototropism and its later steps with one of the carotenoid regulatory pathways (López-Díaz and Cerdá-Olmedo, 1980). Admittedly, this regulation scheme is tentative, and further evidence elucidating photoregulation of carotenogenesis will probably come from studies using cell-free extracts of the different mutants.

CHAPTER II

MATERIALS AND METHODS

A. Organisms.

Trichophyton mentagrophytes ATCC 26323 and *Trichophyton tonsurans* F-163-79 were used in this investigation. Both strains were kindly provided by Dr. J. Rippon, University of Chicago. This strain of *T. mentagrophytes*, originally designated SF 306A/68, is a virulent strain isolated from a patient in Vietnam. *Trichophyton tonsurans* was a clinical isolate from an erythematous patch on a lady's chest.

B. Maintenance of the Organisms.

Stock cultures of these organisms were maintained on Sabouraud-dextrose agar (SDA) at room temperature (23-25°C) in the dark. Cultures were transferred every 3 weeks by spreading of dilute aqueous suspensions of microconidia on SDA so that individual colonies derived from single spores were obtained. By this procedure, the granular colony phenotype, rather than the pleomorphic or variant phenotype, was maintained.

C. Media.

Sabouraud-dextrose agar (SDA, Difco Labs) with a shallow overlay of Sabouraud-dextrose broth (SDB, Difco Labs) was used in this investigation. The broth overlay was added 24 h after inoculation. Both media were supplemented with 0.1% sodium acetate (Mallinckrodt). This concentration of sodium acetate suppressed the formation of sterile

variants but had no effect upon pigment accumulation. Since SDA contained 4% glucose, 1% neopeptone, and 1.5% agar, while SDB contained only 2% glucose and 1% neopeptone, SDB was routinely supplemented with an additional 2% glucose (Mallinckrodt).

D. Culture and Growth Conditions.

1. Preparation of the Microconidial Inoculum.

Microconidia were produced and harvested by a slight modification of the method of Hashimoto et al. (1972). The fungus was grown in the dark on SDA at 25°C so that 1-3 individual colonies, each derived from a single spore, were present per plate. After 3 weeks, the microconidia within a colony were dislodged from the agar surface by gently scraping the culture with a sterile metal spatula. The spores (from a maximum of 3 colonies) were transferred to a 50 ml plastic screw cap centrifuge tube containing 20 ml of sterile distilled water and dispersed by vortexing. The suspension was then filtered through 16 layers of sterile gauze (Parke, Davis, and Company) to eliminate hyphae and undispersed masses of conidia. The microconidial filtrate was washed at least 5 times by centrifugation at 1500 x g for 15 min (I.E.C. Model B-20, Rotor #870) and resuspended in sterile distilled water. All steps in the isolation and purification of microconidia were carried out at 4°C.

Microconidia were then suspended in sterile distilled water to a turbidity of 100 Klett units (KU; 2 x 10^6 spores per ml of water) using a number 54 filter (green) in a Klett-Summerson colorimeter. For standard plastic petri dishes (100 x 15 mm), 0.5 ml of the 100 KU

suspension was used as the inoculum. Because of lighting space limitations, it was necessary to use smaller petri dishes in some studies. In order to maintain a relatively constant thin layer of cells in culture, the amount of inoculum varied proportionally with the surface area of the agar.

2. Formation of Arthrospores on Sabouraud's Medium.

A slight modification of the procedure described by Hashimoto and Blumenthal (1977) was used. For studies where a large amount or "batch" of preformed arthrospores was needed, freshly prepared microconidia (1 x 10^6 conidia in aqueous suspension) were inoculated onto a sterile dialysis membrane (Union Carbide), which had been placed on SDA containing 0.1% sodium acetate (SDA-acetate). In order to remove any preservatives and impurities, the dialysis membrane, approx 9 cm in diam, had been previously boiled for 15 min in a solution of 0.01 M ethylenediamine tetraacetic acid (EDTA, Mallinckrodt) with 0.1 M sodium bicarbonate (Sigma Chem. Co.). After thorough washing with distilled water, the membrane was sterilized by autoclaving in a small volume of water before aseptic placement on 20 ml of solidified agar medium contained within a standard size petri dish (100 x 15 mm). The inoculated plates were incubated for 24 h at 37°C in the dark to allow maximal microconidial germination, which had been previously shown to be inhibited by visible light (Buchníček, 1974). During this period, the germinating microconidia became attached to the dialysis membrane. Each plate was then gently overlaid with 5.0 ml of SDB-acetate. The plates were subsequently placed in humidity chambers and incubated in

the dark at 37°C for various time periods, depending upon the particular study.

In experiments where cultures were to be irradiated, each petri dish was sealed with parafilm (American Can Corp.) after the broth overlay, and subsequently exposed to light. Petri dishes wrapped in aluminum foil served as dark controls and were placed next to irradiated cultures. Some studies required the removal or replacement of aluminum foil which surrounded the culture. When smaller cultures were used, an aqueous suspension of 3×10^5 microconidia was inoculated onto a dialysis membrane, approx 5 cm in diam, which had been placed on SDA-acetate (8.0 ml) in a plastic petri dish (60 x 15 mm, Falcon). After incubation in the dark at 37°C for 24 h, the cultures were overlaid with 1.8 ml of SDB-acetate. They were immediately sealed with parafilm and incubated at 37°C for designated time periods. Dark controls were wrapped in aluminum foil. In all studies, temperature was monitored directly beneath the light source at the culture level by measurement with a calibrated Model 46 Tele-thermometer (Yellow Springs Instrument Co.).

3. Quantitation of Arthrospore Formation.

The degree of arthrosporulation was determined according to the method of Timberlake and Turian (1975). Cells were removed from the dialysis membrane and examined microscopically using an oil immersion objective. The percentage of hyphal tips containing arthrospores was determined after examining 200 tips.

4. Dry Weight Determination.

The dry wt of washed cells was determined by vacuum desiccation of samples in predried, tared glass vials over phosphorous pentoxide (P_2O_5, Mallinckrodt) at 25°C until constant weight was achieved.

5. Endotrophic Carotenoid Accumulation.

To partially elucidate the mechanism by which visible light suppresses carotenogenesis, arthrospore suspensions were incubated in the absence of exogenous carbon and nitrogen sources. Batch cultures of T. mentagrophytes were grown in the dark on Sabouraud's medium at 37°C for a total of 5 days. The arthrospores were readily removed from the dialysis membranes by a sterile spatula. Under dim white light (5-15 lux), the harvested arthrospores were thoroughly washed with sterile distilled water by gravity filtration on a cellulose filter (Qualitative #1, Whatman) and suspended in sterile sodium phosphate buffer (0.1 M, pH 6.8) at a concentration of approx 10 mg dry wt of spores per ml of buffer. This is referred to as the batch cell suspension. The arthrospore suspension was gently agitated on a rotary shaker (Lab-Line Instruments, Inc.) at 50 rpm in the dark for 1 h at 25°C. After incubation, the spores were further rinsed with buffer (100 X the original volume of suspension) on a sterile Millipore filter (0.45 µm pore size, 4.7 mm diam, Millipore Corp.) and resuspended in buffer at the concentration mentioned above. A 1 ml aliquot of the spore suspension was pipetted into a small petri dish (60 x 15 mm), and 3 ml of buffer was subsequently added. After gentle swirling, the arthrospores settled to the bottom of the dish and formed a thin,

uniform layer of cells. The petri dish containing the arthrospore suspension was incubated under the appropriate intensity of light for 24 h at 37°C. The dark controls were samples which were wrapped in aluminum foil and incubated under the same conditions. In some studies, batch suspensions were prepared from cultures of less mature arthrospores. Some experiments required longer periods of incubations at 37°C.

6. Photobleaching of Pigment in Arthrospores under Carotenogenic Arrest.

Carotenoid photobleaching in whole arthrospores was examined under conditions where de novo synthesis of these pigments was arrested. Two different methods of arrest were employed: 1. cold incubation at 4°C; and 2. pretreatment with formaldehyde before incubation at 37°C.

For photobleaching in the cold, SDA-acetate cultures of arthrospores were shifted from 37°C to 4°C and exposed to light for various periods of time. In some studies, the effect of different intensities of light was examined.

Since it is known that cold temperature may alter the properties of many lipids, the action of visible light on intracellular carotenoids was examined at 37°C where it was necessary to chemically arrest carotenogenesis in arthrospores. The experimental approach was to determine several intensities of light which did not cause photobleaching and to ascertain whether there was a reduction of endotrophic carotenoid accumulation at these specific intensities.

To determine nonbleaching intensities of light, batch cell suspensions (10 mg dry wt spores/ml 0.1 M sodium phosphate buffer, pH 6.8) were prepared as described for endotrophic carotenoid accumulation. Formaldehyde (37% aqueous solution containing 10-15% methyl alcohol, Mallinckrodt), which had been diluted with sodium phosphate buffer, was added to the spore suspension so that the final concentration of formaldehyde was 2.0%. Treatment with formaldehyde lasted for 1 h at 25°C in the dark. After treatment, the spores were rinsed, suspended in buffer, and dispersed to form a thin layer in a small petri dish, exactly as in the endotrophic system. Individual samples were incubated for 24 h under a range of predetermined intensities of light at 37°C. Pigment levels of irradiated cells and dark controls were measured by the procedure mentioned in Materials and Methods, Section G 1. In subsequent studies, portions from the same batch of arthrospore suspension were used to concurrently determine nonbleaching doses of visible light (formaldehyde pretreatment) and reduction of endotrophic carotenoid accumulation under these doses at 37°C. Thus any pigmentation differences resulting from one batch of spores to another were avoided.

7. Germination under Light of Arthrospores Containing Different Amounts of Pigment.

Arthrospores were formed using the standard dialysis membrane technique with Sabouraud's medium. After a broth overlay, each culture was sealed with parafilm and was individually wrapped in aluminum foil. Cultures were incubated in the dark at 37°C for a total of 72 h. At

this time, sets of these cultures were further grown for an additional 5 days under each of the following conditions: 1. 37°C, dark; 2. 39°C, dark; 3. 37°C, blue light (500 lux); and 4. 37°C, blue light (100 lux). The arthrospores from each set of cultures were aseptically harvested, filtered through 16 layers of sterile gauze to remove residual hyphae and long chains of spores, and washed 5 X with cold (4°C) sterile distilled water. Pigment content was determined by the single culture method described in Materials and Methods, Section G 1. The purified arthrospores were stored in tightly-sealed test tubes at -20°C for several days. Under these conditions, neither the viability nor the pigmentation of arthrospores was affected.

Germination of arthrospores was monitored by a procedure previously described for the microconidia of T. mentagrophytes (C. D. Wu, Master's thesis, Loyola University of Chicago, 1973). Initially, arthrospores with altered pigmentation, due to the above cultural manipulations, were suspended in SDB (4% glucose) at a concentration of 10 KU (2×10^5 spores per ml of broth). One drop of suspension was inoculated onto a sterile microculture slide containing SDA. The culture slide had been prepared on the day of use in the following way: a glass microscope slide (1 x 3 in) was placed on a U-tube support in a glass petri dish and was sterilized by autoclaving. A few drops of molten sterile SDA were subsequently placed on the slide, and a sterile glass coverslip (22 x 22 mm) was immediately placed on the agar to flatten the surface. After solidification of the agar, the coverglass was removed, and sterile water was placed in the bottom

of the petri dish to prevent excessive drying of the agar. During preparation, petri dishes were kept closed as much as possible.

After inoculation, the cultures, which remained in the closed petri dishes, were continuously irradiated with blue light (1,000 lux, 16 W/m^2) at 37°C. Dark controls were petri dishes wrapped in aluminum foil. Cultures were incubated for 5 h, which had been previously determined to be the minimum time needed for complete germination in the dark of fully-pigmented arthrospores (formed in darkness at 37°C). For germination assessment, the percentages of arthrospores developing visible germ tubes were estimated microscopically by counting a total of 200 arthrospores (Hashimoto and Blumenthal, 1977).

E. Light Sources and Irradiation Procedures.

Three different types of fluorescent light were used in this investigation. To study the suppressive effect of white light on carotenogenesis, cultures were grown or incubated under various intensities produced by two parallel General Electric (GE) Cool White fluorescent tubes (F-158T8-CW, 15-W). When a large number of cultures were required, a series of parallel GE Cool White fluorescent tubes (F-45T10-CW High-output, 40-W) were used. Blue light was produced by two parallel Westinghouse Special Blue fluorescent tubes (F-20 T 12/BB, 20-W). Red light was produced by two parallel GE red fluorescent tubes (F-258T8-R, 20-W). Different intensities of light were achieved by adjusting the distance between the sample and the light source. In all light studies, dark controls were placed at the same level as irradiated samples.

The intensity of light reaching the surface of the petri dishes was measured by a Luna-Pro sbc photometer (Gossen Division, Berkey Marketing Co.) or a Model 65A radiometer (Yellow Springs Instrument Co.) with a Y.S.I. 6551 probe which had a constant output in the wavelength range of 0.3-2.7 μm. The radiometer and probe were equilibrated for 24 h at the specific temperature before light intensities were measured. Since continual access to the radiometer was not possible, standard curves measuring light intensity in photometric units (lux) and in energy units (W/m^2) were constructed. Emission spectra of red and blue lamps were measured with a Model 2400 digital photometer (Gamma Scientific, Inc.) equipped with a Model 700-31 monochrometer and V.T.M. 700-31 grating.

Experiments on photobleaching of carotenoids in cell-free extracts and isolated pigment granules were performed in test tubes (10 x 75 mm, borosilicate, Kimble Products) which were capped with parafilm and contained 1.5 ml of sample suspended in HEPES-EDTA buffer, pH 7.4 (see Materials and Methods, Section F). The tubes were positioned almost horizontally and irradiated for the designated periods of time at either $4^{o}C$ or $37^{o}C$. Dark controls were tubes wrapped in aluminum foil. For studies where extracts or granules were irradiated in an atmosphere of reduced oxygen, samples were placed in Thunberg tubes, and air was replaced by nitrogen gas (N_2, Medical Grade, Ohio Medical Products) through 3 successive evacuations of the tube with a house vacuum line followed by introduction of the gas.

F. Preparation of Cell-free Extracts and Isolation of Pigment Granules.

A modification of the procedure described by Riley and Bramley (1976) was used. Cytoplasmic extracts and carotenoid-containing granules were isolated from mature arthrospores (8 days, dark) as follows: rinsed arthrospores from approx 150 cultures were suspended in cold (4°C) 10% sorbitol (J. T. Baker Chem. Co.), 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Sigma Chem. Co.), and 1 mM EDTA, pH 7.4. The arthrospores were subsequently dispersed by brief sonication (2 sec burst, #3 setting) in a Model S-75 sonifier (Branson Instruments, Inc.) equipped with a standard microtip horn. Following dispersal, the spores (approx 2 g dry wt per ml of buffered sorbitol) were broken in a Model J5-598A French pressure cell press (1 in piston, American Instrument Co.) at 40,000 psi with a flow rate of approx 15 drops per min into a prechilled glass centrifuge tube. The broken spore homogenate was centrifuged at 1,500 x g for 15 min at 4°C to remove whole arthrospores and broken cell wall fragments. The resultant supernatant was considered the cell-free extract.

For pigment granule isolation, the supernatant was subsequently placed in 60 ml cellulose nitrate centrifuge tubes (Beckman Instruments, Inc.) and gently overlaid with an equal volume of 5% sorbitol in HEPES-EDTA buffer. The carotenoid-containing granules were isolated by flotation centrifugation at 24,500 rpm (100,000 x g) for 60 min at 4°C using a Model L5-65 ultracentrifuge with an SW 25.2 rotor (Beckman Instruments, Inc.). The granules, which had migrated to the top of

the gradient, were removed with a Pasteur pipette. All steps involved in the pigment granule isolation were performed under dim white light (5-15 lux). Fig. 2 summarizes the procedure for isolation of carotenoid-containing granules.

G. Pigment Extraction and Quantitation of Total Carotenoids.

1. Whole Cells.

In some studies, the cells from several identical cultures were combined and used to determine a data point. At the designated times, cells were removed from the dialysis membranes with a spatula and placed in a 30 ml Corex centrifuge tube (Corning Glass Works). The harvested cells were washed in cold, sterile distilled water 3 X by means of centrifugation (1,500 x g, 15 min). The pigments from each sample were extracted in the dark with methanol (Eastman Organic Chemicals). Depending upon the amount of pigment, some samples needed repeated extractions until the cells appeared totally white. For each sample, the extracts were combined, and the total volume of the extract was recorded. The absorbance at 460 nm, the wavelength of maximum absorption for methanol extracts of total carotenoids, was measured spectrophotometrically (see Materials and Methods, Section J) as recommended by Liaaen-Jensen and Jensen (1971). The extract was then recombined with the corresponding extracted cell material and evaporated in the dark in a Thelco Model 81 water bath (Precision Scientific) at 75°C. The dry wt of samples was subsequently determined. The pigmentation index (PI) was expressed as:

$$\frac{A_{460} \times v}{d}$$

Fig. 2. Procedure for the isolation of carotenoid-containing granules from arthrospores of T. mentagrophytes.

Arthrospores suspended in 50 mM HEPES containing 10% sorbitol and 1 mM EDTA

↓

Disruption in a French press (40,000 psi)

↓

Centrifugation at 1,500 x g for 15 min at 4°C

↓

Supernatant overlaid with an equal volume of 5% buffered sorbitol

↓

Flotation centrifugation at 100,000 x g for 60 min at 4°C

↓

Granules, which form a thin film at the top of the gradient, removed by Pasteur pipette

where A_{460} is the absorbance of the methanol extract at 460 nm, light path 10 mm, v is the volume in ml of the extract, and d is the weight of the dry biomass (mg). Unless otherwise stated, pigmentation was expressed relative to the PI obtained from dark-grown arthrospores (8 days old). Since the same number of cultures were used for each sample within an experiment, d also was a quantitative measurement of growth or mass at a particular developmental stage.

Because the above pigment quantitation involved a long processing time, carotenoid levels from single cultures (approx 10 mg dry wt cells) were determined as follows: the cells were removed from the dialysis membrane of a culture by a spatula, placed on a Millipore filter (0.45 μm pore size, 4.7 cm diam, Millipore Corp.), and rinsed with 30 ml of cold, sterile distilled water. After all the water had passed through the filter, vacuum was retained for an additional 30 sec. The cells were then gently scraped off the filter and placed in a predried, tared 20 ml glass scintillation vial (Scientific Products). Each vial was immediately capped and weighed. Dry weight was calculated from wet weight by dividing by 9.01, a factor which had been previously determined. The moist cells were then extracted with methanol in the dark. Pigmentation index and growth (dry wt) were determined as above.

For cell suspensions used in endotrophic carotenoid accumulation and carotenogenic arrest, the contents of one petri dish were removed by Pasteur pipette, placed on a Millipore filter, and processed as mentioned for single cultures. In most studies, pigmentation was

expressed as a percentage relative to the amount of total pigment obtained from dark controls after 24 h of incubation at 37°C. Since the numbers of cells used in each petri dish were the same within an experiment, pigmentation was sometimes expressed as A_{460} of the methanol extract (4.0 ml).

2. Cell-free Extracts and Isolated Pigment Granules.

The pigment levels of extracts and granules were determined as follows: the contents of tubes containing these cell-free fractions (see Materials and Methods, Section E) were rinsed and collected on solvent resistant teflon (fluoropore) filters (0.5 μm pore size, 2.5 cm diam, Millipore Corp.) which had been pretreated with methanol and water. The filters, which retained subcellular carotenoid fractions, were placed in scintillation vials containing 2.0 ml of methanol. After 4 h of incubation in the dark at room temperature, the absorbance of each methanol extract was read spectrophotometrically at 460 nm. The quantity of pigment remaining after irradiation was expressed as the percentage absorbance of the initial control.

H. Isolation and Identification of Individual Carotenoids.

Methanolic extracts derived from rinsed whole cells (at least 50 mg dry wt) were saponified with 15% potassium hydroxide (KOH, Mallinckrodt) for 18 h in the dark at room temperature. Preliminary studies indicated that carotenoids were not destroyed by this treatment. The pigments were collected in hexane (Mallinckrodt) by phase separation (Davies, 1965). The epiphase fraction containing the pigments was concentrated under vacuum in the dark. To remove sterols,

the concentrated pigment solution was stored for several hours at -17°C and subsequently decanted.

For isolated pigment granules, fluoropore filters (Millipore Corp.) containing the rinsed granules were submerged directly into hexane. After total extraction of the pigments from the granules, the filters were removed. The extracts were further condensed in the dark, and sterols were removed as mentioned above.

The concentrated carotenoid solution in hexane was applied by capillary pipette to silica gel 60 pre-coated thin-layer glass plates (#5767, EM Laboratories, Inc.) with fluorescent indicator F-254. Development of the thin-layer plates was carried out at 25°C in the dark using a solvent system of hexane-benzene (10:1.5) previously described by Hashimoto et al. (1978). Carotenoids extracted from dark-grown arthrospores (8 days, dark) served as reference standards.

Colorless carotenoids on the chromatograms were visualized by illumination with a Model R52 UV Mineral light (Ultraviolet Products Inc.) equipped with filters #20118 and #20119 for long- and short-wavelength UV, respectively. Photographic records of thin-layer chromatograms were made on Ektachrome 160 (tungsten) film (Eastman Kodak Co.) with a Nikon FM camera (Nippon Kogaku K.K.) equipped with a micro-Nikkor 50 mm lens and a Kodak #81 A filter.

After development, individual carotenoids bound to the silica gel matrix were scraped off the plate, while still damp, and eluted from the gel with methanol. The extracted pigments were transferred into hexane by phase separation (Davies, 1965).

I. Chemical Characterization of Isolated Pigment Granules.

The isolated granules, which were placed in 15 ml Corex glass centrifuge tubes, were rinsed 5 X with cold, sterile distilled water by flotation centrifugation (4°C) at 19,000 rpm (15,000 x g) in a Model J2-21 centrifuge with a JA 20 rotor (Beckman Instrument Co.). After the final rinse, the granules were removed from the top of the water surface with a Pasteur pipette. Except in the case of carotenoid analysis (see below), the concentrated granule suspension was placed in predried, tared Corex test tubes, shell frozen in an acetone-carbon dioxide bath, and freeze-dried in a Model 10-145-MRBA lyophilizer (The Virtis Co., Inc.) for 18 h in the dark. Samples were further dried to constant weight under P_2O_5 in a vacuum desiccator (house line) at room temperature (23-25°C) and stored in the dark under these conditions.

1. Carotenoids.

Since γ-carotene was found to be the predominant carotenoid within the granules, the total amount of carotenoids within the pigment granules was determined as the amount of γ-carotene per dry wt granules. Immediately after rinsing (see above), freshly prepared granules were suspended in H_2O (approx 20 ml) and gently vortexed (Omni Mixer, Sorvall, Inc.) to make a uniform suspension. Small volumes (2.0-5.0 ml) of the granule suspension were individually collected on solvent resistant teflon (fluoropore) filters (0.5 μm pore size, 2.5 cm diam, Millipore Corp.). The filters had been premoistened with methanol and rinsed with a small amount (2.0 ml) of H_2O in the filtration apparatus

before use. After filtration, each filter with the granules was placed in a scintillation vial containing 2.0 ml of hexane. The vials were tightly capped and placed in the dark at room temperature for 4 h so that the carotenoids became totally extracted from the granules. Absorbances of the hexane extracts were recorded at 460 nm and normalized to 1 ml of the original granule suspension.

To determine the dry wt of granules in these studies, samples (6.0-7.0 ml) of the original granule suspension were collected on filters (0.45 μm pore size, HAWP, Millipore Corp.) which had been predried and tared together in 15 ml Corex centrifuge tubes. The damp filters containing the pigment granules were replaced in their corresponding test tubes and lyophilized in the dark overnight. Each tube containing the filter and granules was then dried under vacuum desiccation over P_2O_5 to constant weight. A weight control consisted of a filter through which a small volume of water was passed and treated as above. After the normalization of dry wt to 1 ml of the original granule suspension, the quantitative determination of pigmentation was calculated from the extinction coefficient $E^{1\%}_{1cm}$ of γ-carotene (in hexane) which was 2720 (Goodwin, 1955).

2. Sugar.

The anthrone method was used for quantitation of sugars present in the pigment granules (Morris, 1948). Dried preweighed samples (approx 200 μg) were placed in Pyrex test tubes (13 x 100 mm, Corning Glass Works) containing 1.5 ml of double distilled deionized water and 3.0 ml of anthrone reagent, which was prepared as a fresh solution of

0.2% anthrone (MCB Manufacturing Chemists, Inc.) in concentrated sulfuric acid (Mallinckrodt). The mixture was boiled in a water bath for 10 min along with D-glucose standards. After cooling, the absorbance at 620 nm was recorded.

3. Protein.

Protein content was estimated by the method of Lowry et al. (1951) using crystalline bovine serum albumin (Sigma Chem. Co.) as a standard. The Folin Ciocalteu reagent was supplied by Anderson Laboratories, Inc.

4. Lipid.

Solvent extractable lipids were determined gravimetrically by a modified method of Bligh and Dyer (1959). Two ml of methanol was added to a dried, preweighed sample (5.0 mg), and the mixture was dispersed in a sonic bath (Heat Systems Ultrasonics). The mixture was incubated in the dark for 30 min at room temperature. One ml of chloroform (MCB Manufacturing Chemists, Inc.) was subsequently added, and the mixture was extracted for an additional 30 min. After further addition of 3.0 ml of chloroform, samples were incubated for 1 h. The final ratio of chloroform to methanol was 2:1 (v,v). Nonextractable particulate material was removed by centrifugation at 54,000 x g for 1 h. The supernate was carefully removed, and the pellet was reextracted exactly as mentioned above. The extracts of a sample were combined, placed in a predried tared aluminum planchet, evaporated at room temperature, and dried to constant weight under vacuum over P_2O_5.

Before evaporation, visible and ultraviolet (UV) absorption

spectra were recorded for sample extracts to check for sterol presence. Ergosterol (Sigma Chem. Co.) which was dissolved in chloroform-methanol 2:1 (v,v) served as the standard. Absorbances of standards and samples were recorded at 280 nm.

5. Phosphorous.

Phosphorous was determined by the phosphate procedure of Chen et al. (1956). Predried granule samples (100-200 µg) were suspended in 4.0 ml of double distilled deionized H_2O and were sonicated until uniformly suspended. Each sample, which had been placed in Pyrex test tubes, was ashed by the addition of concentrated H_2SO_4 (4 drops) and by subsequent heating in a sand bath until the white fumes of sulfur trioxide appeared. Two drops of perchloric acid (J. T. Baker, 72% w/v) were added to each tube, and the sample was heated in a Bunsen burner flame until clearing. After cooling, the sample was diluted to 4.0 ml with H_2O. Two ml of the reagent mix solution [6 N H_2SO_4, H_2O, 2.5% aqueous ammonium molybdate (J. T. Baker), and 10% aqueous ascorbic acid (Sigma Chem. Co.); 1:2:1:1 (v,v,v,v)] was added, and the samples were incubated at 37°C in a water bath for 2 h. After cooling, absorbances were measured spectrophotometrically at 820 nm. A phosphorous standard solution (Sigma Chem. Co.) containing 20 µg of phosphorous (as potassium dihydrogen phosphate, KH_2PO_4) per ml was diluted 1:1 and used in construction of the standard curve.

J. Spectrophotometry.

Absorption spectra of either total or individual carotenoids (in hexane) were examined in a Cary 15 spectrophotometer (Varian Instru-

ments Division). Derivative spectra were recorded with a Perkin-Elmer Model 320 spectrophotometer. Absorbance determinations at specific wavelengths were measured in a Model 2000 multiple sample spectrophotometer (Gilford Instrument Laboratories, Inc.).

K. Cytological Techniques.

1. Light Microscopy.

Light microscopic procedures were performed with a Nikon microscope which had both phase contrast and plain objectives. Photomicrographs were made on Plus-X panchromatic film or Ektachrome 160 tungsten color film (Eastman Kodak Co.) using a Nikon M-35S camera equipped with an automatic exposure system attached to the microscope. The carotenoid-containing granules within arthrospores were checked for the presence of lipid by staining with Sudan black B (Sigma Chem. Co.) in 70% ethanol (American Scientific Products, 90%) (Burdon, 1946).

2. Transmission Electron Microscopy (TEM).

Whole cells were fixed and embedded as previously described (Samsonoff et al., 1971). In some studies, arthrospores were fixed in 4% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8, for 24 h at room temperature. The fixed cells were rinsed and postfixed in 2% osmium tetroxide in the same buffer for 24 h at room temperature. After dehydration through a graded acetone series, cells were infiltrated by gradual replacement with an Epon mixture (812/815 = 1), and the final cell-resin mixture was polymerized at 60°C for 48 h.

Pigment granule films, isolated by flotation centrifugation through 5% sorbitol in HEPES-EDTA buffer, were fixed and embedded as

described earlier (Samsonoff et al., 1971). This procedure was subsequently modified so that granules were rapidly dehydrated (1 min intervals) through either a graded acetone series or a methanol series followed by propylene oxide. In some studies, fixed granules were embedded in Noble agar (Difco) before dehydration.

Alternatively, the granule film was stabilized with 70% sorbitol in HEPES-EDTA buffer for 1 h at 4°C and subsequently fixed with 2% osmium tetroxide in Kellenberger buffer, pH 7.0, for 4 h at room temperature. The granules were collected on a Millipore filter (0.45 μm), briefly rinsed with double distilled deionized water, and dried on the filter under vacuum desiccation over P_2O_5 for 18 h. The filter was directly submerged in Epon resin with one fresh resin change after 24 h. The granules were scraped off the filter and placed in BEEM capsules (Ernest F. Fullum, Inc.) containing resin. The Epon mixture was subsequently polymerized at 60°C for 48 h.

Thin sections were cut with a diamond knife (E. I. Du Pont De Nemours and Co., Inc.) on a Model 4800 A Ultratome microtome (LKB Produkter AB) and mounted on copper grids (300 mesh, Ernest F. Fullum, Inc.) coated with 0.25% formvar (Electron Microscopy Sciences). The grids were stained with a saturated solution of uranyl acetate (Polysciences) for 30 min followed with alkaline lead citrate (Reynolds, 1963) for 10 min or with lead citrate alone (10 min). Sections were examined with either an RCA EMU-3 transmission electron microscope operating at 50 kV or an Hitachi HU11A electron microscope at 50 or 75 kV.

L. Chemicals.

All chemicals used in this investigation were of analytical reagent quality. All solvents were either chromatographic or spectroscopic grade and were used without further purification.

M. Analysis of Data.

Averages were represented with a plus and minus (±) standard error of the mean (SE). The determination of the statistical significance of pigmentation differences was done by using the Student's t test for small independent samples. In arthrospore germination studies, studies, the Chi-square (χ^2) statistic was used. A difference was considered significant if the probability (p) value was less than 0.05. In some experiments, the exact p values were determined by use of a TI Model 59 Programmable calculator (Texas Instruments, Inc.) with an Applied Statistics module (program St-21). The slopes of intensity (lux versus W/m^2) standard curves were determined from the best fit line (least squares method), and the Pearson product-moment correlation coefficient (r) was used as an index of correlation between the set of data points. In other studies which determined the best fit curves and lines for the effect of light intensity on carotenoid accumulation, calculations were done on an Amdahl 470 computer (I.B.M. Corp.) using bivariate data transforms and the nonlinear least squares method.

CHAPTER III

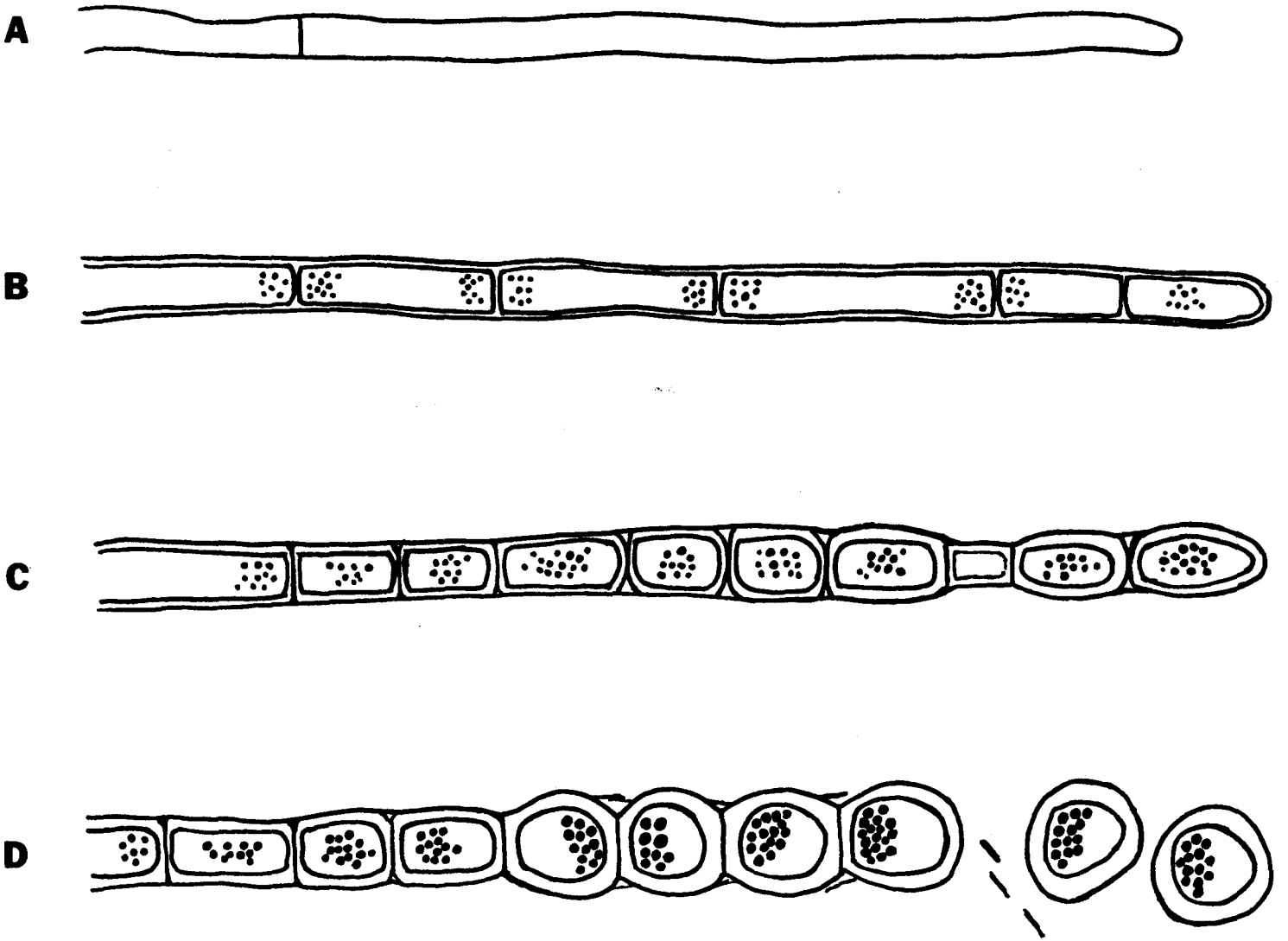
RESULTS

A. Structural Aspects of Carotenoid Formation in Arthrospores.

1. Development of Pigment Granules during Arthrosporulation.

When grown on SDA agar in continuous darkness at 37°C, T. mentagrophytes formed arthrospores by the thallic-arthric mode of ontogeny, that is, by fragmentation of pre-existing hyphae. As mentioned in the Introduction, carotenoid pigments appeared to be localized in intracellular granules of arthrospores. The light microscopic appearance of pigment granules during arthrosporulation in T. mentagrophytes is schematically depicted in Fig. 3. Pigment granules were not observed in hyphae (Fig. 3A). Intracellular clusters of pale yellow granules became evident only after sporulation septa began to be formed (Fig. 3B). These granules were readily stained with Sudan black B (see below), thus confirming their lipid nature. As arthrosporulation commenced, there was increased septal deposition, and pigment granules became more conspicuous, acquiring a yellow-orange hue (Fig. 3C). Completion of septation normally marked the beginning of arthrospore maturation with cylindrical spores assuming more spherical shapes. In mature arthrospores, pigment granules were bright orange and tended to cluster subapically (Fig. 3D). During maturation, arthrospores continuously accumulated intracellular carotenoids (Hashimoto et al., 1978).

Fig. 3. Schematic representation of pigment granule appearance and arrangement in arthrosporulating T. mentagrophytes based on light micrographs. A. Vegetative hypha. Pigment granules are absent. B. Early stage of arthrosporulation. Septation has initiated, and clusters of pale yellow granules are usually localized at opposite poles of a cell compartment. C. Completion of arthrospore septation. Single clusters of yellow-orange granules are loosely organized in arthrospores. D. Mature arthrospores. Compact clusters of orange granules become localized subapically in swollen, spherical arthrospores.


A
B
C
D

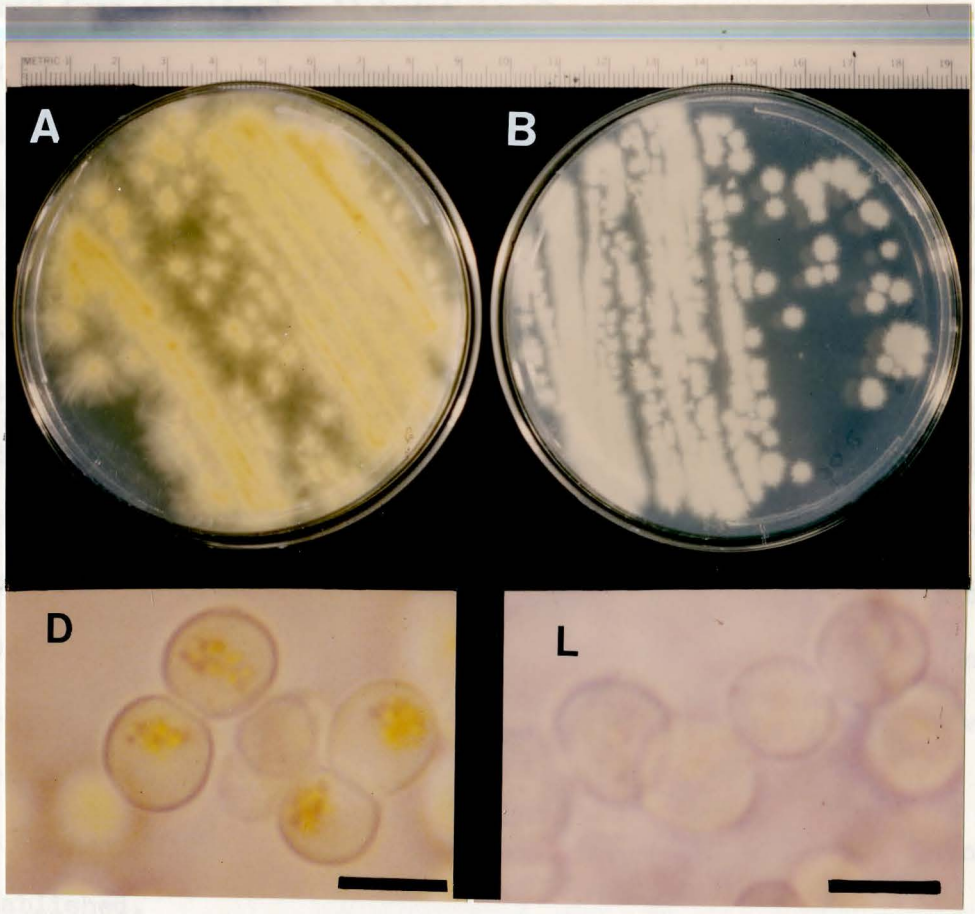
It was observed that cultures of arthrosporulating T. mentagrophytes which were exposed to continuous white light at 37°C contained considerably less pigment than their dark-grown counterparts (Fig. 4, A and B). Light micrographs of arthrospores from these cultures revealed no detectable pigment granules in irradiated arthrospores (Fig. 4, D and L). However, these illuminated arthrospores were found to contain intracellular lipid granules when stained with Sudan black B (data not shown). In the above study, intracellular pigment granules were not discernible during any stage of development under light.

Arthrospores of T. mentagrophytes formed at 39°C on SDA in the dark were almost completely devoid of carotenoids, and pigment granules were not observed. The development, shape, and germination rates of these spores, however, appeared normal (Table 4). At lower temperatures (25°C or 30°C), T. mentagrophytes hyphae had low rates of transformation into arthrospores, and carotenoids were not detected under the cultural conditions mentioned in Materials and Methods (Emyanitoff and Hashimoto, 1979; Mock and Hashimoto, unpublished data). Furthermore, a shift of arthrosporulating cultures, which as yet did not contain large amounts of pigment, from 37°C to 25°C or 30°C resulted in the eventual germination of many arthrospores, and pigment granules were absent.

2. Morphology of Pigment Granules in Situ

To further assess the effect of light on pigment granule formation, a study of granule development in situ during arthrosporulation was done by using both light microscopy and TEM. Several problems

Fig. 4. Effect of white light on pigmentation of T. mentagrophytes arthrospores. Plate A is an SDA culture of arthrospores grown in continual darkness for 8 days at 37°C. Plate B contains arthrospores formed in constant white light (1,000 lux) under otherwise identical conditions. Beneath each plate is a light micrograph of arthrospores harvested from the respective dark (D)- and light (L)-exposed cultures. Bars represent 5 µm.


A
B
D
L

were encountered in the ultrastructural investigation. Using the standard preservation techniques for whole cells mentioned in Materials and Methods, we observed considerable variation in pigment granule density (gray to black) in thin sections of unstained arthrospores prepared from identical cell batches. Because of the well-known difficulties associated with obtaining good preservation of fungi for TEM, a number of different fixation, dehydration, and embedding techniques were tested. The results of these experiments also indicated that, although pigment granules were usually of the same density in a particular sample, considerable variation existed in the degree of density from one batch of cells to another. A further obstacle in the elucidation of pigment granule ultrastructure was the observation that the conventional 10 min poststain with Reynold's lead citrate caused many of the granules to become altered, apparently through a loss of osmiophilic or electron dense material. In some samples, poststaining with lead for only 1 min was sufficient to at least partially remove the granule contents. The exact reason for variation of granule density in arthrospores through preservation or by staining could not be established.

In dark-grown arthrospores, pigment granules appeared during arthrospore septation. Since their initial color was barely discernible, cells were routinely stained with Sudan black B for light microscopy. This lipid specific stain formed dense deposits mainly at the pigment granule sites. It was observed that septation in many arthrosporulating cells occurred by division of the pre-existing cell into

approximate halves. In anticipation of cell division, clusters of pigment granules became localized at opposite poles of a cell compartment which was about to septate (Fig. 5A, P). Thin sections (stained) revealed that the pigment granules assumed roughly spherical shapes and were either gray or mottled (P in Fig. 5B). Although not shown, coalescence of granules occasionally occurred. Upon completion of septation, each arthrospore contained a single cluster of pigment granules within its cytoplasm (Fig. 6A, P). Electron micrographs of arthrospores at the same developmental stage demonstrated that the intracellular granules (P) were spherical and in close proximity to one another (Fig. 6B). In Fig. 6B, the density of pigment granules had not been altered by staining and probably reflects a more accurate example of a well-preserved cytoplasm. During arthrospore maturation, pigment granules formed a more compact cluster within the arthrospore cytoplasm (Fig. 7A, P). A surrounding membrane or envelope became apparent in those granules whose contents were partially lost by lead citrate staining (Fig. 7B, arrow).

When viewed at the ultrastructural level and by light microscopy with Sudan black B, no differences were observed in the size, shape, and position of lipid granules of light- and dark-grown arthrospores (data not shown). Although the difficulties in the ultrastructural interpretation of pigment granules <u>in situ</u> have been enumerated, the present observations suggest that light probably did not restrict the formation of lipid granules in arthrospores but rather suppressed the accumulation of pigment within them.

Fig. 5. In situ appearance of pigment granules of dark-grown T. mentagrophytes during arthrospore septation. A. Light micrograph of lipid-stained cells. B. Electron micrograph of thin-sectioned cells at the same period of development (3 days). Pigment granules (P) are located at both poles of a cell compartment prior to further septation. As a result of staining with lead citrate, granule contents in the thin section were partially lost. Bars represent 5 μm.

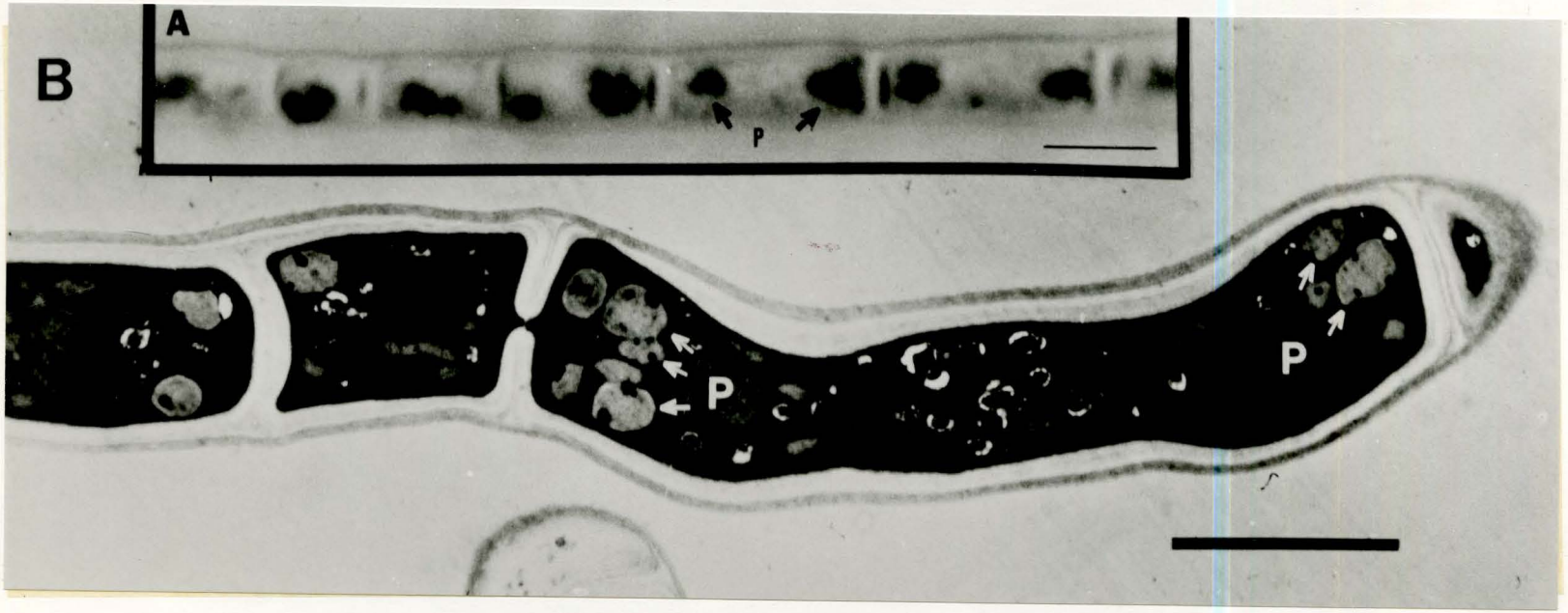

A
B
P
P
P

Fig. 6. *In situ* appearance of pigment granules of dark-grown *T. mentagrophytes* after completion of arthrospore septation. A. Light micrograph of lipid-stained cells. B. Electron micrograph of thin-sectioned arthrospores (5 days). Pigment granules (P) form a single aggregate in the spore cytoplasm. Note that the pigment granules in the thin section have not been altered by lead citrate staining. Bar in A represents 5 μm.

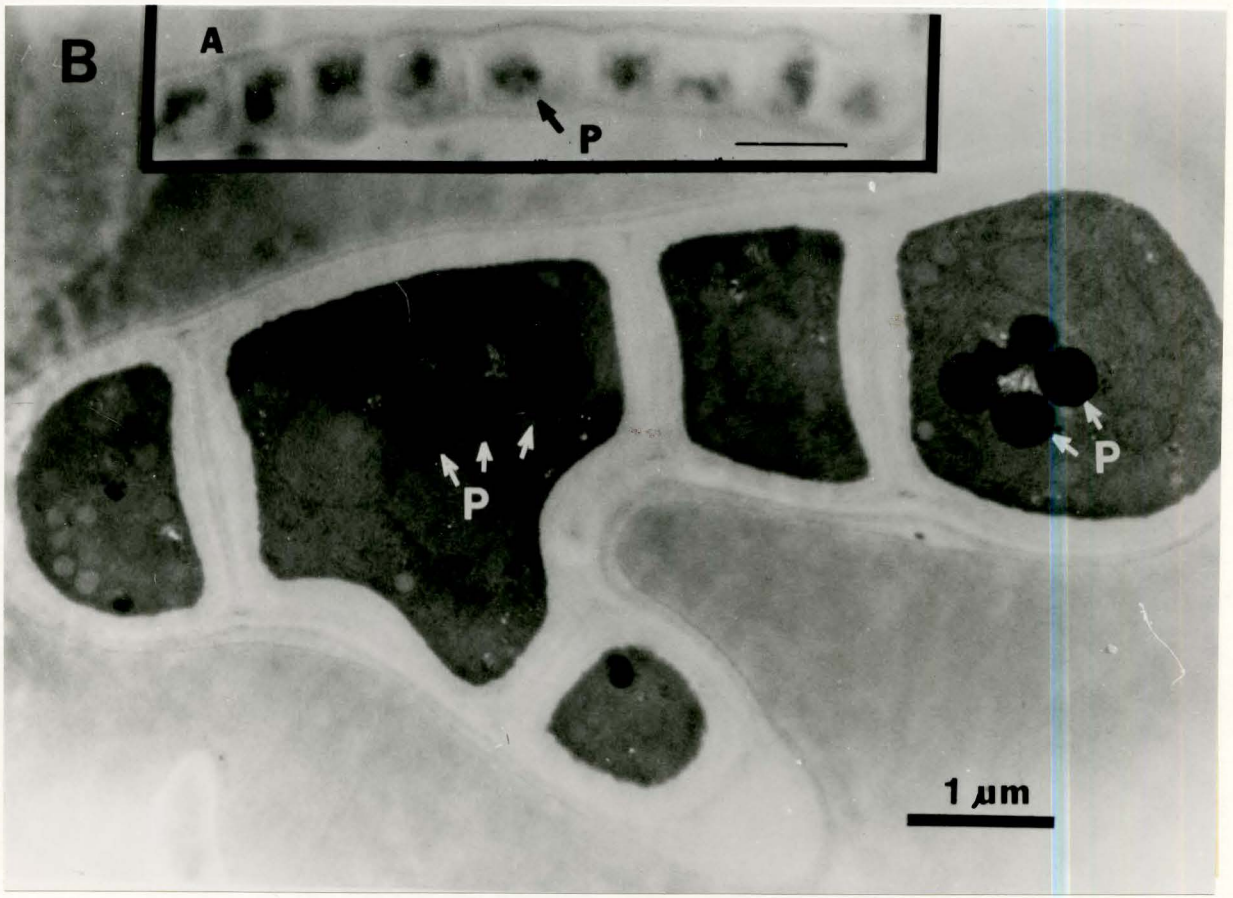
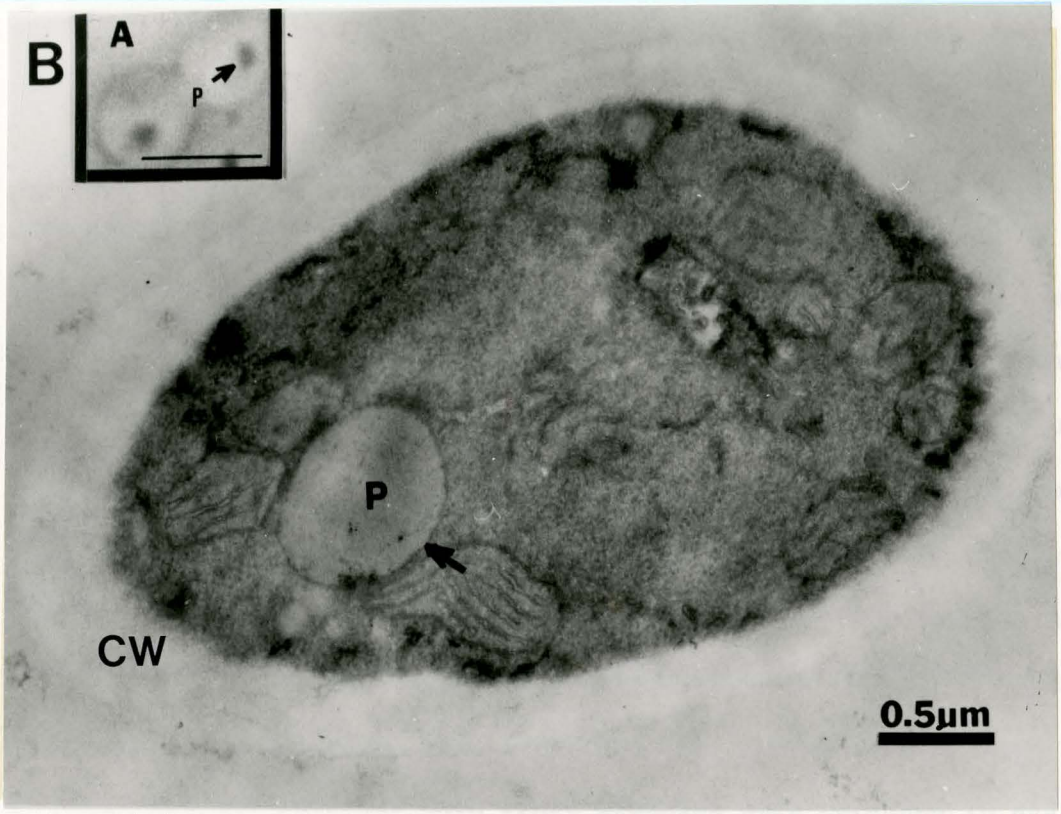

B
A
P
P
P
1 µm

Fig. 7. In situ appearance of pigment granules in mature arthrospores of T. mentagrophytes. A. Light micrograph of lipid-stained arthrospores. B. Electron micrograph of a thin-sectioned arthrospore (8 days, dark). Arrow indicates a single-layered membrane which surrounds a pigment granule (P) whose contents are almost completely lost after lead citrate staining. CW: cell wall. Bar in A represents 5 µm.

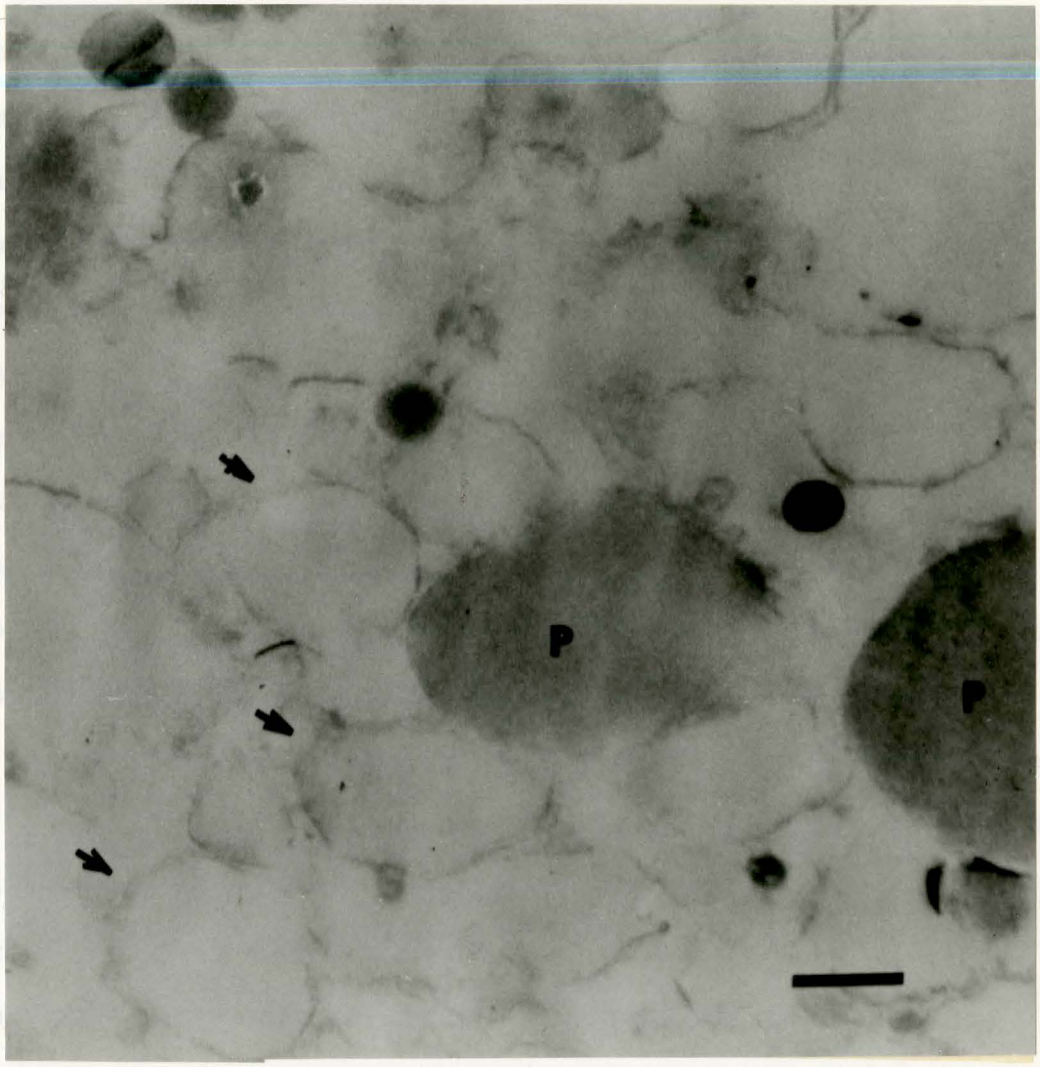

B
A
P
P
CW
0.5µm

3. Ultrastructure and Chemical Characterization of Isolated Pigment Granules.

As mentioned above, lead citrate altered the density of pigment granules in situ. Therefore, unstained thin sections of isolated pigment granules from arthrospores (8 days, dark) were initially examined. In preliminary studies, it was found that conventional whole cell preservation of lipid granule films yielded membranous structures of irregular and varied sizes. Since pigment granules appeared to contain large amounts of lipid as evidenced by their staining and sedimentation characteristics, it was likely that the standard acetone dehydration series of 1 h intervals caused a total loss of intragranular contents. If each dehydration step was reduced to 1 min, membranous ghosts still appeared, regardless of an acetone or a methanol-propylene oxide dehydration series (Fig. 8, arrows). In these preparations, however, some moderately electron dense granules were observed (Fig. 8, P). These granules appeared to be surrounded by a fragmented single-layered membrane. The number of granules with matrices did not increase when fixed granules were embedded in agar blocks before solvent dehdydration. These observations suggest that the isolated pigment granules from T. mentagrophytes arthrospores do not contain a large amount of unsaturated lipid, which would be highly crosslinked by osmium tetroxide, and consequently less likely to be solubilized during solvent dehydration.

To achieve better preservation of isolated pigment granules, an alternative method for water removal was used. In this procedure,

Fig. 8. Electron micrograph of isolated pigment granules which were rapidly dehydrated in acetone. Many empty membranous ghosts (arrows) are present. A few granules (P) are electron dense. This thin section is unstained. Bar represents 0.1 μm.


P
P

solvent dehydration was replaced by drying fixed granule films under vacuum with P_2O_5. As a result, pigment granules were not distorted and had moderately dense matrices (Fig. 9). Relatively minor amounts of membranous and other non-lipid contamination were noted in the isolated granule preparations. The majority of granules, which were roughly spherical, had diameters of less than 1 μm. In Fig. 9, several granules (P) appear to be undergoing fusion. Although not shown, a few granules had diameters which exceeded 1 μm and were larger than those observed in intact cells. These kinds of particles probably represent lipid granules which coalesced during cell breakage and separation. Unit or trilamellar membranes did not surround the pigment granules; however, a thin layer of slightly denser material coated the granule matrix (Fig. 9, arrows). Staining thin sections of isolated granules with Reynold's lead citrate for 10 min did not alter granule density (Figs. 10A and 10B).

From the above results, it is clear that there are similarities as well as differences in the electron microscopic properties of isolated and *in situ* pigment granules from mature arthrospores (8 day, dark). Their resemblance with respect to size, shape, and possible membrane enclosure became apparent only when fixed pigment granule films were dried by vacuum desiccation before embedding in resin.

The contents of isolated pigment granules (fixed) were easily removed by solvent dehydration even though several protective measures were used for lipid retention. In contrast, unstained pigment granules *in situ* appeared to retain most of their lipid matrix. This retention

Fig. 9. Thin section of unstained pigment granules which were dried under vacuum. Some of the granule membranes appear fragmented (arrows). Several granules (P) seem to be undergoing fusion. Bar represents 0.1 μm.

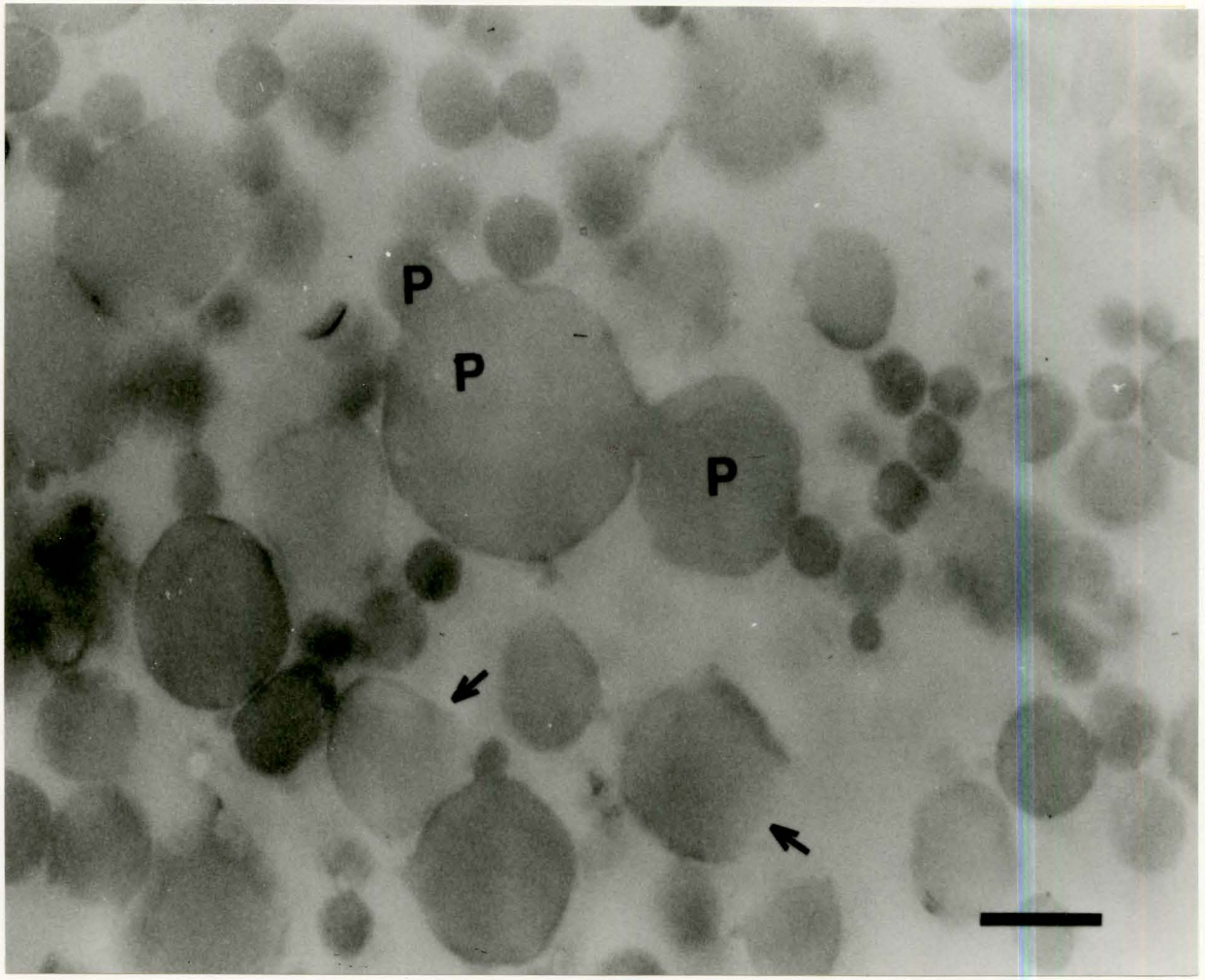
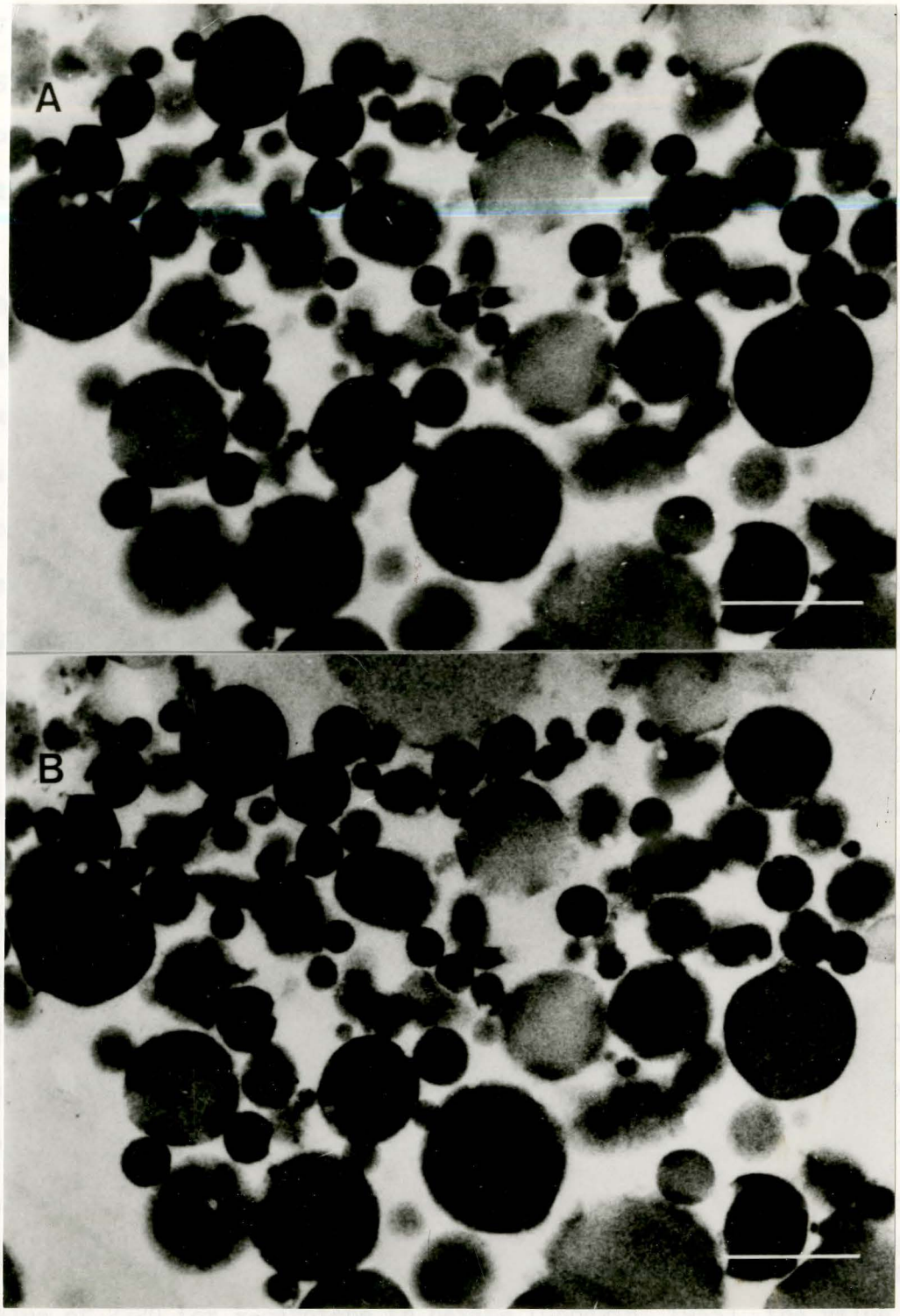

P
P
P

Fig. 10. Effect of alkaline lead citrate staining on the density of thin-sectioned pigment granules. A. Before staining. B. After staining. Bar represents 0.5 µm.


A
B

is probably due to an increased stabilization of granules within the cytoplasmic milieu. However, a reduced penetration of preservative agents through the relatively thick arthrospore wall cannot be discounted. Inadequate cell preservation may also account for the density alteration of granules stained with alkaline lead citrate.

Analyses of isolated granules from mature, pigmented arthrospores are presented in Table 1. All data are given as percentage values derived from mg quantities of isolated granules. As shown in Table 1, pigment granules were composed mainly of solvent extractable lipid with smaller amounts of carbohydrate and protein. These major components accounted for approx 100% of the granule dry wt. The granules contained relatively small amounts of ergosterol and phosphate (probably phospholipid), further suggesting an absence of contaminating membrane fragments.

The floating lipid layer of isolated pigment granules had an orange color. The visible absorption spectrum of extracts (in hexane) from the granules closely matched that obtained from corresponding whole arthrospores (Fig. 11). In addition, thin-layer chromatograms of total carotenoids from both extracts revealed that isolated granules contained the same carotenoids in approx equal proportions as whole cells (Fig. 11, insert). (For a detailed account of individual carotenoids isolated from T. mentagrophytes, see Fig. 18.) The carotenoid content of isolated granules was determined from the amount of γ-carotene, the predominant carotenoid of arthrospores. Based on the $E^{1\%}_{1cm}$ value of 2720 for γ-carotene in hexane (Goodwin, 1955), the value of

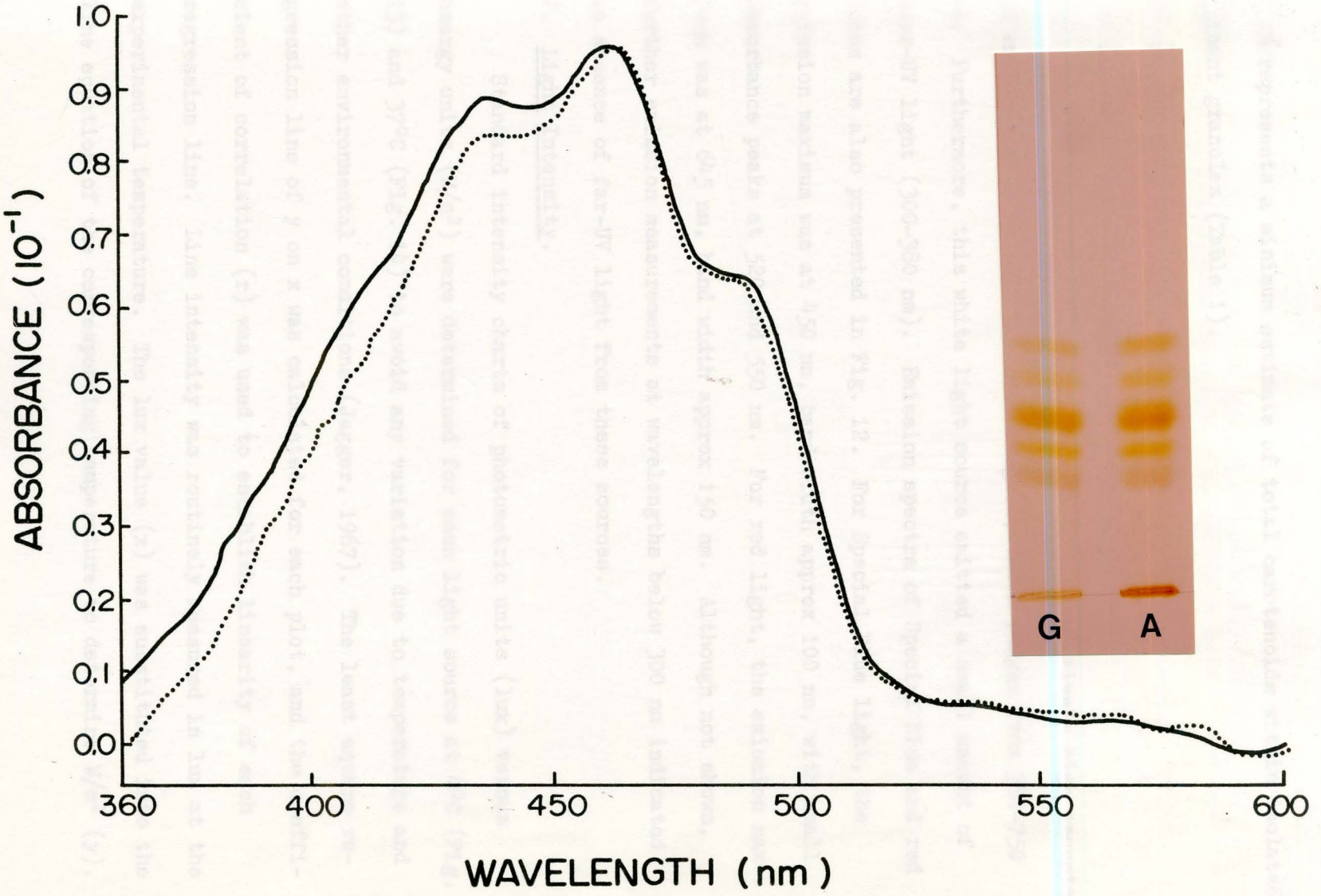
Table 1. Chemical composition of isolated carotenoid-containing granules from mature, pigmented arthrospores of T. mentagrophytes.

Component	Amount (%)[a]
Protein	7.2[b]
Sugar (as glucose)	11.9
Phosphorous	0.2
Lipid	81.5
Sterol (as ergosterol)	0.7
Carotenoids (as γ-carotene)	0.2

[a]Composition is expressed as % granule dry wt.

[b]Each value is the mean of at least duplicate samples.

Fig. 11. Absorption spectra (in hexane) of carotenoids extracted from isolated (·····) pigment granules and dark-grown (———) arthrospores (8 days old). Spectra were normalized at the maximum peak of absorption. Insert is a thin-layer chromatogram of individual carotenoids from each sample. Symbols: G, isolated pigment granules; A, whole arthrospores.


ABSORBANCE (10^{-1})
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
360
400
450
500
550
600
WAVELENGTH (nm)
G
A

0.2% represents a minimum estimate of total carotenoids within isolated pigment granules (Table 1).

B. Determination of Lighting Condition.

1. Emission Spectrum of Individual Light Source.

As shown in Fig. 12, Cool White tubes had relatively high amounts of emission throughout the visible region, which ranges from 380-750 nm. Furthermore, this white light source emitted a small amount of near-UV light (300-380 nm). Emission spectra of Special Blue and red tubes are also presented in Fig. 12. For Special Blue light, the emission maximum was at 450 nm, band width approx 100 nm, with small absorbance peaks at 520 and 550 nm. For red light, the emission maximum was at 645 nm, band width approx 150 nm. Although not shown, further emission measurements at wavelengths below 300 nm indicated an absence of far-UV light from these sources.

2. Light Intensity.

Standard intensity charts of photometric units (lux) versus energy units (W/m^2) were determined for each light source at 4°C (Fig. 13) and 37°C (Fig. 14) to avoid any variation due to temperature and other environmental conditions (Jagger, 1967). The least square regression line of y on x was calculated for each plot, and the coefficient of correlation (r) was used to establish linearity of each regression line. Line intensity was routinely measured in lux at the experimental temperature. The lux value (x) was substituted into the line equation of the corresponding temperature to determine W/m^2 (y).

Fig. 12. Spectral power distribution of lamps. Emission spectrum of Cool White fluorescent light courtesy of General Electric Co., 1970. The intensities of Special Blue and red fluorescent light sources were determined as described in Materials and Methods. Relative intensities are plotted on a normalized scale and are, therefore, not comparable.

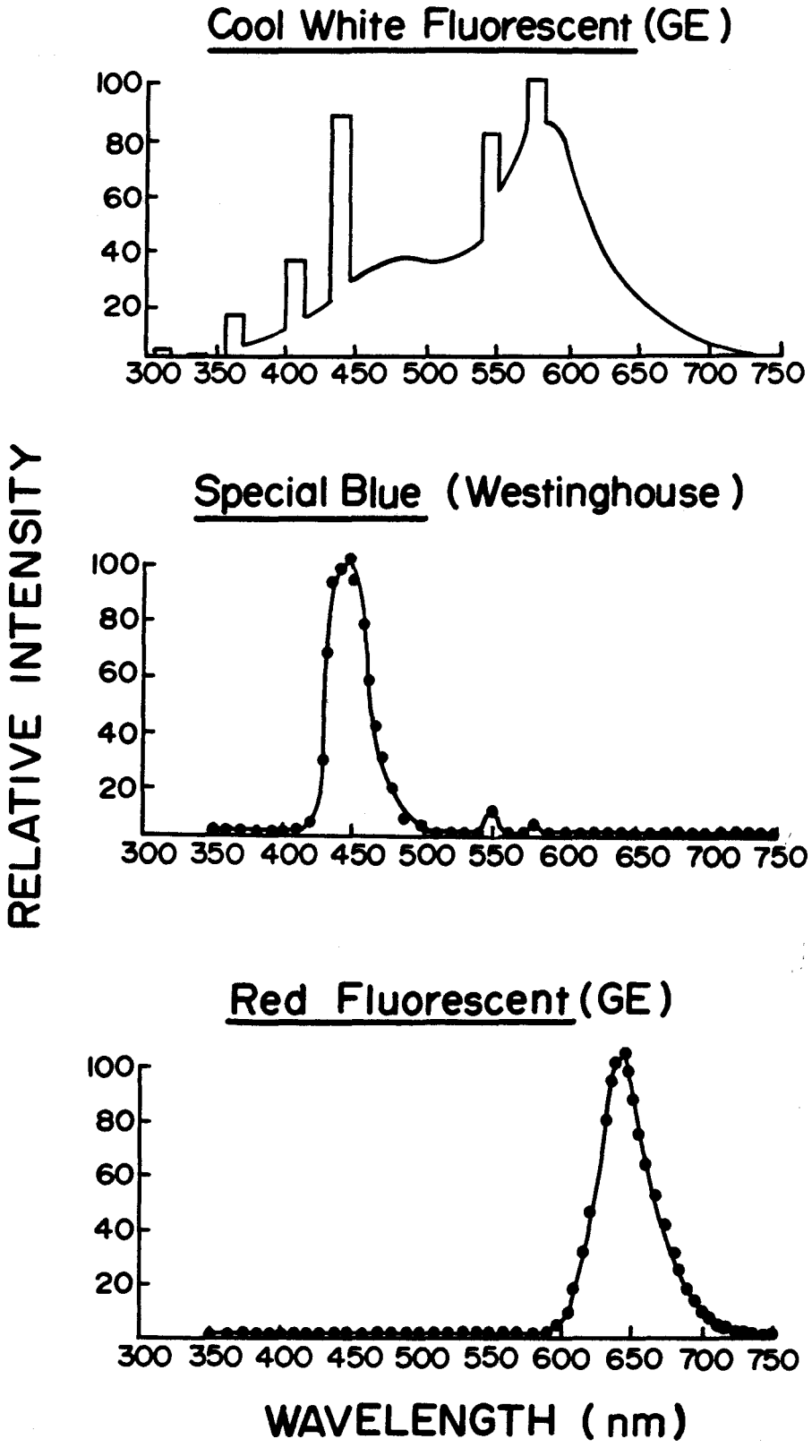

Cool White Fluorescent (GE)
100
80
60
40
20
300 350 400 450 500 550 600 650 700 750
Special Blue (Westinghouse)
100
80
60
40
20
300 350 400 450 500 550 600 650 700 750
RELATIVE INTENSITY
Red Fluorescent (GE)
100
80
60
40
20
300 350 400 450 500 550 600 650 700 750
WAVELENGTH (nm)

Fig. 13. Standard curves correlating illuminance (lux) with irradiance (W/m^2) of fluorescent tubes at 4^oC. Each point is the avg of 2 values. The slope and y-intercept of each line were determined by the method of least squares. Coefficient of correlation = r. Symbols: ●, Special Blue; △, red; x, Cool White.

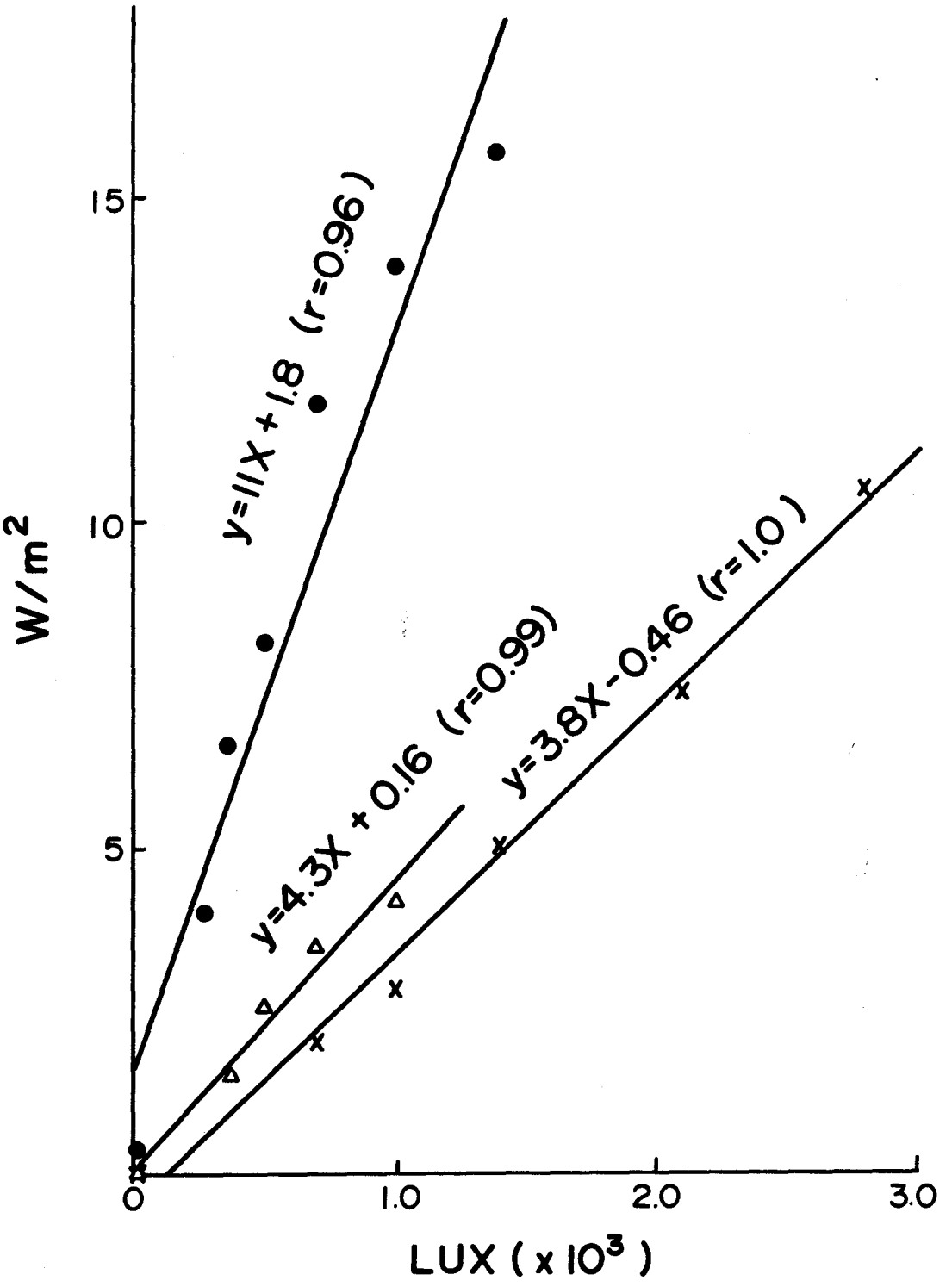
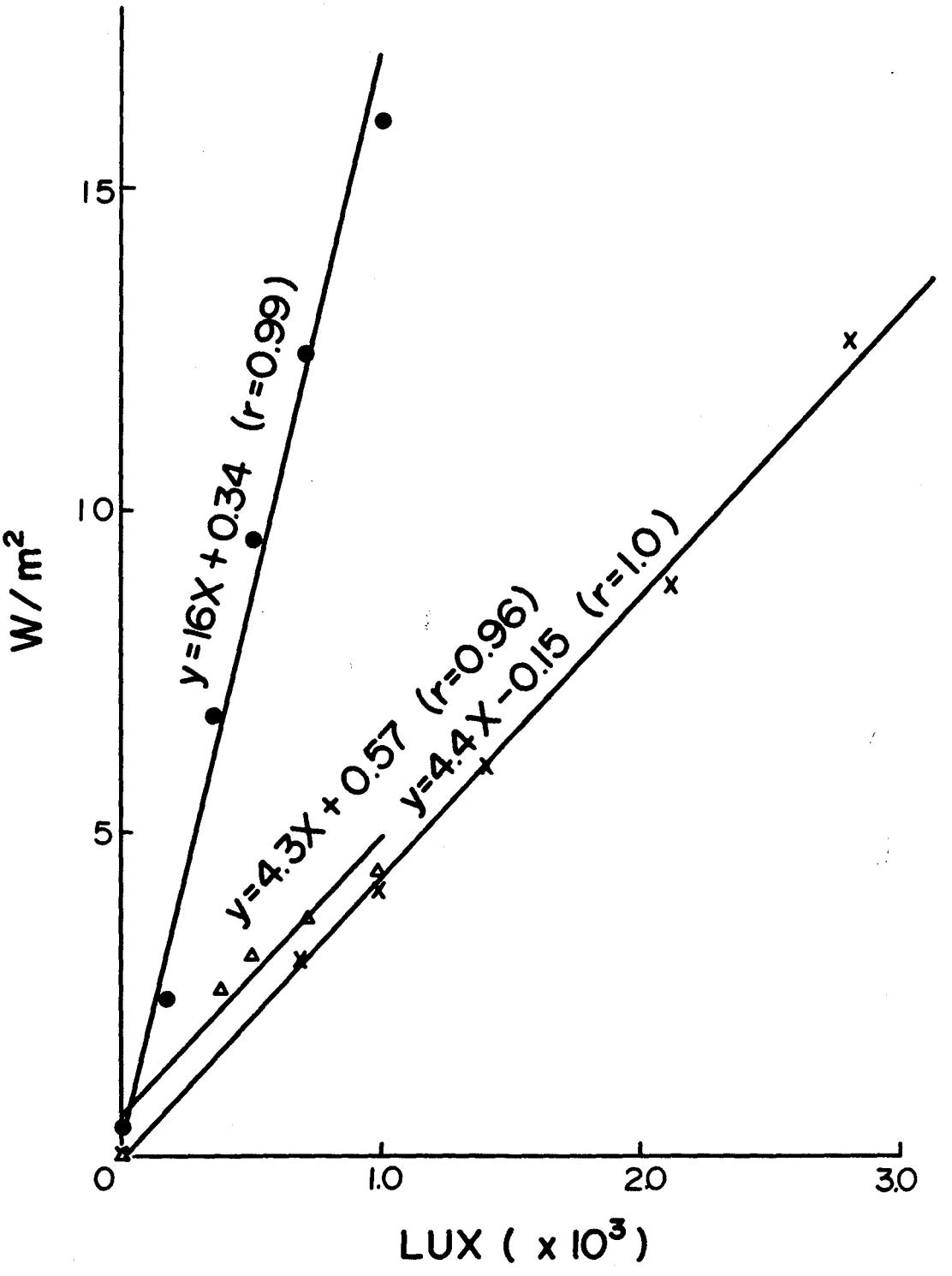

W/m²
15
10
5
0
1.0
2.0
3.0
LUX (x 10³)
y=11X + 1.8 (r=0.96)
y=4.3X + 0.16 (r=0.99)
y=3.8X-0.46 (r=1.0)

Fig. 14. Standard curves correlating illuminance (lux) with irradiance (W/m^2) of fluorescent tubes at 37°C. Each point is the avg of two values. The slope and y-intercept of each line were determined by method of least squares. Coefficient of correlation = r. Symbols: ●, Special Blue; △, red; x, Cool White.


W/m²
15
10
5
0
1.0
2.0
3.0
LUX (x 10³)
y=16X+0.34 (r=0.99)
y=4.3X+0.57 (r=0.96)
y=4.4X-0.15 (r=1.0)

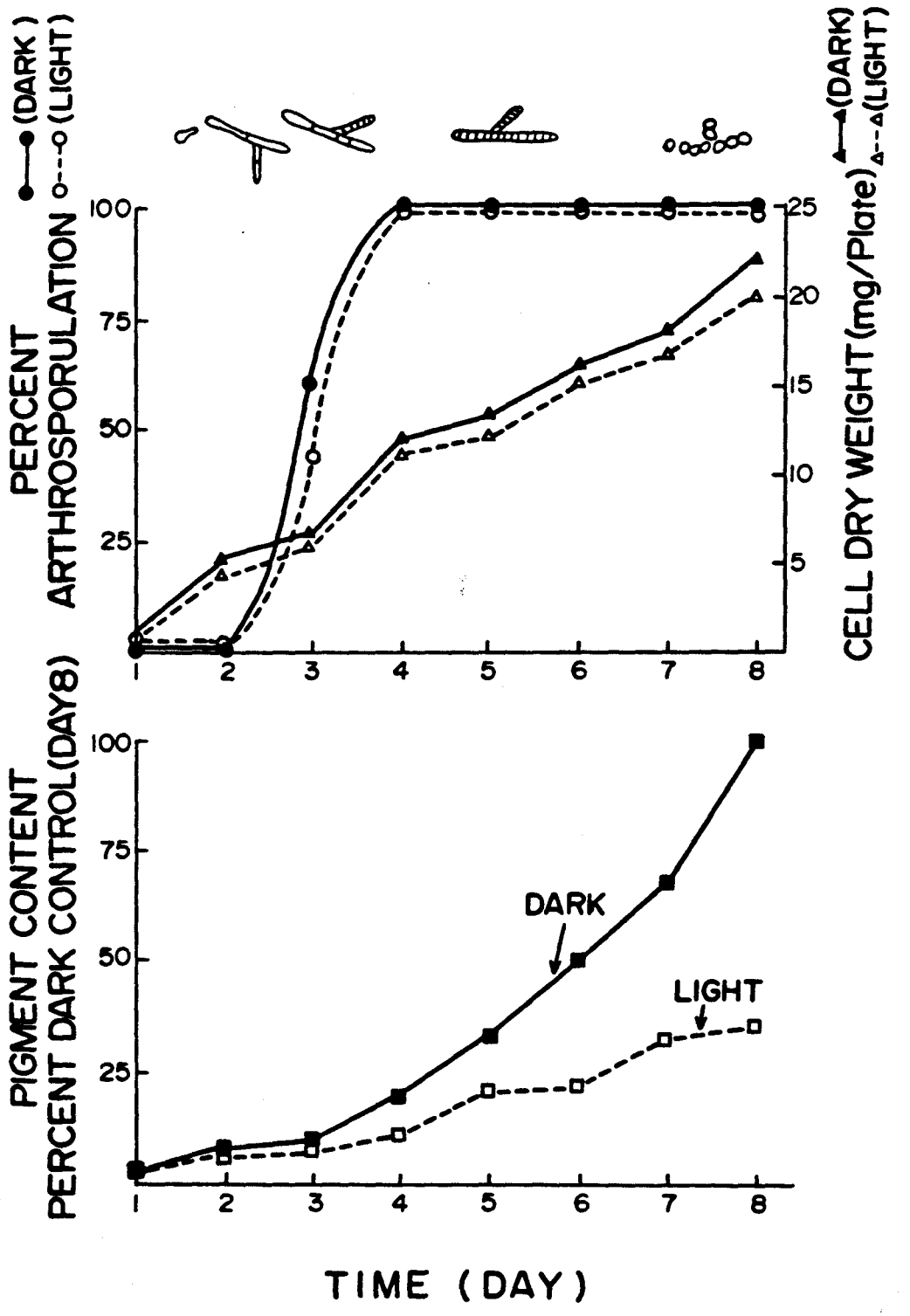
C. Visible Light Effect on Carotenoid Accumulation in T. mentagrophytes Undergoing Arthrosporulation on SDA-acetate.

1. Effect of Continuous White Light.

To characterize the light-promoted reduction of carotenoid accumulation in T. mentagrophytes, several other parameters in addition to pigment content were examined for sensitivity to visible light. Fig. 15 is a representative time course study of the effect of continuous white light (1,000 lux) on arthrosporulation, growth (dry wt), and carotenoid accumulation throughout hyphal and arthrospore development at 37°C. Under the conditions of this study, continuous irradiation of T. mentagrophytes under white light did not appreciably alter the rate of arthrosporulation in comparison with dark controls. At the end of 8 days, virtually all the hyphae were converted to arthrospores. With the exception of pigment granules, there were also no major differences in the light microscopic appearance of irradiated and dark-grown cells of equal ages throughout the period of investigation (data not shown). Consequently, the stages of development which are schematically depicted at the top of Fig. 15 represent either lighting condition.

Irradiation of T. mentagrophytes under continuous white light did not affect either hyphal growth or the additional dry wt increase (primarily due to inner wall thickening) associated with the development of mature arthrospores (Fig. 15). In continual darkness, T. mentagrophytes began to accumulate carotenoids after the onset of arthrosporulation, and pigmentation continually increased throughout

Fig. 15. Effect of continuous white light on carotenoid accumulation, growth, and arthrosporulation of T. mentagrophytes at 37°C. To permit maximal microconidial germination, all cultures were grown in the dark on SDA-acetate for 24 h at 37°C. At day 1, one-half of the cultural population was exposed to continuous white light (1,000 lux, 4 W/m^2), while the other half remained in continuous darkness. Light- and dark-exposed cultures were harvested and assayed for the above parameters as described in Materials and Methods. For dry wt and pigment determinations, each point is the avg of 10 plates. The developmental stage of T. mentagrophytes is indicated at the top of the Fig. Symbols: △------, mg dry wt/plate in light; ▲———, mg dry wt/plate in darkness; o------, % arthrosporulation in light; ●———, % arthrosporulation in darkness; □------, relative pigmentation/plate in light; ■———, relative pigmentation/plate in darkness.


PERCENT ARTHROSPORULATION
●—● (DARK)
o---o (LIGHT)
CELL DRY WEIGHT (mg/Plate)
▲—▲(DARK)
△--△(LIGHT)
PIGMENT CONTENT
PERCENT DARK CONTROL(DAY8)
DARK
LIGHT
TIME (DAY)

arthrospore maturation, reaching a maximum level at 8 days. After this period, arthrospores still continued to accumulate carotenoids; however hyphal variants emerged and rapidly overgrew the culture. The accumulation of carotenoids in irradiated spores was initiated at approx the same time as in the dark. In contrast, pigmentation of light-exposed cells was always reduced and did not appear to increase continually throughout the duration of irradiation.

Since the carotenoid content of cells is based on both dry wt and the absorbance of the total carotenoid extract, pigmentation increase as a function of time must be considered a conservative estimate in cases where there is also a concomitant increase in cellular dry wt. Therefore, small increases in carotenoid content within sporulating cultures might go undetected, and it is possible that the amount of carotenoids in irradiated arthrospores might steadily increase during development, albeit at low levels.

It is important to point out that arthrospores do not uniformly accumulate high levels of carotenoids on SDA unless they are formed from several fairly uniform layers of hyphae. In the present study, relatively thin layers of arthrospores developing on dialysis membranes were used for light studies. Because of the spore wall thickness and possible shading effect of the pigments themselves, it is therefore likely that each successive layer of cells received a slightly lower intensity of light.

At the end of 8 days, mature arthrospores which were formed under constant irradiation by white light (4 W/m^2) contained approx

65% less pigment than corresponding dark controls (Fig. 15). Although the colored carotenoid content of irradiated spores was considerably reduced, only minor differences were observed in the visible-light absorption spectra of total carotenoid extracts (in hexane) from light-exposed and dark-grown arthrospores (8 day) (Fig. 16). Both extracts exhibited absorption maxima at 410 (shoulder), 437, 460 (maximum peak), and 485 nm (shoulder). Derivative spectra which often enhance the resolution of an absorption spectrum can also discriminate sharper features which may be masked. Second derivative spectra of the pigment extracts (in hexane) from above (Fig. 16) suggested no major qualitative differences existed in the total carotenoids of either sample within the visible-light wavelength region (Fig. 17). Some variation, however, was noted in the near-UV range of 360-380 nm. Thin-layer chromatographic and spectroscopic properties of isolated carotenoids from light-exposed and dark-grown arthrospores (day 8) are shown in Fig. 18. The same colored carotenoids, originally identified by Hashimoto et al., 1978, were demonstrated in both types of spores. Based on the relative size of each pigment spot, colored carotenoids appeared to be present in approx the same ratio under light or darkness. Although not shown, there were no differences in the relative quantities of the colorless carotenoids phytoene (R_f 0.51; absorption peaks, 277, 286, 297) and phytofluene (R_f 0.45; absorption peaks, 330, 347, 368). In addition, the chromatographic data suggested that γ-carotene was the predominant carotenoid within arthrospores.

When considered together, the spectroscopic and chromatographic

Fig. 16. Absorption spectra (in hexane) of carotenoids extracted from arthrospores (8 days) formed under continuous white light (------) or in darkness (———). Carotenoids were extracted from equal quantities of cells (7 mg) for each sample. The intensity of the light source used to illuminate cultures was 1,000 lux (4 W/m^2).

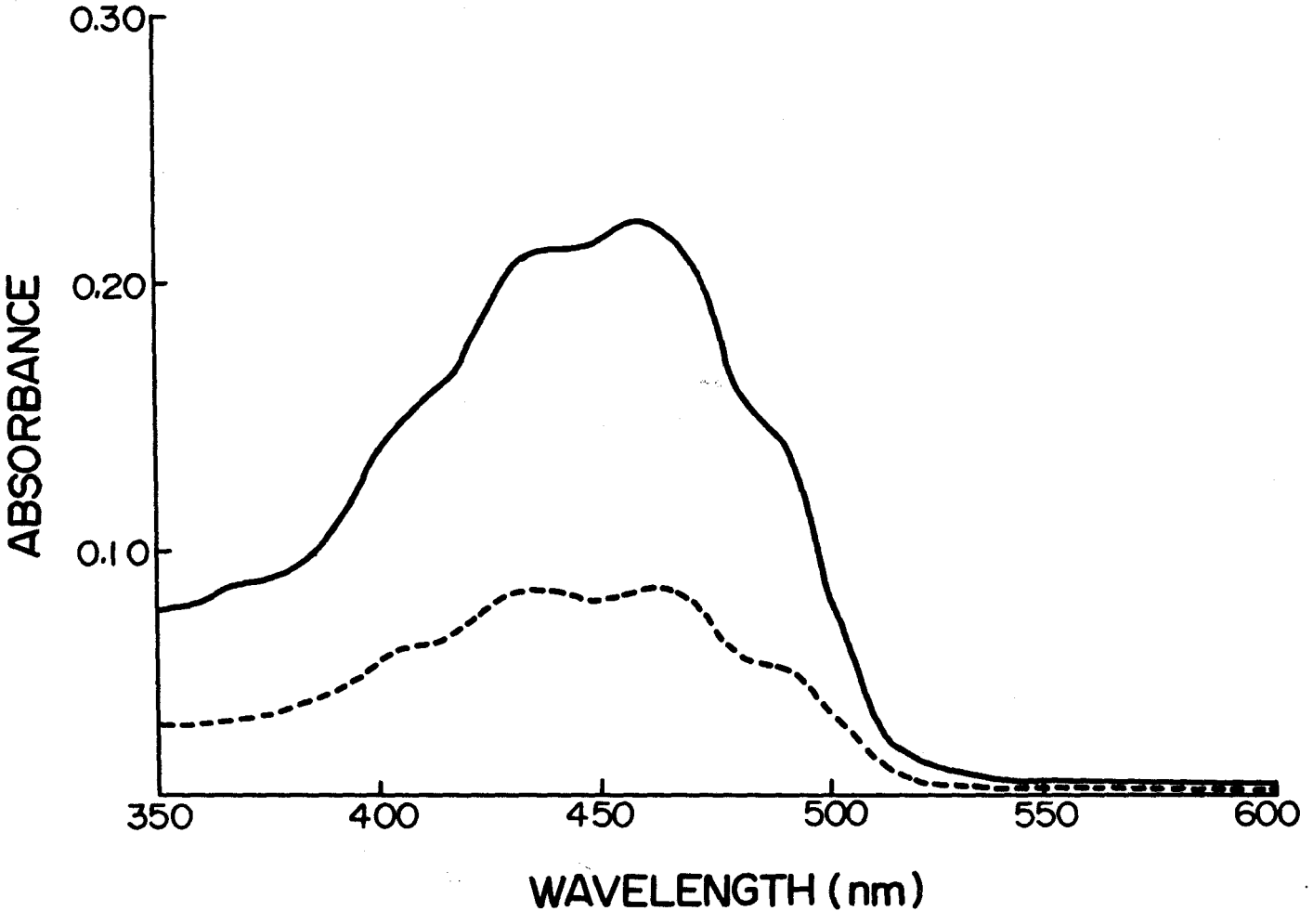

0.30
0.20
0.10
ABSORBANCE
350
400
450
500
550
600
WAVELENGTH (nm)

Fig. 17. Second derivative spectra of carotenoids extracted from light- (------) and dark-grown (———) arthrospores (8 days). The intensity of white light used to continuously illuminate cultures was 1,000 lux (4 W/m^2). Scan speed of spectrophotometer = 120 nm/min.

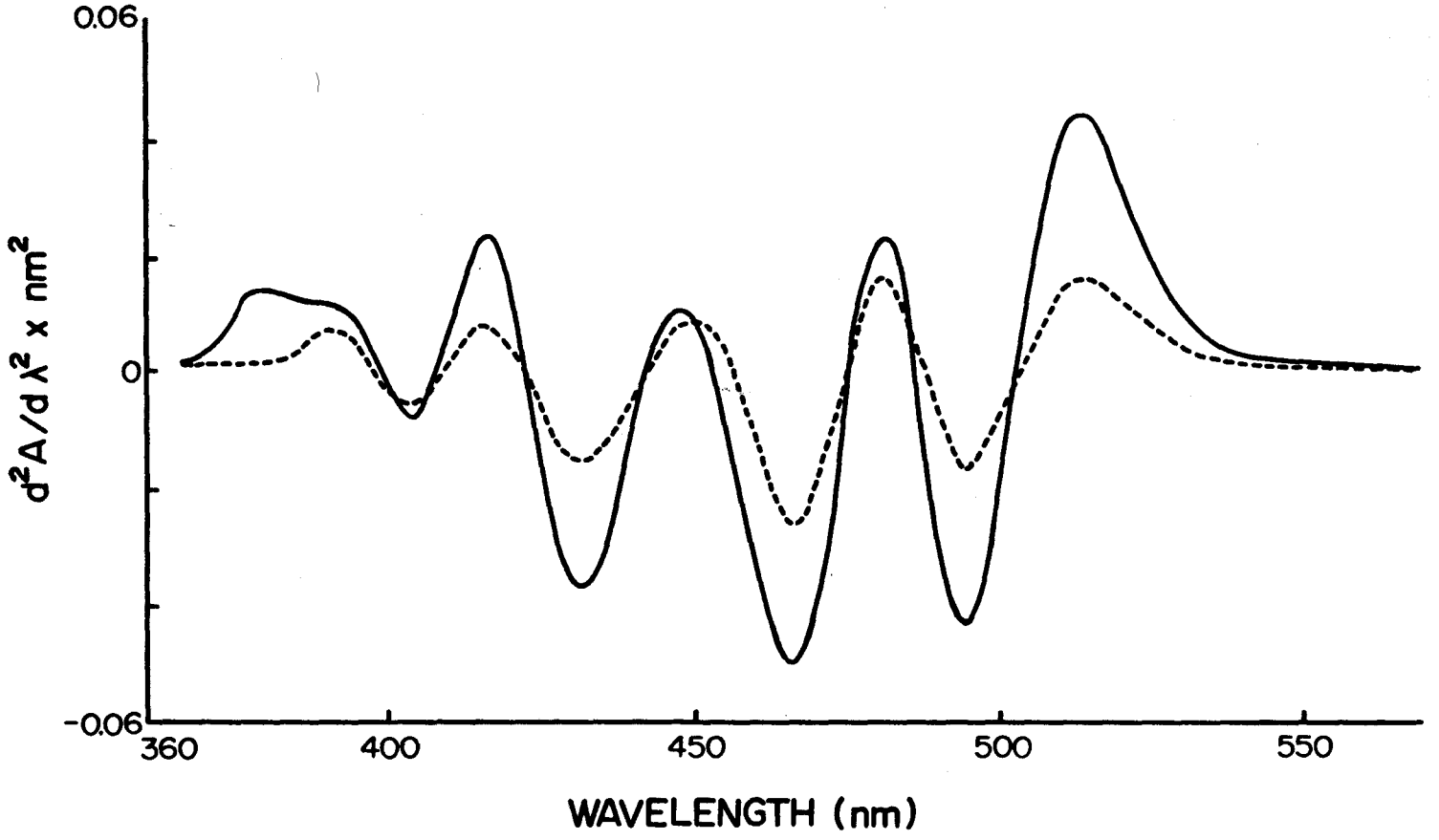
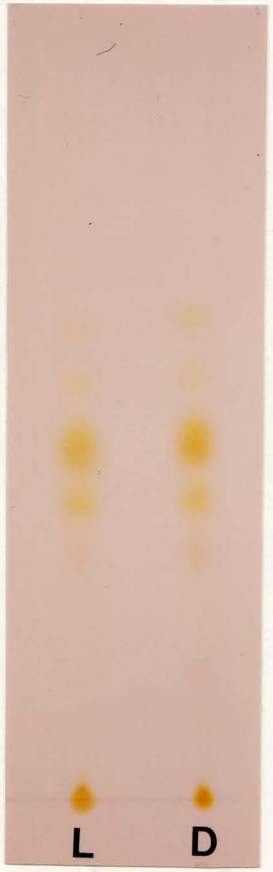

0.06
0
-0.06
$d^2A/d\lambda^2 \times nm^2$
360
400
450
500
550
WAVELENGTH (nm)

Fig. 18. Thin-layer chromatographic and spectroscopic characteristics of colored carotenoids isolated from saponified epiphase fractions of light- (L) and dark-grown (D) arthrospores (8 days). The intensity of white light used to continuously illuminate cultures was 1,000 lux (4 W/m^2). Chromatogram (thin-layer plate of silica gel 60) was developed in hexane-benzene (10:1.5) at 25°C in the dark. The major absorption peak of each carotenoid is underlined.



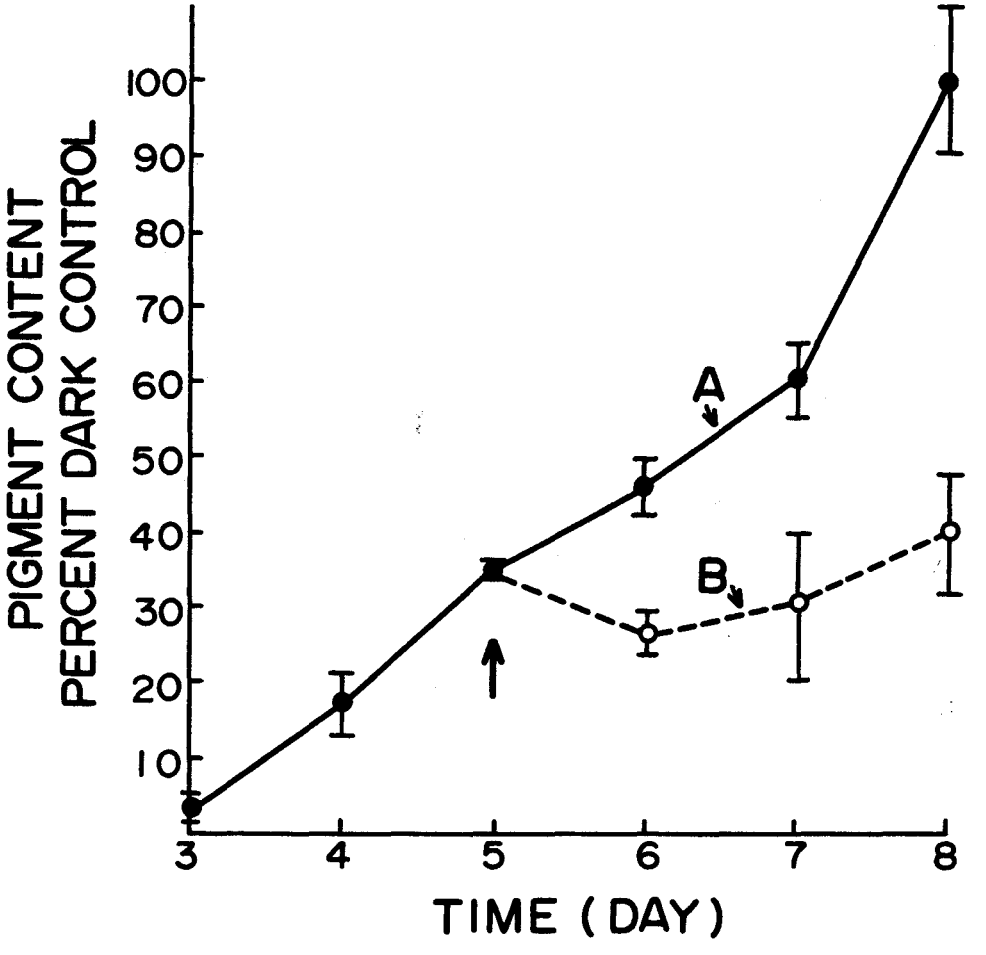
Color	R_f	Absorption maxima in hexane (nm)	Identification
orange	0.49	428, <u>450</u>, 477	β-carotene
yellow	0.44	426, <u>446</u>, 467	unidentified spot
orange	0.38	432, <u>458</u>, 488	γ-carotene
yellow	0.31	417, <u>437</u>, 467	neurosporene
pink	0.25	444, <u>469</u>, 498	lycopene
orange		404, <u>424</u>, 445	unidentified spot

results of above indicate that white light (4 W/m^2) causes quantitative, rather than qualitative, differences in the carotenoids of arthrosporulating T. mentagrophytes. Although continuous white light suppresses the amount of carotenoid accumulation in arthrospores, neither growth nor arthrosporulation are affected. From the data obtained, it is difficult to deduce the mechanism which is responsible for this light-promoted pigment reduction in arthrospores. It is clear, however, that light could act as an inhibitor of carotenogenesis and/or as a destroyer of synthesized pigments.

2. Dark to White Light Shift.

When irradiation with white light (4 W/m^2) was initiated during arthrospore maturation (day 5, 6, 7, or 8), when carotenoids were already being synthesized, pigment accumulation was also suppressed. In Fig. 19, cultures of dark-grown arthrospores (5 day) were transferred to white light (4 W/m^2) and continuously irradiated for an additional 3 days (day 5-8). In comparison with dark controls (Fig. 19A), the pigment content of light-exposed arthrospores (Fig. 19B) was significantly reduced after 24 h of irradiation. Arthrosporulation and growth were not affected by light. Beyond 24 h, pigmentation in irradiated spores remained relatively low, while carotenoid levels of spores in continous darkness increased considerably. Therefore, the pigmentation differences between light- and dark-grown spores became larger as irradiation progressed. In conjunction with Fig. 15, these results also suggest that white light suppresses carotenoid accumulation throughout arthrosporulation.

Fig. 19. Suppression of carotenoid accumulation by a dark to white light shift during arthrosporulation in T. mentagrophytes at 37°C. A. Dark. B. Light. Arrow indicates time of transfer to white light (1,000 lux, 4 W/m^2). Each point is the mean of 2 experiments, each done in duplicate (± SE), and represents % PI of dark control at day 8.


PIGMENT CONTENT
PERCENT DARK CONTROL
100
90
80
70
60
50
40
30
20
10
A
B
3
4
5
6
7
8
TIME (DAY)

3. Release of Light-mediated Suppression.

The suppressive effect of white light on carotenoid accumulation in arthrospores was reversible. Fig. 20 illustrates that irradiated cultures (6 day), which were removed from light and incubated in the dark for an additional 2 days, had a significant increase in the amount of carotenoid accumulation when compared with continuously irradiated arthrospores of the same age. Based on these results, it appears that white light does not cause a permanent alteration in those processes which are involved with carotenoid accumulation.

4. Effect of Intensity of White Light.

To determine whether suppression of carotenoid accumulation was dependent upon light dosage, arthrosporulating cultures were continuously irradiated (day 3-8) with various intensities of white light. Fig. 21 is a representative study which illustrates the effect of different intensities of white light on carotenoid accumulation in arthrospores at 8 days. The results of the same experiment are expressed in two ways in Fig. 21: in the insert, light intensity was measured in lux units; whereas, in the major figure, energy measurements (W/m^2) were used. Regardless of the type of intensity measurement, the data clearly indicate that an inverse relationship exists between pigment content and light intensity extending to 32 W/m^2 (7,200 lux). Computer analysis of the data by nonlinear regression further demonstrates that the suppressive effect of different light intensities is exponentially related to the amount of carotenoid accumulation as defined by the best fit equation:

Fig. 20. Release of light-mediated suppression of carotenoid accumulation in arthrosporulating T. mentagrophytes at 37°C. Arrow indicates time of a white light to dark shift. Each point is the mean of 3 values (± SE) and represents % PI of dark control at day 8. Symbols: ●———, continual darkness; □------, continual white light (1,000 lux, 4 W/m^2); ■———, white light (1,000 lux) from days 3-6 and dark from days 6-8.

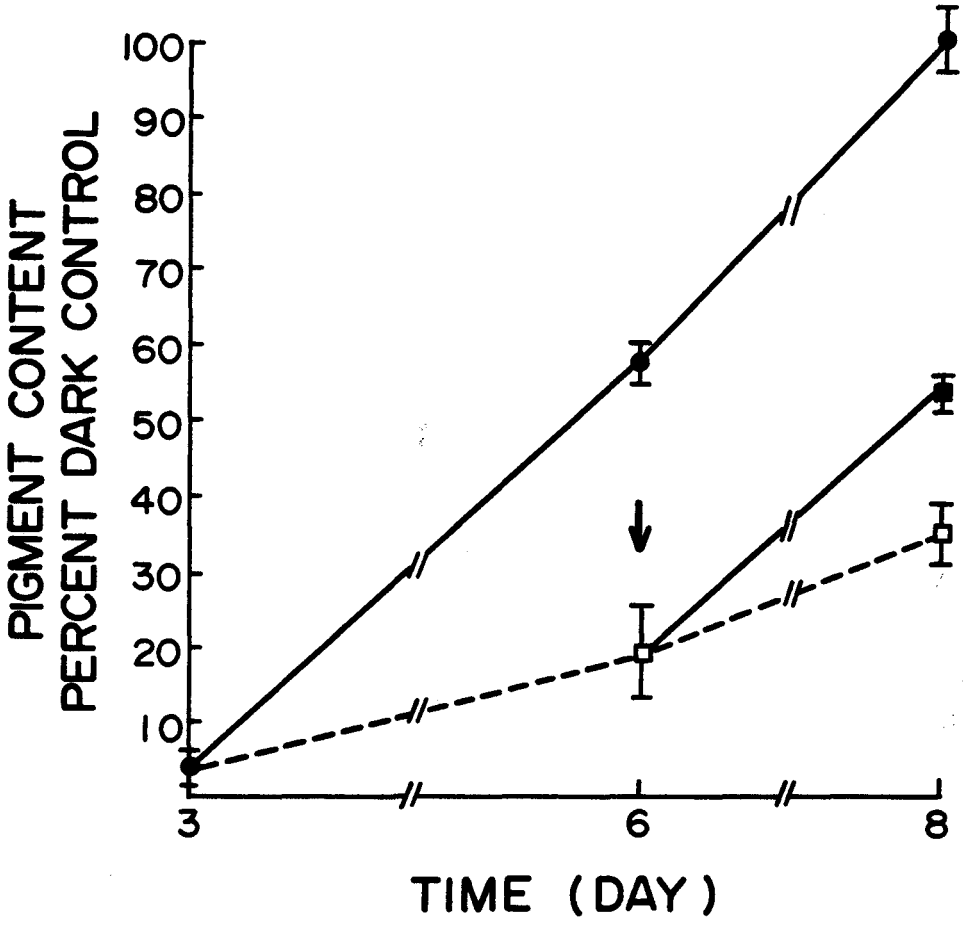
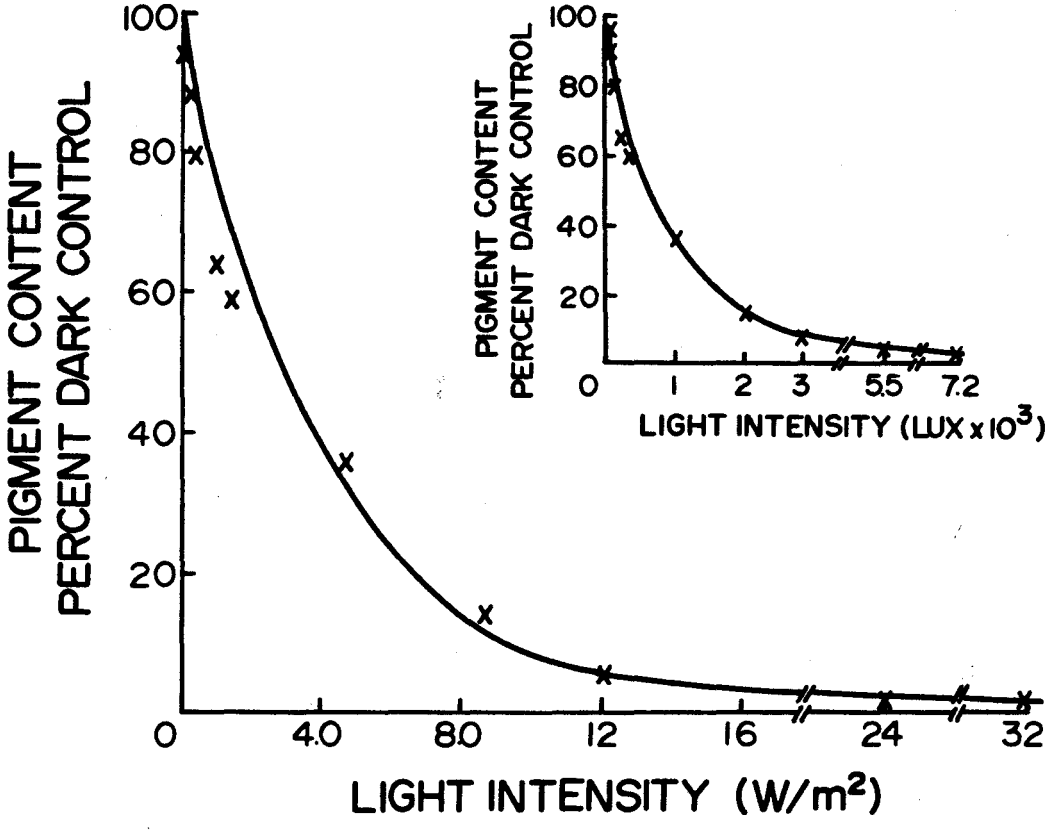

PIGMENT CONTENT
PERCENT DARK CONTROL
100
90
80
70
60
50
40
30
20
10
3
6
8
TIME (DAY)

Fig. 21. Effect of intensity of white light on carotenoid accumulation in T. mentagrophytes arthrospores at 37°C. Cultures were grown in the dark for 3 days and then continuously irradiated with a specific intensity of white light for an additional 5 days. Data points represent % PI of the respective dark control at day 8. Insert is a plot of the same data except light intensity has been converted from W/m^2 to lux.


PIGMENT CONTENT
PERCENT DARK CONTROL
100
80
60
40
20
0
4.0
8.0
12
16
24
32
LIGHT INTENSITY (W/m²)
PIGMENT CONTENT
PERCENT DARK CONTROL
100
80
60
40
20
0
1
2
3
5.5
7.2
LIGHT INTENSITY (LUX x 10³)

$$y = 90\ e^{-0.23x} \quad (r = 0.99)$$

where y = pigmentation (% dark control), x = intensity of white light (W/m^2), and e = base of ln.

In comparison with dark controls, neither growth nor arthrosporulation were affected at the light intensities employed. It is interesting to note that even under the lowest intensity attainable in this study, white light suppressed carotenoid accumulation in T. mentagrophytes. Furthermore, pigmentation was almost completely absent in light-exposed arthrospores when the intensity of white light exceeded 24 W/m^2 (Fig. 21).

5. Is Carotenogenesis Photoinducible in T. mentagrophytes?

Even though a variety of cultural conditions were tested, carotenoids were not detected in the hyphae of T. mentagrophytes. It is likely that carotenogenesis was normally repressed or noninduced during this developmental stage; however, it is also possible that the exact physiological requirements for pigmentation were not satisfied. Brief periods of irradiation with white light (4 W/m^2) had no effect upon carotenoid accumulation in resulting arthrospores (Table 2). Thus, unlike Neurospora or Phycomyces, carotenogenesis in T. mentagrophytes is not photoinduced or stimualted by visible light.

6. Effect of Different Intensities of Blue and Red Light.

Many fungal photoresponses including photoinduction of carotenogenesis are mediated by visible light from the blue wavelength region. In order to better characterize light-mediated suppression of carotenogenesis in T. mentagrophytes, the effects of different wavelengths of

Table 2. Effect of irradiation of hyphae on subsequent carotenoid accumulation during arthrosporogenesis of T. mentagrophytes.

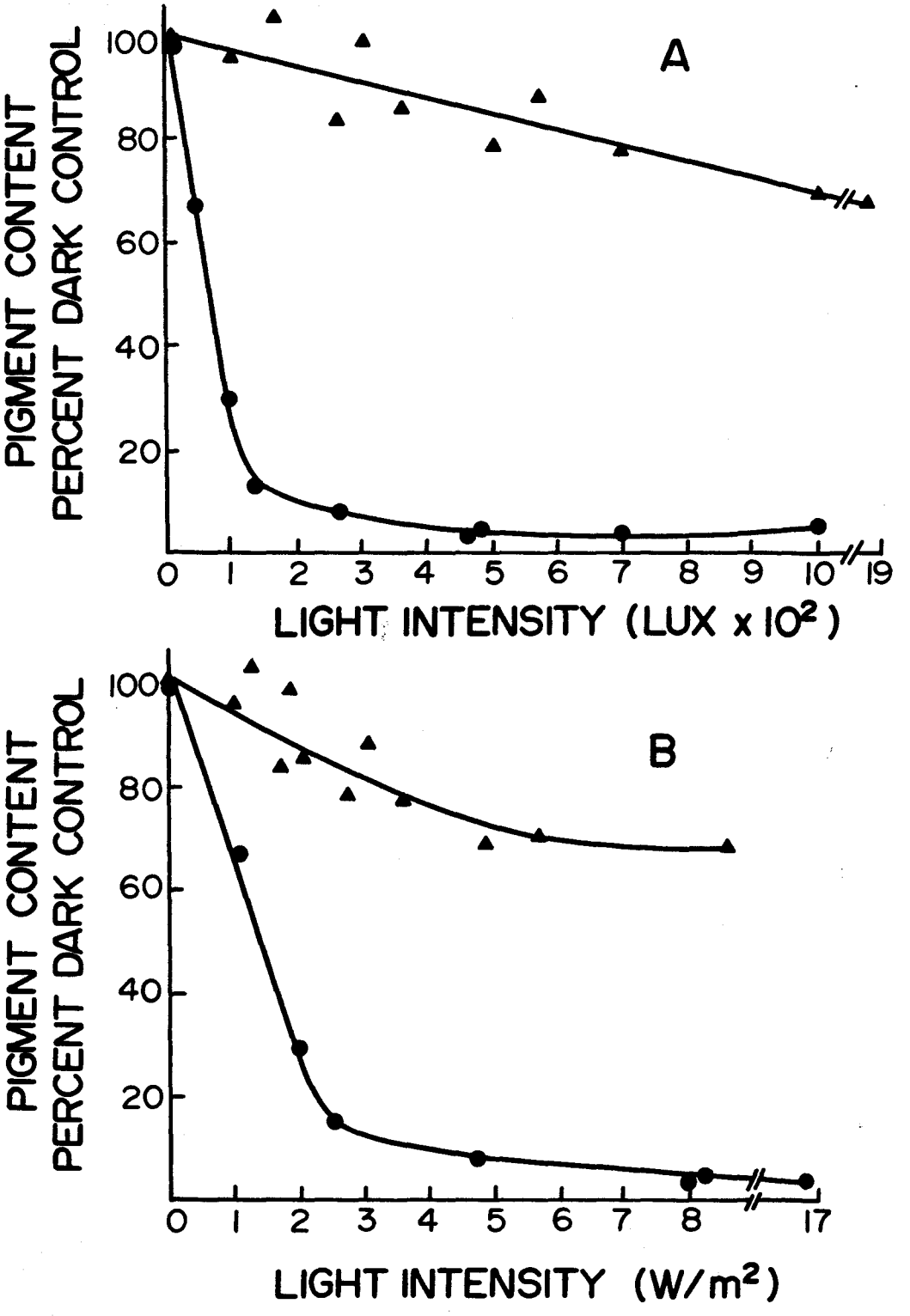
Duration of irradiation (min)	Pigmentation index x 10^3	Percent dark control
0	52	100.0
5	52	100.0
30	46	88.5
60	45	86.5
300	47	90.3
1440	54	104.0

Cultures of T. mentagrophytes hyphae (2 days old) were irradiated with white light (1,000 lux, 4 W/m^2) at 37°C for various time periods. The plates were further incubated in the dark at 37°C, and pigmentation was quantitated 72 h after the start of irradiation. Each value is the average of 2 cultures from 1 experiment.

light were investigated. Two relatively pure sources of broad band colored light were selected. These were red and Special Blue fluorescent tubes (see Results, Section B 1, for emission spectra). Preliminary experiments indicated that, in comparison with dark controls, continuous illumination with red or blue light (1,000 lux) caused a decrease in the levels of colored carotenoids in arthrosporulating cultures. Fig. 22 is a representative study of the effect of different intensities of continuous red or blue light (day 3-8) on arthrospore pigmentation at day 8. The results of the same experiment are expressed in lux (Fig. 22A) or W/m^2 (Fig. 22B). For both light sources, the decrease in pigmentation was intensity dependent. Furthermore, reduction of carotenoid accumulation was much greater under blue light. Computer analysis of these data by nonlinear regression indicates that the suppressive effects of different intensities of red and blue light are exponentially and inversely related to the amount of carotenoids accumulated. The best fit curves are defined as $y = 130e^{-0.72x}$ ($r = 0.99$) for blue light (intensity range of 0-17 W/m^2) and $y = 100e^{-0.62x}$ ($r = 0.87$) for red light (intensity range of 0-9 W/m^2) where y = pigmentation (% dark control); x = intensity of light (W/m^2); and e = base of ln. At these intensities, neither red nor blue light significantly affected growth or arthrosporulation of T. mentagrophytes on SDA-acetate at 37°C.

To determine the effect of blue or red light on pigment composition, carotenoids were extracted and isolated from batch cultures of arthrospores grown at selected light intensities under the experimental

Fig. 22. Effect of intensity of red (▲) and blue (●) light on carotenoid accumulation in T. mentagrophytes arthrospores at 37°C. Light intensity is expressed as either lux (A) or W/m^2 (B). Cultures were grown in the dark for 3 days and then continuously irradiated for an additional 5 days. Data points are % PI of the respective dark controls at 8 days.


PIGMENT CONTENT
PERCENT DARK CONTROL
100
80
60
40
20
A
0 1 2 3 4 5 6 7 8 9 10 19
LIGHT INTENSITY (LUX x 10²)
PIGMENT CONTENT
PERCENT DARK CONTROL
100
80
60
40
20
B
0 1 2 3 4 5 6 7 8 17
LIGHT INTENSITY (W/m²)

conditions used in Fig. 22. Although pigment accumulation in arthrospores was suppressed under red light (approx 5 W/m^2) as compared to dark controls (Fig. 22), the data of Fig. 23 suggest that no single carotenoid was selectively decreased. There were also no observable differences in the relative amounts of the colorless carotenoids phytoene and phytofluene (data not shown).

Visible light absorption spectra of total carotenoids (in hexane) from arthrospores grown in the dark and under continuous blue light at intensities of 1 W/m^2 and 2 W/m^2 are presented in Fig. 24. When compared with dark controls (Fig. 24A), the major peaks of the absorption spectra from irradiated samples (Fig. 24B and 24C) decreased proportionately as light intensity was increased. Although not shown, thin-layer chromatograms of colored carotenoids from these extracts revealed no qualitative differences in pigment between light- and dark-grown arthrospores. Visualization of the same chromatograms with far-UV light demonstrated that blue light caused an increase in the amount of material which migrated at the phytoene level (Fig. 24, insert, A, B, and C). Ultraviolet absorption spectra (in hexane) of this material from the chromatograms in Fig. 24 indicated that, besides phytoene, irradiated spores contained an additional unknown component (data not shown). The above results suggest that red and, to a larger extent, blue light cause a quantitative reduction in the colored carotenoids of T. mentagrophytes arthrospores.

To further elucidate the rather unique suppressive effect of red light on carotenogenesis, higher intensities of light were tested.

Fig. 23. Absorption spectra (in hexane) of carotenoids extracted from arthrospores (8 days old) formed in continuous red light (------) or in the dark (———). The intensity of red light used to illuminate the cultures was 1,000 lux (approx 5 W/m^2). Spectra were normalized at the peak of maximum absorption. Insert is a thin-layer chromatogram of individual carotenoids isolated from the red light- (L) or dark-exposed (D) arthrospores.

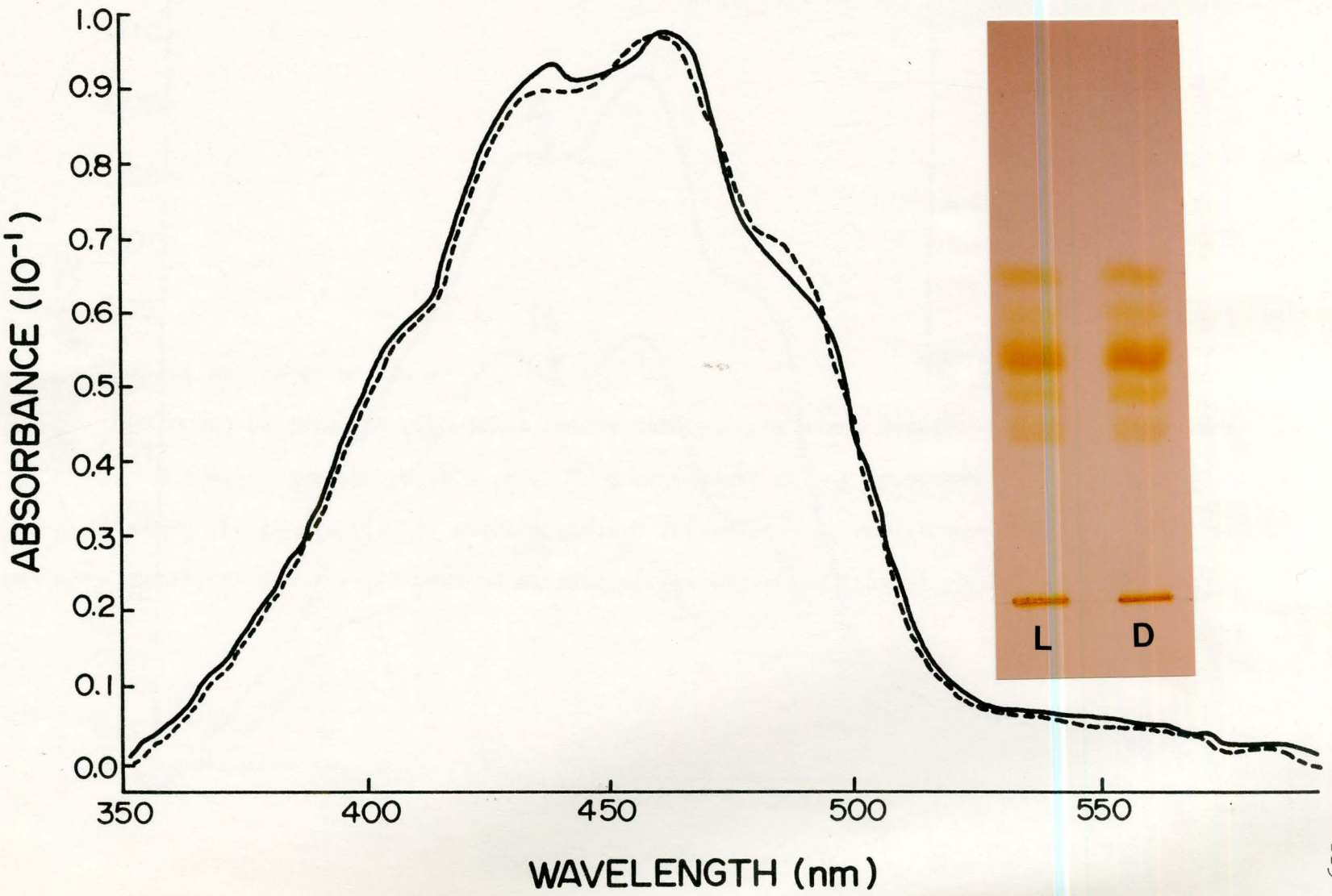
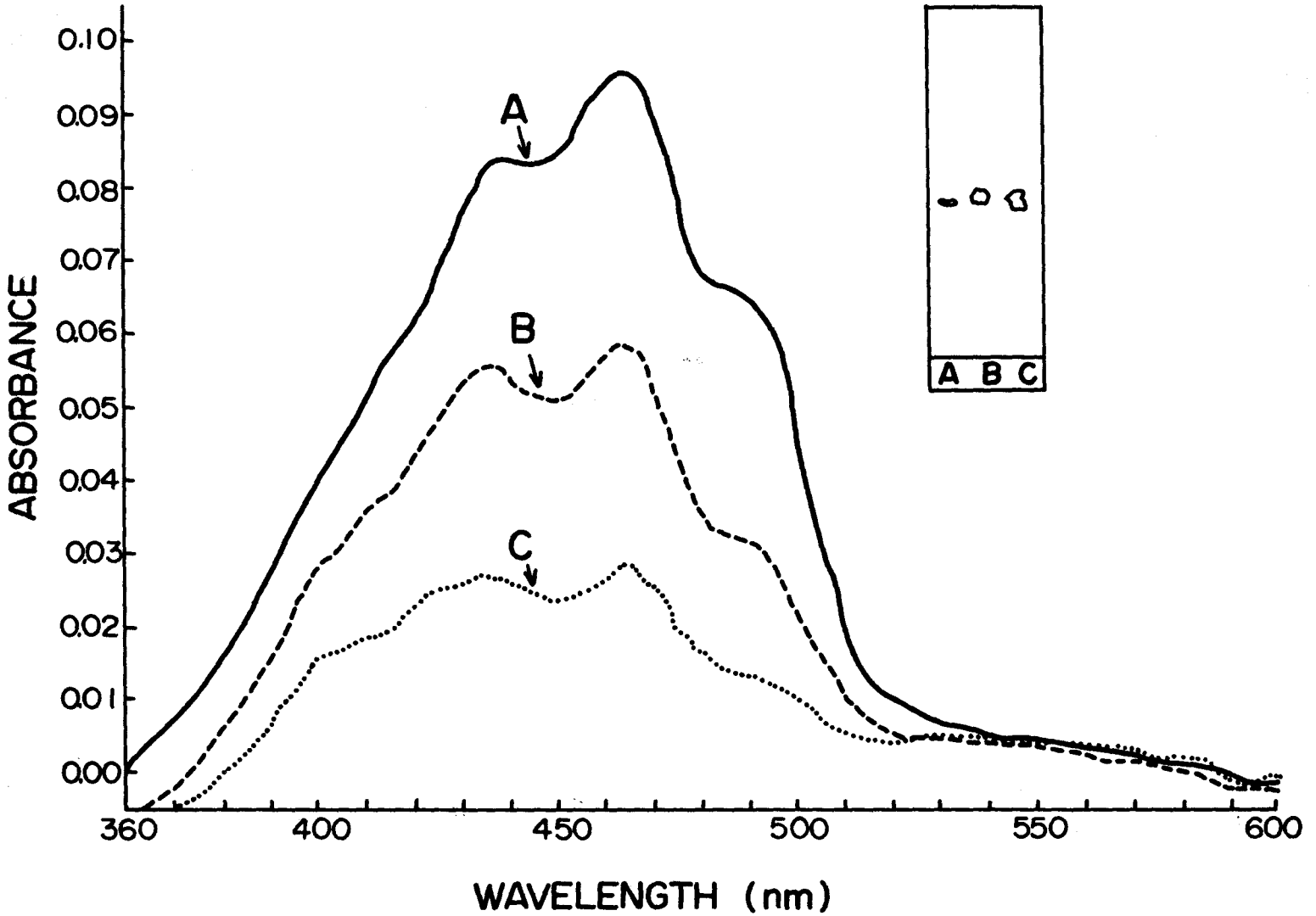

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE (10⁻¹)
350
400
450
500
550
WAVELENGTH (nm)
L
D

Fig. 24. Absorption spectra (in hexane) of carotenoids extracted from arthrospores (8 days old) formed in the dark (A) or under different intensities of continuous blue light (B, 1 W/m^2; C, 2 W/m^2). The insert is a tracing of colorless, far-UV absorbing material (predominantly phytoene) isolated by thin-layer chromatography from equal quantities of arthrospores (80 mg dry wt) in A, B, and C.


0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
ABSORBANCE
A
B
C
360
400
450
500
550
600
WAVELENGTH (nm)
A B C

Unfortunately, at the distances required to achieve these intensities, the bulbs emitted large amounts of heat which could not be dissipated. Since carotenogenesis in dark-grown arthrospores was almost totally inhibited at 39°C, temperature was constantly monitored and maintained at 37°C.

7. Dark to Red or Blue Light Shift.

We examined the effect of red and blue light on the pigment content of arthrospores which were already actively accumulating carotenoids. In these experiments, cultures of dark-grown arthrospores (day 5) were shifted to either red or blue light (1,000 lux) and continuously irradiated for an additional 3 days (day 5-8). As demonstrated in Fig. 25, carotenoid accumulation in arthrospores transferred to red light (Fig. 25A) was nearly identical with dark controls (Fig. 25B). On the other hand, a shift to blue light resulted in the reduction of pigmentation (Fig. 26B) in comparison with dark controls (Fig. 26A). Furthermore, the A_{460} of total carotenoids (in methanol) from equal numbers of arthrospores at day 5 (initial control) and after 1, 2, and 3 days of irradiation (Fig. 26B, day 6, 7, and 8), progressively decreased, thus suggesting that blue light caused photodestruction or bleaching of carotenoids.

8. Effect of Intensity of White Light on Carotenogenesis in T. tonsurans Arthrospores.

Attempts to identify carotenoids in other dermatophytes led to the discovery that carotenogenesis was associated with arthrosporulation in Trichophyton tonsurans, another agent of ringworm. Carotenoids

Fig. 25. Effect of a dark to red light shift on carotenoid accumulation during arthrosporulation in T. mentagrophytes at 37°C. A. Dark. B. Light. Arrow indicates time of shift to red light (1,000 lux, approx 5 W/m^2). Data points represent % PI of dark control at day 8.

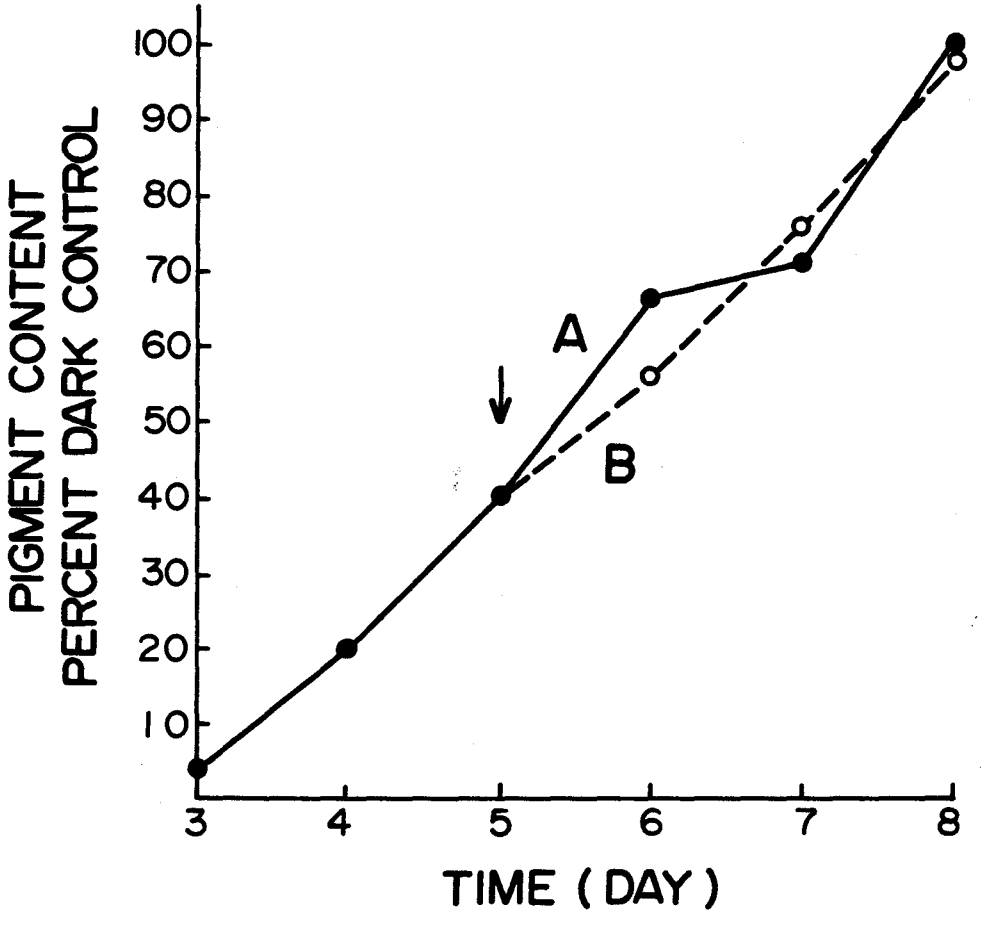
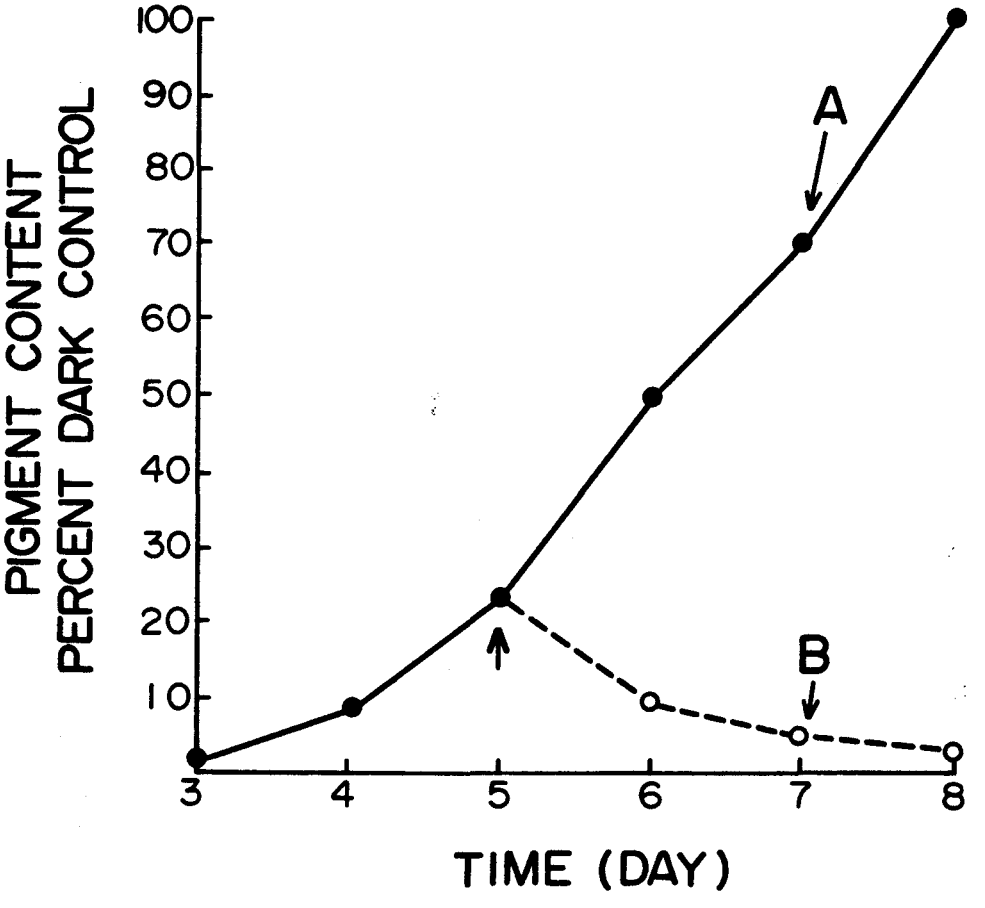

PIGMENT CONTENT
PERCENT DARK CONTROL
100
90
80
70
60
50
40
30
20
10
3
4
5
6
7
8
TIME (DAY)
A
B

Fig. 26. Suppression of carotenoid accumulation by a dark to blue light shift during arthrosporulation in T. mentagrophytes at 37°C. A. Dark. B. Light. Arrow indicates time of shift to blue light (1,000 lux, 16 W/m^2). Data points represent % PI of dark control at day 8.


PIGMENT CONTENT
PERCENT DARK CONTROL
100
90
80
70
60
50
40
30
20
10
3
4
5
6
7
8
TIME (DAY)
A
B

accumulated in lipid granules during arthrosporulation. In addition, continuous irradiation with white light (day 3-8) at 37°C was found to suppress carotenoid accumulation in an intensity-dependent manner (Table 3). Although identical cultural conditions were used, arthrosporulation of T. tonsurans occurred more slowly than in T. mentagrophytes. This difference may account for the observation that, at 1,000 lux (4 W/m^2), suppression of carotenoid accumulation is approx two-fold greater in T. mentagrophytes (compare Fig. 21 and Table 3).

D. Photobleaching of Carotenoids in T. mentagrophytes.

1. Whole Arthrospores.

Several possibilities existed for the light-promoted reduction of carotenoid accumulation within arthrospores. As previously suggested for other fungi, the two most likely explanations include the photobleaching of carotenoid pigments and inhibition of their biosynthesis (Cantino and Horenstein, 1956; Chu and Lilly, 1960; Gribanovski-Sassu and Foppen, 1969). Photobleaching of carotenoids in whole arthrospores was studied under conditions in which de novo synthesis of these pigments was arrested. A preliminary study indicated that, when dark-grown cultures of arthrosporulating T. mentagrophytes were shifted to 4°C and further incubated under darkness, carotenoid biosynthesis was halted, regardless of the time of shift (Fig. 27). In addition, growth or dry wt increase was totally arrested in the cold (data not shown).

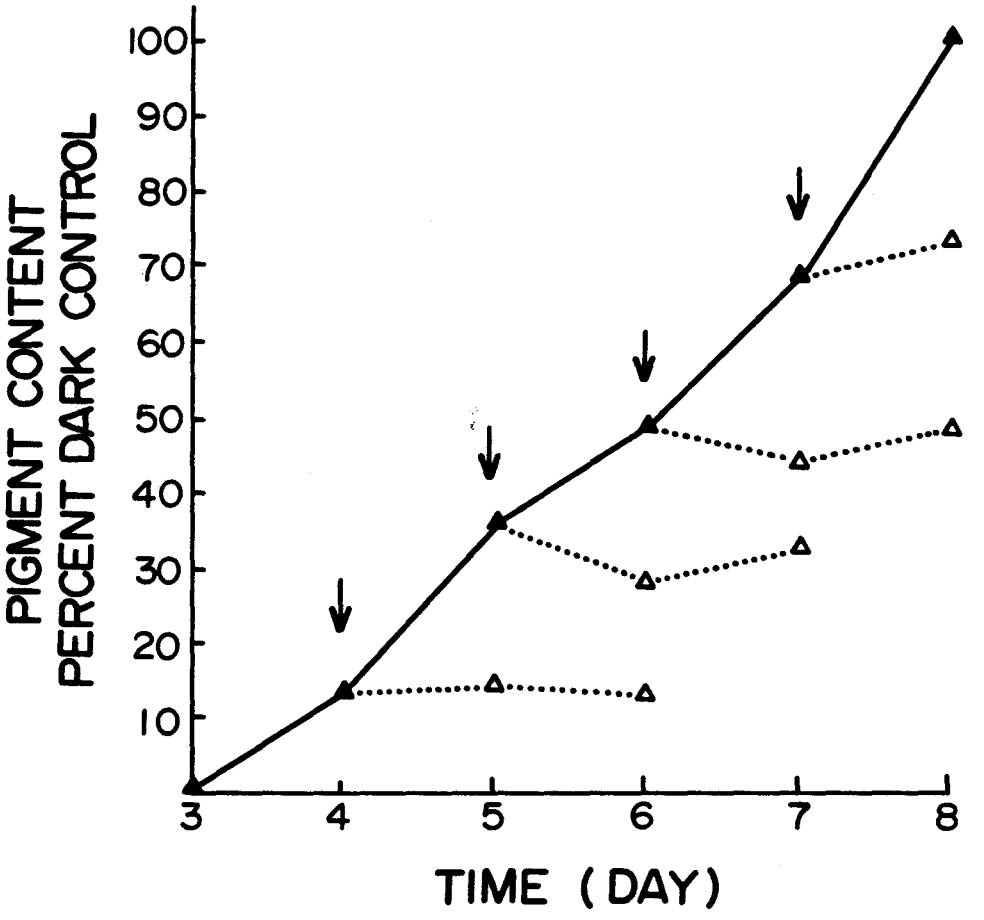
Cultures of mature, fully-pigmented arthrospores (8 day) which were placed under white light (1,000 lux) after a shift to 4°C ex-

Table 3. Effect of intensity of white light on carotenoid accumulation in T. tonsurans arthrospores (8 days old) at 37°C.

Intensity of light			Pigment content	
lux	W/m^2		PI x 10^3	% dark control
360	1.4		70.9[a]	74.1
1000	4.2		58.0	60.6
1400	6.0		28.5	29.8
0	0	(dark control)	95.6	

[a]Each value is the average of duplicate samples from 1 experiment.

Fig. 27. Inhibition of carotenoid accumulation in T. mentagrophytes at low temperature (4°C). Arrows indicate times when cultures of dark-grown arthrospores at 37°C (▲———) were shifted to 4°C (Δ·····) in the dark. All data points are the avg of duplicate samples (range of fluctuation = 11.5) and represent % PI of dark control (37°C) at day 8.


PIGMENT CONTENT
PERCENT DARK CONTROL
100
90
80
70
60
50
40
30
20
10
3
4
5
6
7
8
TIME (DAY)

hibited a gradual loss of pigment (Fig. 28B) in comparison with dark controls (Fig. 28A). The amount of carotenoid reduction was directly related to the duration of irradiation. After 8 days of continuous irradiation by white light at 4°C, arthrospores contained approx 40% of their initial pigmentation levels. Microscopic examination of irradiated and nonirradiated spores revealed that white light caused bleaching of pigment granules within the arthrospores.

In further studies, blue light was substituted for white light. Cultures of dark-grown arthrospores (day 5) were shifted to 4°C and continuously irradiated under different intensities of blue light for 24 h at 4°C. The results of this experiment are expressed in lux units and W/m^2 (Fig. 29A and 29B). It was observed that the degree of bleaching of intracellular carotenoids varied directly with the intensity of blue light at 4°C. Since the activities of most enzymes are reduced at 4°C, it is likely that photobleaching of intracellular carotenoids by white or blue light was due to physical or chemical processes rather than an enzymatic modification of the pigments.

2. Cell-free Extracts.

In contrast to intact arthrospores, carotenoids in cell-free homogenates were rapidly photobleached by continuous white light (1,000 lux) at 4°C (Fig. 30). After 6 h, irradiated cytoplasmic extracts lost 80% of their initial pigment, while dark controls were unaffected. Within 24 h, dark controls had begun to lose pigment. Under conditions of reduced oxygen levels, irradiated cytoplasmic extracts followed a similar time course of carotenoid photobleaching

Fig. 28. Bleaching of intracellular carotenoids by white light at 4°C. Dark-grown cultures of fully-pigmented arthrospores (8 days at 37°C) were transferred to 4°C and incubated under the specified conditions. A. Dark. B. White light (1,000 lux). All data points represent % PI of arthrospores at 0 time.

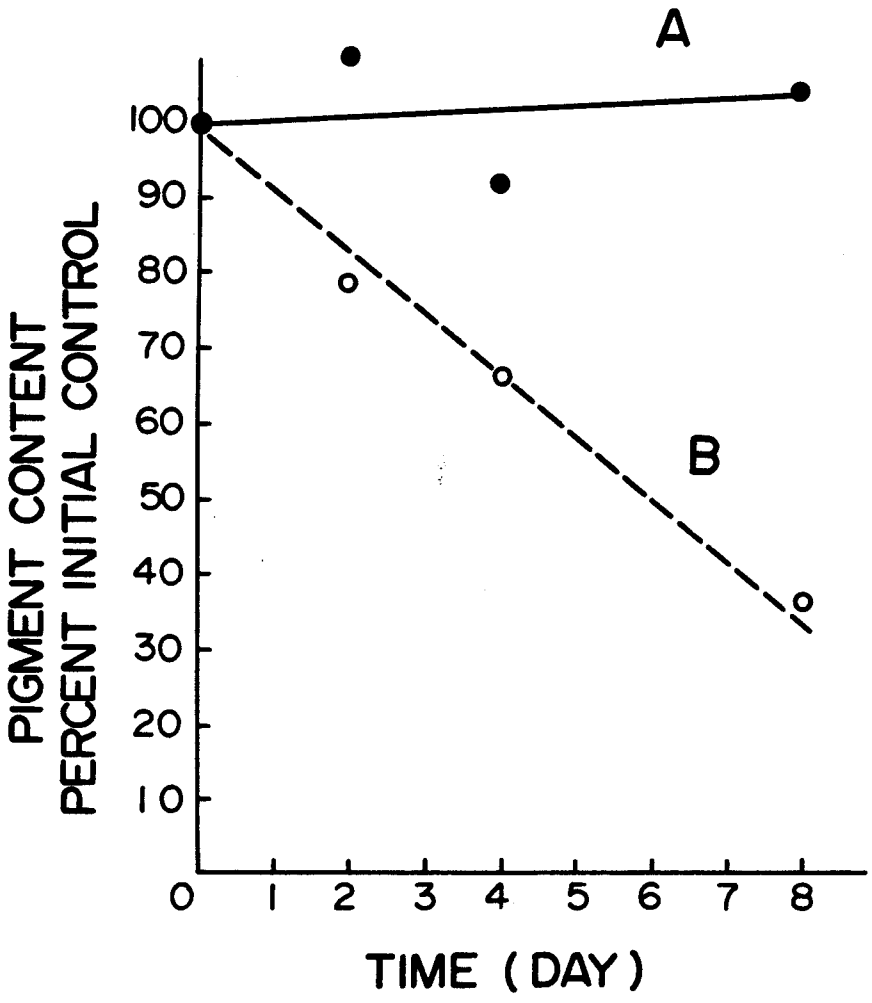
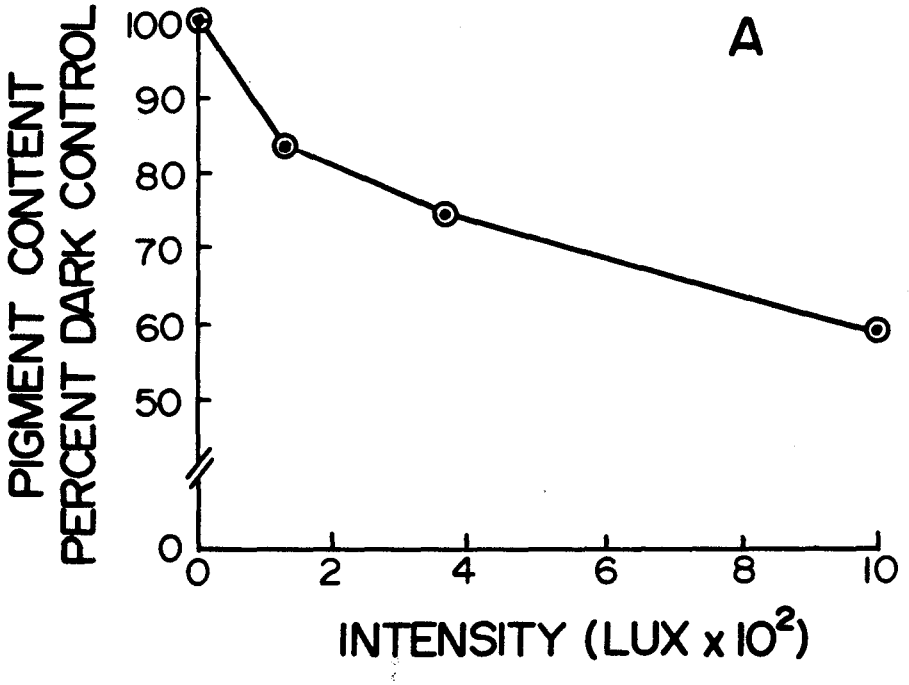

A
B
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
PIGMENT CONTENT
PERCENT INITIAL CONTROL
TIME (DAY)

Fig. 29. Dose-dependent bleaching of intracellular carotenoids by blue light at 4°C. Cultures of dark-grown arthrospores (5 days at 37°C) were transferred to 4°C and irradiated with the designated intensities of blue light for 24 h. Light intensity is expressed as lux (A) and W/m^2 (B). All data points represent % PI of the dark control (cultures of arthrospores shifted to 4°C and incubated for 24 h in the dark).


A
PIGMENT CONTENT
PERCENT DARK CONTROL
100
90
80
70
60
50
0
0
2
4
6
8
10
INTENSITY (LUX x 10²)

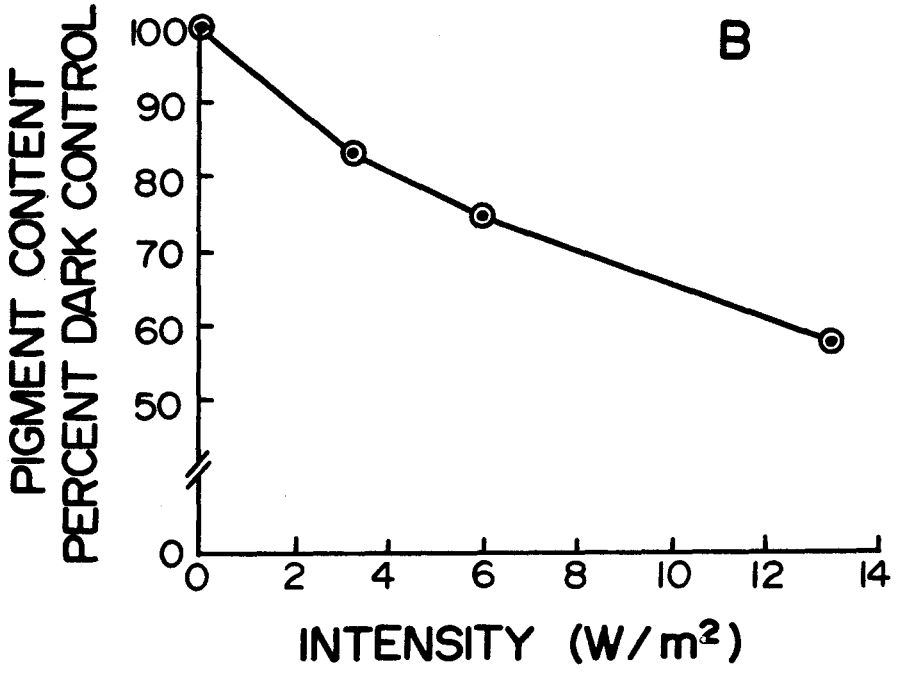
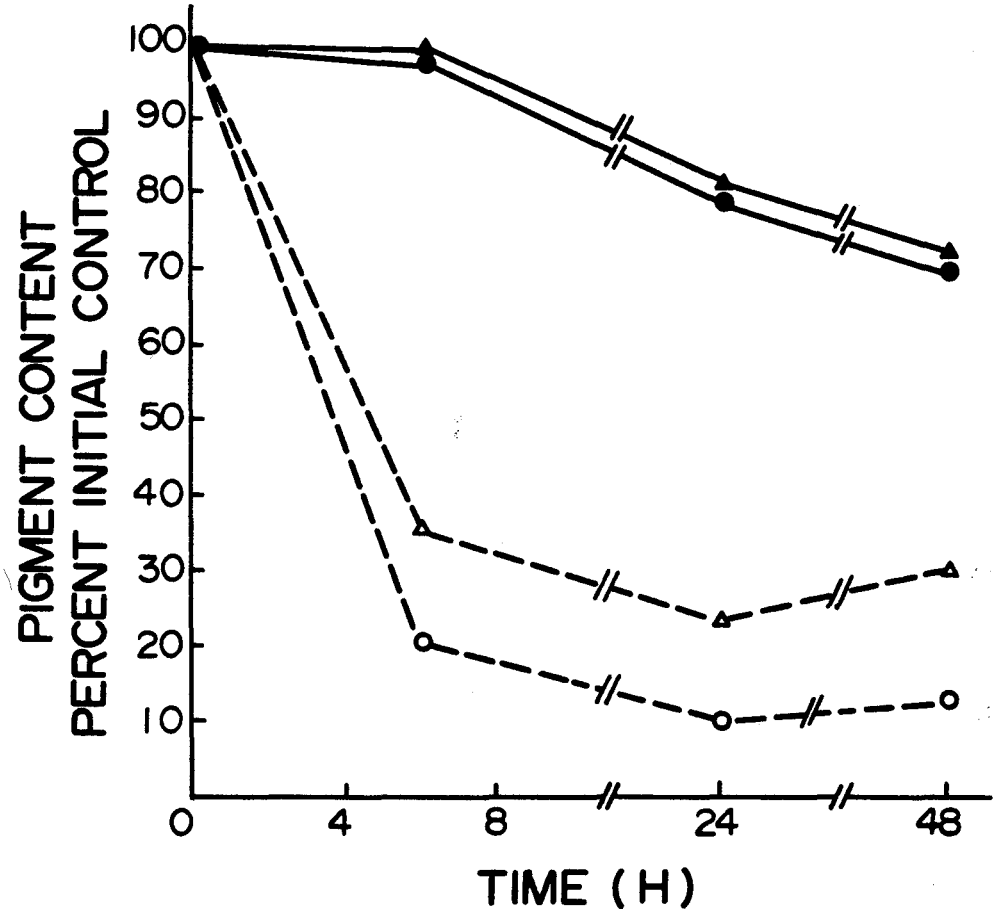

B
PIGMENT CONTENT
PERCENT DARK CONTROL
100
90
80
70
60
50
0
0
2
4
6
8
10
12
14
INTENSITY (W/m²)

Fig. 30. Effect of white light on carotenoid bleaching of cell-free extracts under reduced oxygen levels at 4°C. Extracts were prepared from arthrospores previously grown in the dark at 37°C for 8 days. All data points represent % pigmentation of initial extract (0 time). Symbols: o------, white light (1,000 lux) in air; ●———, dark in air; △------, white light in N_2; ▲———, dark in N_2.


PIGMENT CONTENT
PERCENT INITIAL CONTROL
100
90
80
70
60
50
40
30
20
10
0
4
8
24
48
TIME (H)

as their aerobic counterparts (Fig. 30). However, at the periods tested (6 h, 24 h, and 48 h), they consistently contained approx 15% more pigment.

3. Isolated Pigment Granules.

When suspended in HEPES-EDTA buffer, isolated pigment granules did not synthesize carotenoids and thus offered an additional system for the study of carotenoid photobleaching. Carotenoids in isolated granules from fully-pigmented arthrospores were highly sensitive to photoalteration. As shown in Fig. 31, continuous white light (1,000 lux) caused bleaching of granule carotenoids at both 4°C and 37°C. After 24 h of illumination, colored carotneoids were almost completely absent from pigment granules incubated at either temperature. The loss of pigment in granules illuminated at 37°C was not entirely due to light. During incubation at 37°C, dark controls also lost considerable amounts of pigment (Fig. 31). To minimize any enzymatic alteration of carotenoid pigments, suspensions of isolated pigment granules were preboiled for 5 min in the dark before initiation of illumination. Although continuous white light caused bleaching of carotenoids within preheated granules, some of the carotenoids associated with the granules were destroyed by the boiling process itself. Therefore, the amount of pigment reduction attributable to white light could not be accurately determined at 37°C.

When pigment granules were illuminated at 4°C in an atmosphere of reduced oxygen, photobleaching of carotenoids was initially less rapid than in air (Fig. 32). After 16 h of incubation at 4°C, however,

Fig. 31. Bleaching effect of white light on pigment granules isolated from T. mentagrophytes arthrospores. Granules were isolated from dark-grown arthrospores (8 days at 37°C) as described in Materials and Methods. Intensity of white light was 1,000 lux. All data points represent % pigmentation of the initial granule isolate (0 time). Symbols: ●———, 37°C; x———, 4°C.

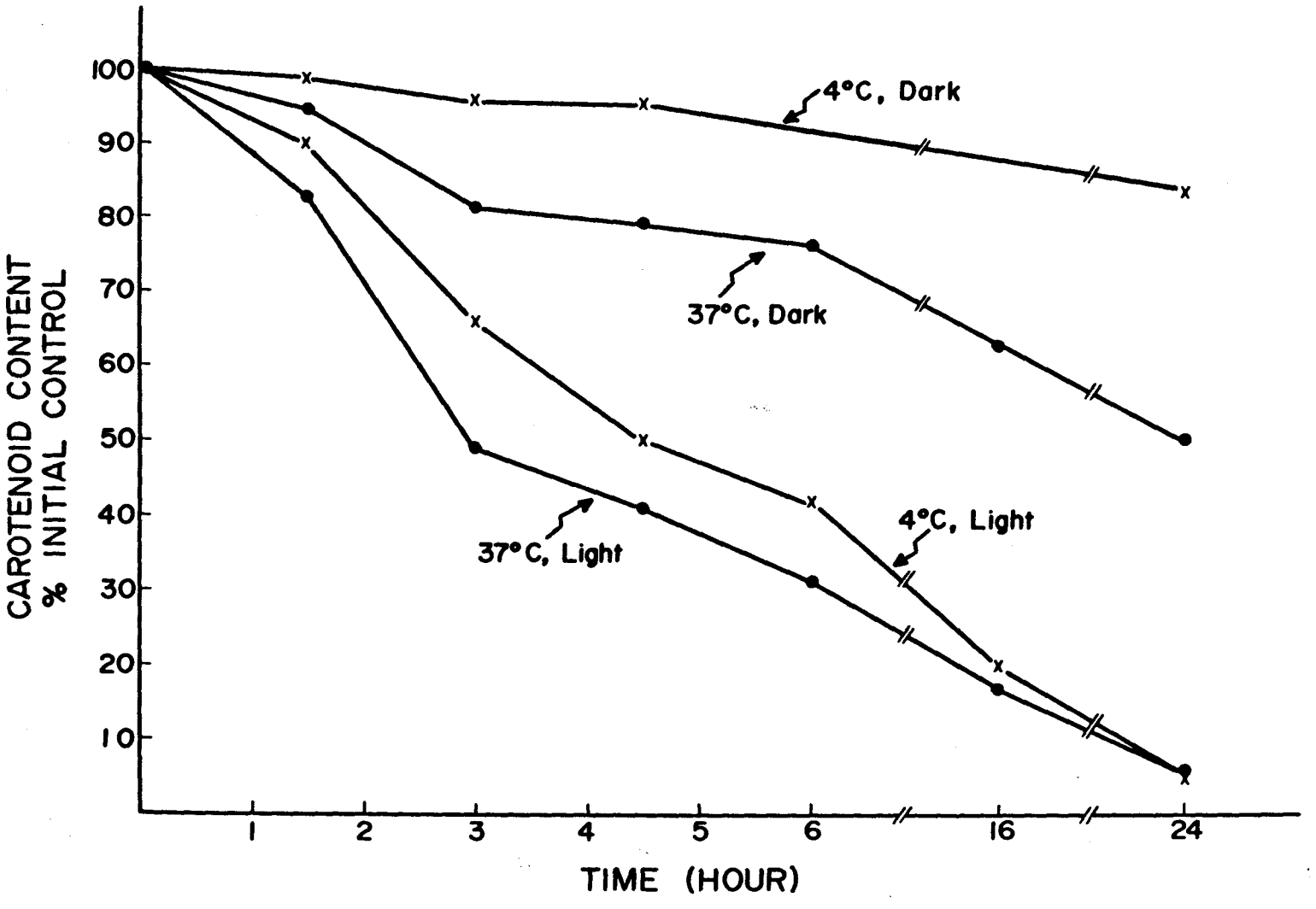
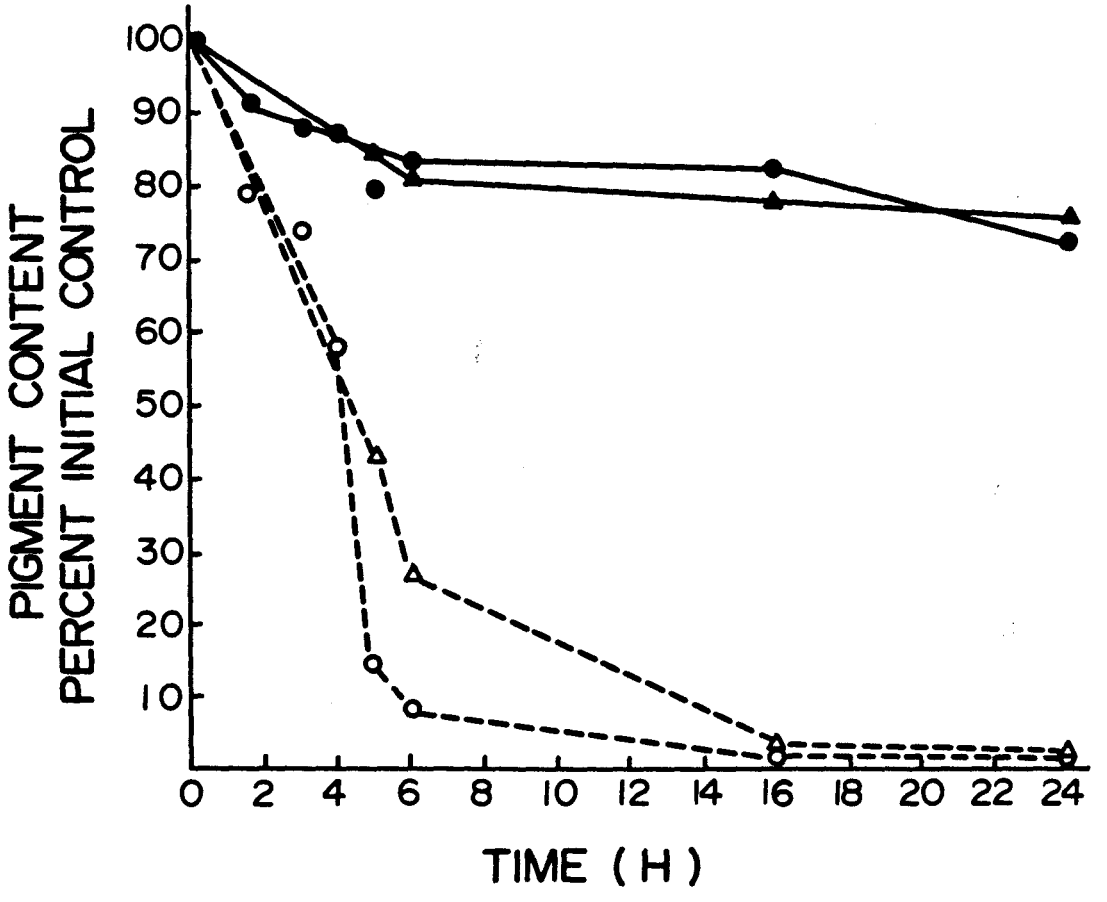

CAROTENOID CONTENT
% INITIAL CONTROL
100
90
80
70
60
50
40
30
20
10
4°C, Dark
37°C, Dark
4°C, Light
37°C, Light
1
2
3
4
5
6
16
24
TIME (HOUR)

Fig. 32. Effect of white light on carotenoid bleaching of isolated pigment granules under reduced oxygen levels at 4°C. Pigment granules were isolated from dark-grown arthrospores (8 days at 37°C) as mentioned in Materials and Methods. All data points represent % pigmentation of initial granule isolate (0 time). Symbols: o------, white light (1,000 lux) in air; ●———, dark in air; △------, white light (1,000 lux) in N_2; ▲———, dark in N_2.


PIGMENT CONTENT
PERCENT INITIAL CONTROL
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (H)

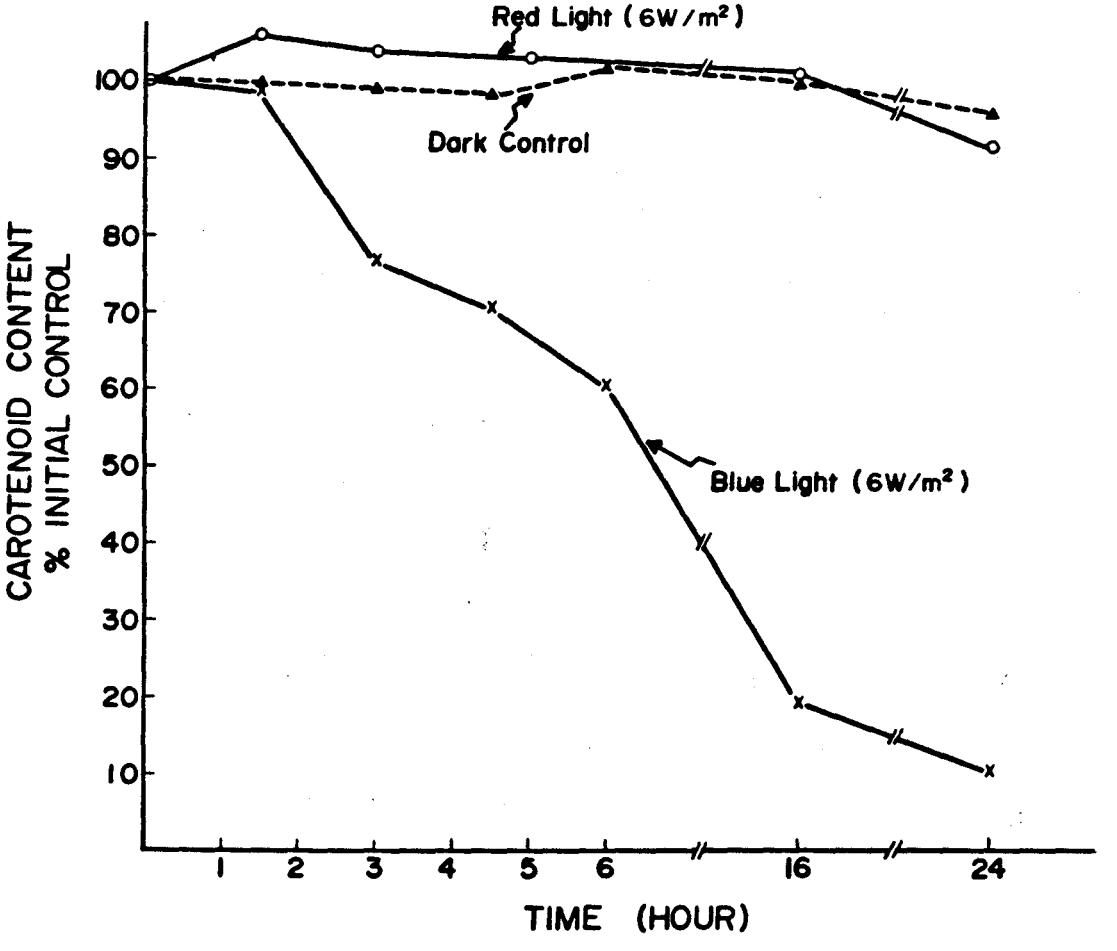
granules illuminated in air or N_2 had almost identical amounts of pigment. Although most studies were conducted in HEPES-EDTA buffer (pH 7.4), aqueous suspensions (pH 5.8) of isolated pigment granules also became rapidly bleached upon illumination with continuous white light (1,000 lux). By 24 h, dark controls had begun to lose significant amounts of pigment.

To further determine which wavelengths of visible light caused photobleaching of carotenoids, isolated pigment granules were continuously irradiated with red or blue light (6 W/m^2) at 4°C. As shown in Fig. 33, blue light caused a fairly rapid loss of carotenoids associated with isolated granules. Red light, however, had no effect on pigmentation of isolated granules for at least 24 h at 4°C. These results suggest that shorter wavelengths of visible light are primarily responsible for photobleaching of carotenoids in isolated pigment granules.

E. Endotrophic Carotenoid Accumulation under Visible Light.

The data of the preceding section strongly suggest that photobleaching of carotenoids could occur within arthrospores. Further studies were undertaken to determine the effect of visible light on carotenoid biosynthesis in arthrospores of T. mentagrophytes. Several synthetic and semisynthetic media were screened for their ability to support carotenogenesis in T. mentagrophytes. The following media were examined: Bacto-Yeast Nitrogen Base supplemented with 0.5% glucose; Bacto-Yeast Carbon Base supplemented with Basic Medium Amino Acids with L-glutamine; Dulbecco's Modified Eagle Medium, the basal

Fig. 33. Effect of red or blue light on carotenoid bleaching of isolated pigment granules from T. mentagrophytes arthrospores at 4°C. Pigment granules were isolated from dark-grown arthrospores (8 days at 37°C) as described in Materials and Methods. All data points represent % pigmentation of initial granule isolate (0 time). Symbols: ▲, dark; o, red light (6 W/m^2); x, blue light (6 W/m^2).


Red Light (6W/m²)
Dark Control
Blue Light (6W/m²)
CAROTENOID CONTENT
% INITIAL CONTROL
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
16
24
TIME (HOUR)

defined medium of Merz et al. (1972) supplemented with 15 mM L-leucine, and 2% Bacto-Vitamin-Free Casamino Acids. Since low rates of arthrosporulation and barely detectable levels of carotenoids were observed, an alternative approach was used.

Preliminary experiments indicated that, if immature arthrospores of T. mentagrophytes were thoroughly washed and suspended in sodium phosphate buffer (0.1 M, pH 6.8), they continued to accumulate carotenoids in the absence of exogenous carbon and nitrogen sources, that is, they accumulated carotenoids endotrophically. For arthrospores transferred to buffer after 5 days of growth, the levels of carotenoids increased approx 35% after 24 h of incubation in the dark at 37°C. Continuous irradiation by white or blue light during this period suppressed endotrophic carotenoid accumulation. Microscopically, no autolysis of arthrospores was observed in either light or darkness.

In the following study, visible light intensities which did not cause bleaching of intracellular carotenoids at 37°C were determined. It was further observed whether endotrophic carotenoid accumulation could be reduced at these specific light intensities.

1. Nonbleaching Intensities of White and Blue Light.

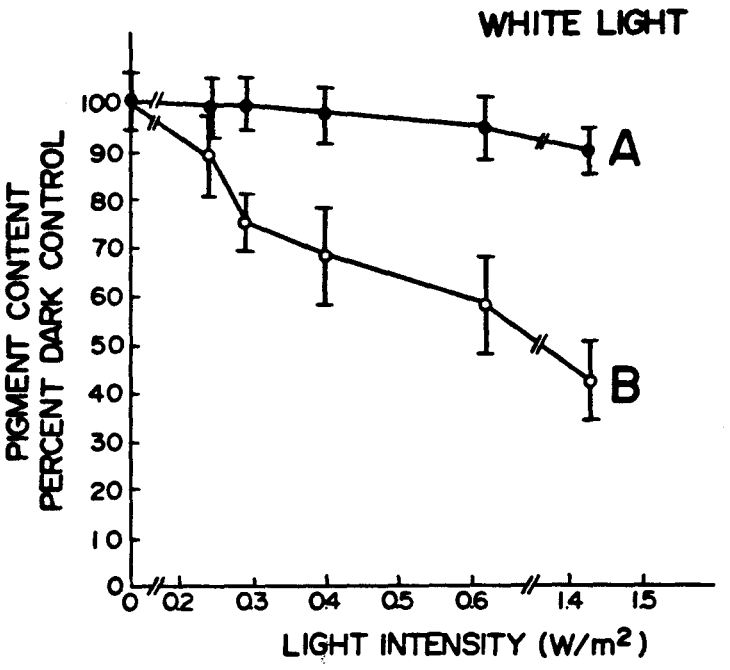
Endotrophic carotenoid accumulation was arrested when rinsed arthrospores were treated for 1 h with 2% formaldehyde before incubation in buffer. By use of this treatment method, several nonbleaching intensities of visible light were determined for each light source (see Materials and Methods for exact protocol). In this assay system, intracellular pigment bleaching was considered negligible if the

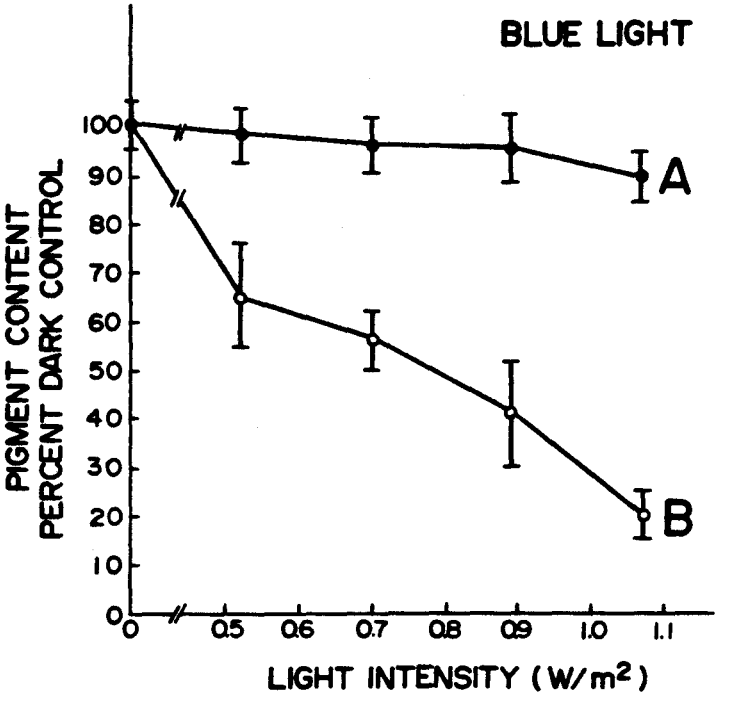
differences between the carotenoid content of irradiated samples and dark controls were not significant at a level of $p > 0.10$, as determined by the Student's t test. Subsequent experiments were designed so that half of an arthrospore batch was treated with formaldehyde, while the remaining half was treated with buffer for the same period of time. Both populations of cells were then rinsed and further incubated in buffer, as almost a unicellular layer, under the predetermined lighting conditions. As shown in Fig. 34, exposure of arthrosporulating T. mentagrophytes to low nonbleaching intensities of continuous white or blue light (Fig. 34A) caused a significant reduction in endotrophic carotenoid accumulation in comparison with dark controls (Fig. 34B). With the exception of white light at an intensity of 0.24 W/m^2, the differences between pigmentation levels of irradiated samples and dark controls were significant at a level of at least $p < 0.02$. In the case of either white light (0.29 W/m^2 or higher) or blue light (0.52 W/m^2 or higher), the degree of suppression of endotrophic carotenoid accumulation was found to be inversely related to light dosage. Based on the above results, it is highly likely that factors other than the physical or chemical processes of carotenoid photobleaching were contributing to the suppression of pigment accumulation in continuously irradiated arthrospores.

2. Effect of Red Light.

Although not shown, continuous red light at intensities as high as 5.0 W/m^2 did not suppress endotrophic carotenoid accumulation in arthrospores (5 day) under the conditions mentioned in the previous

Fig. 34.. Effect of nonbleaching doses of white or blue light on carotenoids accumulating endotrophically in T. mentagrophytes arthrospores at 37°C. Dark-grown arthrospores (5 days at 37°C) were rinsed and treated with 0.1 M sodium phosphate buffer (pH 6.8) or buffered 2% formaldehyde for 1 h at 25°C. After treatment, cells were thoroughly rinsed and placed in buffer under continuous white or blue light at the specified intensities for 24 h at 37°C. A. Arthrospores in which carotenoid synthesis was arrested by 2% formaldehyde. B. Arthrospores synthesizing carotenoids endotrophically in Na-phosphate buffer. Each point is the avg of 4 experiments (± SE) and represents % pigmentation of corresponding dark control (treated arthrospores placed in buffer and incubated in the dark for 24 h at 37°C).


WHITE LIGHT
PIGMENT CONTENT
PERCENT DARK CONTROL
100
90
80
70
60
50
40
30
20
10
0
A
B
0
0.2
0.3
0.4
0.5
0.6
1.4
1.5
LIGHT INTENSITY (W/m²)


BLUE LIGHT
PIGMENT CONTENT
PERCENT DARK CONTROL
100
90
80
70
60
50
40
30
20
10
0
A
B
0
0.5
0.6
0.7
0.8
0.9
1.0
1.1
LIGHT INTENSITY (W/m²)

section. To further determine whether endotrophic carotenoid accumulation was sensitive to red light during any particular developmental stage, arthrospores of several different cultural ages were irradiated for extended periods of time. Fig. 35 illustrates that prolonged irradiation with red light had no effect on endotrophic carotenoid accumulation in arthrospores which were transferred to buffer at day 3, 4, or 5 (Fig. 35A, B, and C, respectively) and incubated at 37°C under continuous light.

F. Preliminary Studies on the Role of Carotenoids in Arthrospores.

In many organisms, colored carotenoid pigments are believed to act as protective agents against photodamage due to visible light (Krinsky, 1978). While studying the effect of visible light on carotenogenesis in arthrospores, we found that continuous blue light (1,000 lux) decreased germination of pigmented arthrospores (8 day, dark). To determine whether the presence of intracellular carotenoids in T. mentagrophytes arthrospores might confer a photoprotective advantage during germination, arthrospore populations which contained different levels of carotenoids were tested for their germinative ability under blue light. The culture conditions used to produce these different types of arthrospores and their respective pigmentation levels are shown in Table 4. Arthrospores grown under each of these conditions were judged to be at the same level of maturity by light microscopy. In these studies, the highest levels of colored carotenoids were attained when spores were developed in continuous darkness for 8 days at 37°C on SDA. These "fully-pigmented" arthrospores served as both

Fig. 35. Effect of red light on endotrophic carotenoid accumulation in T. mentagrophytes arthrospores at 37°C. Cultures of dark-grown arthrospores at day 3 (A), day 4 (B), and day 5 (C) were harvested, rinsed, and suspended in Na-phosphate buffer, pH 6.8. Cells were incubated at 37°C under the specified conditions. Each point is the avg of 2 samples and represents the amount of carotenoids derived from the same number of cells. Symbols: ●———, dark; o------, red light (1,000 lux, approx 5 W/m^2).

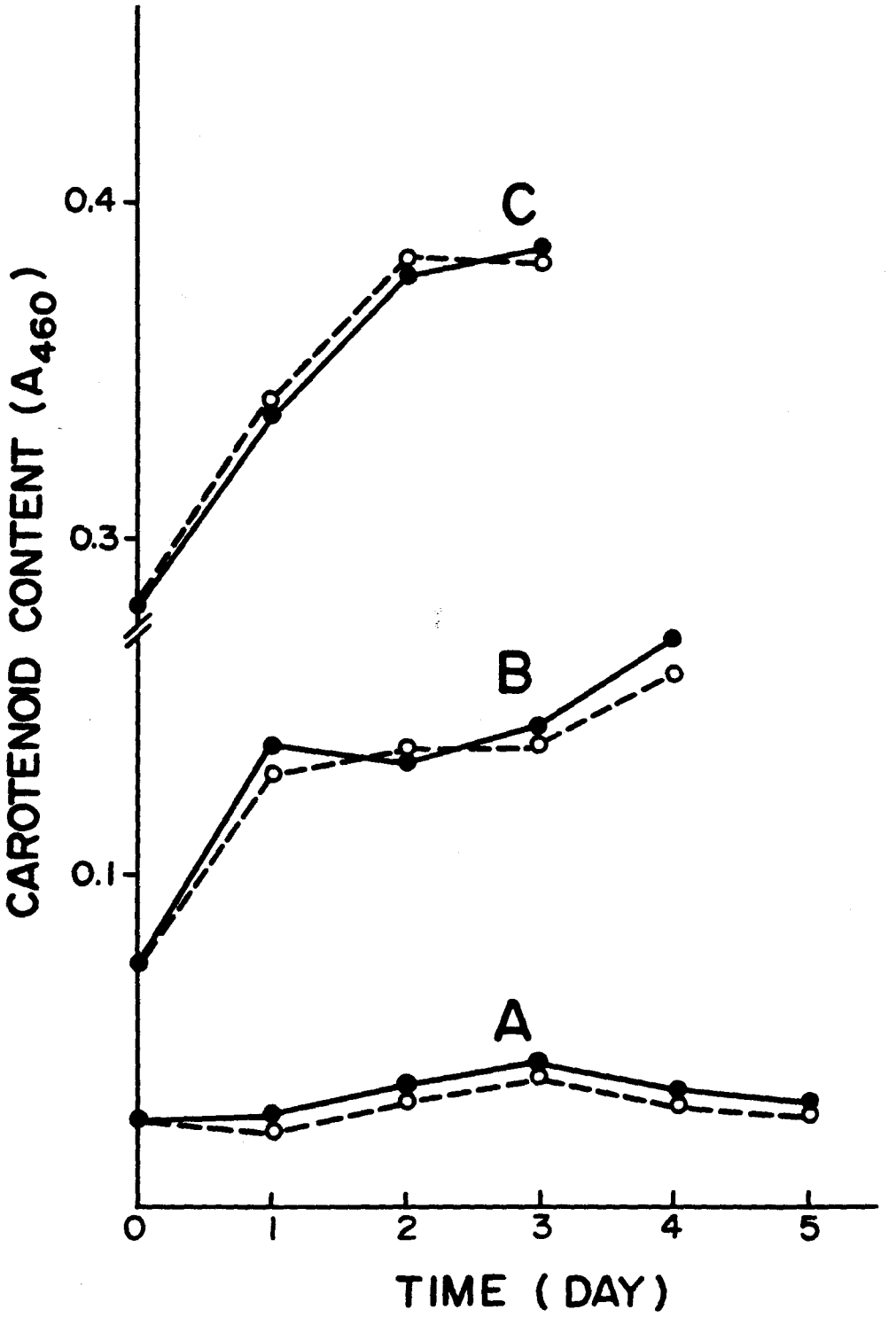

CAROTENOID CONTENT (A_{460})
0.4
0.3
0.1
C
B
A
0
1
2
3
4
5
TIME (DAY)

Table 4. Effect of blue light on germination of T. mentagrophytes arthrospores containing different amounts of pigment[a].

Conditions of arthrospore formation	Pigment content PI x 10^2	% germination dark[b]	% germination light[b]	% dark control[d]
Blue light (500 lux), 37°C	0.9	93	14 ($p < 0.0001$)[c]	15
Blue light (100 lux), 37°C	6.2	96	48 ($p > 0.10$)	50
Dark, 39°C	9.3	93	32 ($p < 0.0001$)	34
Dark, 37°C	37.8	96	50	52

[a]Arthrospores (8 day) were germinated on SDA (microculture) at 37°C either in dark or under continuous illumination by blue light (1,000 lux, 16 W/m^2). % germination was determined at 5 h.

[b]Each value represents the % germination of 200 arthrospores.

[c]The level of significance in % germination under light between the irradiated control (dark, 37°C) and the other types of arthrospores was determined by the text of χ^2.

[d](% germination in light/% germination dark) x 100.

dark and light controls. Under conditions of continual darkness, germination of each spore type was essentially complete after 5 h of incubation at 37°C (Table 4). When the various types of arthrospores were continuously irradiated with blue light (16 W/m^2) for 5 h, arthrospore germination was significantly reduced in comparison with corresponding dark controls. More importantly, the decrease in the percentage of germination under continuous blue light of arthrospores with reduced pigmentation (blue light at 500 lux, 37°C, and dark, 39°C) was significant when compared with irradiated, fully-pigmented arthrospores (dark, 37°C). Although arthrospores formed under blue light (100 lux) at 37°C had reduced pigmentation, their germination rate under continuous blue light was not significantly different than fully-pigmented controls.

CHAPTER IV

DISCUSSION

The present study has revealed some new and significant information concerning the localization of carotenoids and the effect of visible light on carotenogenesis in arthrospores of the dermatophyte T. mentagrophytes. The major findings include: 1. In T. mentagrophytes arthrospores, carotenoids are predominantly localized in discrete cytoplasmic granules; 2. Visible light is not required for the induction or stimulation of carotenoid synthesis; 3. Even at fairly low intensities, visible light causes a reduction of carotenoid accumulation in arthrosporulating T. mentagrophytes; 4. This reduction of carotenoid accumulation is due primarily to blue light; and 5. The light-mediated suppression of carotenogenesis in arthrospores is caused in part by photobleaching of pigment.

There is a great deal of confusion concerning the localization of carotenoids in fungi. It has been found through the use of subcellular fractionation that carotenoids may be localized in one organelle, such as the mitochondrion (Cederberg and Neujahr, 1970) or lipid granule (Mills and Cantino, 1977). On the other hand, both membrane fractions and, to a much larger extent, lipid granules have been shown to contain carotenoids in Phycomyces blakesleeanus (Riley and Bramley, 1976) and in Neurospora crassa (Mitzka-Schnabel and Rau, 1980). Furthermore, polymers of carotenoids, known as the sporo-

pollenins, have been isolated from various fungal cell walls (Furch and Pambor, 1978; Gooday et al., 1973).

Recently Riley and Bramley (1976) have found that carotenoids may be irreversibly transferred from one cell fraction or organelle to another during their isolation by differential centrifugation. The authors recommend gradient centrifugation for the isolation of all subcellular fractions. Based on these observations, it is likely that carotenoids reported to occur in fungal mitochondria, which have been isolated mainly by differential centrifugation, are contaminants (Ruddat and Garber, 1983). Other studies not employing gradient centrifugation will also need to be reevaluated with respect to carotenoid localization.

In agreement with recent results obtained from P. blakesleeanus (Riley and Bramley, 1976), the carotenoids of T. mentagrophytes arthrospores were mostly recovered from the floating lipid layer when cell-free extracts were fractionated by gradient centrifugation (Table 1 and Fig. 11). As shown in Fig. 9, this fraction is essentially composed of lipid granules. Our laboratory has demonstrated that carotenoids are absent from the arthrospore cell wall (Pollack et al., 1983). Whether other cytoplasmic fractions from arthrospores contain carotenoids remains to be determined. As in the case of P. blakesleeanus (Riley and Bramley, 1976), the carotenoid content of these fractions will probably be relatively low.

The chemical composition (Table 1) and morphology (Figs. 9 and 10) of the isolated carotenoid-containing granules from T. mentagro-

phytes arthrospores resemble those reported for zoospores of Blastocladiella emersonii (Mills and Cantino, 1977) and for hyphae of P. blakesleeanus (Riley and Bramley, 1982). Besides being surrounded by a single-layered membrane, pigment granules from these fungi contain greater than 80% lipid and less than 10% protein. In addition, carotenoids account for less than 2.0% of the total granule dry wt in all these fungi. On the other hand, the pigment granules of arthrospores contain anthrone positive material (equivalent to 12% with glucose as a standard), which was not reported for either B. emersonii (Mills and Cantino, 1977) or for P. blakesleeanus (Riley and Bramley, 1982). Furthermore, ergosterol (or free sterol) and phospholipid, which may represent cytoplasmic membrane contaminants, are 5- to 10-fold lower in pigment granules of arthrospores than in the granules from B. emersonii or P. blakesleeanus.

In N. crassa, the major portion of carotenoids appears to be contained in the floating lipid layer, although substantial amounts of pigment were also found in the two membrane fractions (Mitzka-Schnabel and Rau, 1981). The floating lipid layer was recovered, however, only after other cell fractions were isolated by differential centrifugation. As will be recalled, this centrifugation procedure may affect carotenoid localization. Since the lipid fraction of N. crassa was not characterized either morphologically or chemically, any comparison with respect to the pigment granules of arthrospores cannot be made.

The pigment granules of arthrospores appear to fall into a larger category of organelles, called "large lipid granules" (Mills and

Cantino, 1977). These granules, which contain fairly low levels of protein, have been isolated from other fungi and a variety of plants. Carotenoids may be present. Many of these granules, often referred to as spherosomes, appear to be bound by half unit (single-layered) membranes (Wanner et al., 1981; Yatsu and Jacks, 1972). In yeast, this type of membrane, which appears to contain a variety of enzymes, has also been found to surround the central vacuole (tonoplast).

In studies using isolated fractions from cell-free extracts of N. crassa, Mitzka-Schnabel and Rau (1981) demonstrated that maximum carotenogenic activity was localized in two membrane fractions enriched for endoplasmic reticulum, although 60% of the carotenoids were detected in the floating lipid layer. In contrast, Riley and Bramley (1982) suggest that the globules of P. blakesleeanus possess carotenogenic activity. It is unknown where the carotenoid biosynthetic enzymes of T. mentagrophytes are located. The presence of complex membranous structures within pigment granules suggests a possible involvement in carotenoid synthesis (Hashimoto et al., 1978). It is also possible that carotenoid enzymes may reside in the membrane surrounding the pigment granule.

The results of studies using both light microscopy and TEM suggest that the carotenoid-containing granules of T. mentagrophytes may undergo a considerable amount of translocation during arthrosporulation (Figs. 5, 6, and 7). Wanner et al. (1981) have recently proposed that the formation of lipid bodies in N. crassa and various "oil" plants is a membrane process which involves the endoplasmic

reticulum or in some plants, the outer membrane of the plastid envelope. When the lipid granule (spherosome) attains a critical size, it detaches from the membrane. Because the intracellular structure of arthrospores was in many cases indiscernible under TEM, the contribution of membranous elements and other organelles to the formation and translocation of pigment granules in T. mentagrophytes is unknown.

Many fungi, especially spore entities, are difficult to fix and prepare for TEM. This partially accounts for the considerable structural variation which has been observed even for the same microorganism. In Saccharomyces cerevisiae, there are marked differences between lipid granules in situ and isolated lipid granules, even though prepared and stained under identical conditions (Clausen et al., 1974). Isolated granules were found to be uniformly dense, while lipid granules in situ were electron lucent. This difference is attributed to poor osmium fixation in intact yeast cells. As previously reported (R. Emyanitoff, Ph. D. dissertation, Loyola University of Chicago, 1978) and shown in Fig. 7, the in situ appearance of pigment granules of mature arthrospores can become electron lucent as a result of lead citrate staining. In contrast to S. cerevisiae, however, the matrices of isolated pigment granules are almost totally leached even when solvent dehydration was minimized (Fig. 8). In fact, lipid retention in isolated pigment granules from arthrospores was achieved only when dehydration was completely bypassed (Fig. 9). Since Clausen et al. (1974) did not report whether lead citrate staining affected the density of lipid granules in situ, it is difficult to conclude

that the difference in the TEM appearance of isolated pigment granules and pigment granules *in situ* from arthrospores is due solely to poor osmium fixation. It is possible that improper osmium fixation may, in turn, affect the staining properties of pigment granules *in situ*.

In *S. cerevisiae*, the density of lipid granules *in situ* has also been observed to decrease nonuniformly after uranyl-acetate/lead citrate poststaining of fixed, frozen ultra-thin sections (Bauer et al., 1974). It is postulated that this loss results because osmium is adsorbed to the granules, and poststaining acts as a rinse to remove the metal. This type of removal may also be responsible for the loss of pigment granule density observed in arthrospores. On the other hand, isolated pigment granules from arthrospores do not exhibit any alteration after staining with lead citrate (Fig. 10). It should be recalled, however, that these isolated granules were prepared differently than whole arthrospores.

The preparation of artifact-free material for cytological study has for many years eluded electron microscopists. New methods by which steps can be improved or circumvented are constantly being developed. As shown in the case of isolated pigment granules from arthrospores, the latter approach has been successfully applied. Hopefully, newer techniques or modified methods may eventually prove useful for morphological characterization of whole arthrospores.

Studies involving fungal carotenogenesis have mainly concentrated on the formation of carotenoids during the vegetative stage (yeast or hyphal) of development (Davies, 1973; Simpson et al., 1971). As

mentioned in Chapter I, light may be required for significant amounts of carotenoids to be synthesized. Trichophyton mentagrophytes hyphae, however, do not form detectable amounts of carotenoids when grown on SDA-acetate at 37°C in either continuous white light (1,000 lux) or in total darkness (days 2-3, Fig. 15). In fact, these pigments were not demonstrated in hyphae grown under a variety of cultural conditions, including both low and high intensities of light (Mock and Hashimoto, unpublished data).

With the exception of mating studies in several Phycomycetes (Bu'Lock et al., 1976; Ende, 1978), carotenogenesis occurring during fungal sporulation has not been extensively examined. This is probably due to the difficulty of dissociating pigment formation in vegetative cells from that in sporulating structures, both of which are usually in close physical association. In T. mentagrophytes, arthrosporulation involves the transformation of pre-existing hyphae into spores by fragmentation (Fig. 3, R. Emyanitoff, Ph. D. dissertation, Loyola University of Chicago, 1978). Recently, a method has been developed that allows the synchronous and essentially complete transformation of T. mentagrophytes into arthrospores (Hashimoto and Blumenthal, 1977). Utilizing this method, we observed that carotenoid accumulation in T. mentagrophytes does not occur until arthrosporulation has initiated (Fig. 15). This result confirmed the previous finding of Hashimoto et al. (1978) and suggested that carotenogenesis associated with arthrosporulation in T. mentagrophytes was a fairly unique and less problematical system to study the effect of visible light.

As revealed in the present investigation, arthrosporulating T. mentagrophytes produces carotenoids when incubated in total darkness (Fig. 15). Unexpectedly, it was found that continuous white light at virtually any intensity suppresses rather than induces carotenoid accumulation during arthrosporulation (Figs. 15, 19, and 21). In contrast, most carotenogenic fungi produce significantly greater amounts of carotenoids as a result of exposure to visible light (Ruddat and Garber, 1983). In fungi whose carotenogenesis is under strict positive photocontrol, continuous irradiation with visible light leads to a rapid pigmentation increase which, in turn, is followed by a time-dependent linear accumulation of carotenoids (Rau, 1980). Since the amount of pigment in these fungi can increase approx 10-fold, it is likely that any suppressive effect of visible light, such as that observed in T. mentagrophytes, may be masked. That photosuppression of pigmentation may occur in fungi which normally have photoinduced carotenogenesis is supported by the finding that the mutant carA5 carS42 of Phycomyces blakesleeanus has reduced levels of pigment formation in light (López-Díaz and Cerdá-Olmedo, 1980). This mutant totally lacks photoinducible carotenogenesis.

Based on the observations that carotenoids are not detected in hyphae (Fig. 15) and that carotenogenesis is not photoinduced in arthrospores (Table 2; Figs. 15, 19, and 21), it was originally thought that this strain of T. mentagrophytes is analogous to the wc strains of N. crassa (Perkins, 1972; Perkins et al., 1962). The wc strains (white hyphae and orange conidia) are thought to be regulatory mutants

which have defects in photoinduced hyphal carotenogenesis. Since, however, carotenogenesis in Trichophyton tonsurans occurs during the same developmental stages as T. mentagrophytes (Results, Section C 8), it is likely that carotenoid formation may be normally repressed in the hyphae of Trichophyton.

It was also found that the microconidia of both T. mentagrophytes and T. tonsurans accumulate carotenoids when developed in the dark on SDA at 25°C (Mock and Hashimoto, unpublished data). Pigmentation only occurs in fairly old cultures (6 weeks or older). In T. mentagrophytes, these "pigmented" microconidia contain the same carotenoids, although in different proportions, as arthrospores. Since carotenoids are formed in older cultures and associated mainly with swollen microconidia, it is possible that these spores may be activated, as has been previously reported in T. mentagrophytes (Hashimoto et al., 1972). All attempts to promote carotenogenesis in developing or younger microconidia were unsuccessful. Therefore, the relationship between carotenogenesis and microconidiation remains unclear.

From the data presented in Fig. 15, it is apparent that continuous white light has no effect on hyphal growth, the dry wt increase associated with arthrosporulation, nor the arthrosporulation process itself. Pigmentation, however, is constantly suppressed by white light. Furthermore, the finding that carotenoids isolated from light- and dark-grown arthrospores are almost identical (Figs. 16, 17, and 18) suggests that the photosuppression of carotenogenesis in arthrospores is of a quantitative nature with each carotenoid being

proportionally reduced.

Considering that carotenoids are thought to serve as photoprotective agents in fungi, the suppressive effect of visible light on pigmentation is rather "surprising" (Ruddat and Garber, 1983). As reviewed in a previous section (Chapter I), there are only a few fungi which have been shown to have reduced levels of carotenoids under light. Those studies have been confined to examining the effect of visible light on cells in culture. The suppressive effect of white light on carotenoid accumulation has been observed to occur during sporangial formation in a mutant of the aquatic fungus Blastocladiella emersonii (Cantino and Horenstein, 1956) and during mating in the Phycomycetes Choanephora cucurbitarum (Chu and Lilly, 1960) or Blakeslea trispora (Sutter, 1970). It was not mentioned in these studies whether photosuppression of carotenogenesis is associated with hyphae, spore apparati, or both. Furthermore, the effect of white light on sporulation was not indicated. In the present study, however, carotenogenesis in T. mentagrophytes is photosuppressed during arthrosporulation, which is not altered by light (Fig. 15).

It has been reported that there is an inverse relationship between white light intensity and carotenoid accumulation in the hyphae of Epicoccum nigrum (Gribanovski-Sassu and Foppen, 1969) and in the yeasts Rhodotorula glutinis, Sporobolomyces pararoseus, and S. roseus (Bobkova, 1965). In E. nigrum, continuous white light (110-2500 lux) was shown to slightly stimulate mycelial growth, while the dry wt of R. glutinis, S. pararoseus, and S. roseus was significantly reduced at

higher intensities of light (2500 lux). Although a dose-dependent decrease in carotenoid accumulation is also observed in T. mentagrophytes (Fig. 21), increases in hyphal growth and the dry wt associated with arthrosporulation are not significantly affected at light intensities even 5- to 10-fold higher than those used for these other fungi. It should also be pointed out that the qualitative composition of carotenoids in the above-mentioned yeasts was progressively altered with increasing intensities of white light from 150-2500 lux (Bobkova, 1965). On the other hand, these intensities of white light caused a quantitative reduction in all the carotenoids of T. mentagrophytes (data not shown).

When grown under weak intensities of white light (150 lux), S. pararoseus formed more carotenoids than in darkness (Bobkova, 1965). In contrast, T. mentagrophytes has reduced levels of carotenoid accumulation in arthrospores developed under the lowest attainable intensities of white light (approx 40 lux) (Fig. 21). In addition, carotenogenesis in arthrospores is not photoinduced or photostimulated even when the duration of irradiation is considerably shortened (Table 2).

Despite the observed differences in photosuppression of carotenogenesis in the fungi mentioned above, it is important to note that low or moderate intensities of prolonged white light are effective in reducing carotenoid accumulation. In previous studies, the reactions involved in this photosuppression of fungal carotenogenesis were not investigated. Several mechanisms have been postulated (Bobkova, 1965; Cantino and Horenstein, 1956; Chu and Lilly, 1960; Gribanovski-Sassu

and Foppen, 1969; Sutter, 1970). Because of the photolabile nature of carotenoids, some of these workers suggest that the pigments become bleached in fungi which have been subjected to relatively long exposure to visible light. Likewise, visible light may cause inhibition of carotenoid biosynthesis with or without the concomitant photodestruction of pigment. The wavelengths of visible light responsible for suppression of fungal carotenogenesis have not been previously examined. Furthermore, the reactions proposed to cause photosuppression have not been studied using cells under carotenogenic arrest or carotenoid-containing cytoplasmic extracts.

The data obtained in the present study suggest that red and blue light suppress carotenoid accumulation in arthrosporulating T. mentagrophytes quantitatively but not qualitatively (Figs. 22, 23, and 24). The relative percentage of energy that blue and red wavelengths contribute to white light could not be determined. Blue light, in general, has the most effective wavelengths for generating many fungal responses, including photoinduction or photostimulation of carotenogenesis (Tan, 1978). In addition, it has recently been found that blue light affects the enzymatic catabolism of carotenoids in Phycomyces blakesleeanus (Raugei et al., 1982). Since a variety of fungal responses have been shown to be oppositely affected by blue light (Tan, 1978), it was not entirely unexpected that suppression of carotenogenesis in arthrosporulating T. mentagrophytes appears to be due primarily to blue light (Fig. 22).

The data of Figs. 22, 23, and 24 do not reveal the mechanism for

blue light-mediated suppression of carotenogenesis in arthrospores. Since developing arthrospores shifted to higher intensities of blue light (Fig. 26) were observed to contain less carotenoids than at the time of initial transfer, it is possible that at least some of these pigments become photobleached either by chemical or enzymatic reactions. A similar decrease in carotenoid content was also observed in the carA5 carS42 mutant of Phycomyces blakesleeanus; however, pigment reduction was presumed to be due to photolysis (López-Díaz and Cerdá-Olmedo, 1980).

It has been reported by Zechmeister (1962) that visible light catalyzes the formation of colorless cleavage products from carotenoids in solvent solutions. These products are detectable in the UV. As shown in Fig. 24 (insert), blue light causes an increase in UV-absorbing material, which co-migrates with phytoene, in arthrospores. It is unknown whether this material may represent carotenoid photodegradative products.

The suppressive effect of red light on carotenogenesis in T. mentagrophytes is unusual and occurs only in mature arthrospores which have been continuously irradiated during formation on SDA-acetate (compare Figs. 22 and 25). Although it is possible that some early step in the arthrosporulation process may be affected, it is more likely that high fluences of red light, obtainable through prolonged irradiation, are necessary to suppress carotenoid accumulation in arthrosporulating T. mentagrophytes. This question may possibly be resolved through the use of high energy light sources, such as a ruby

laser.

Until fairly recently, convincing reports of fungal photoresponses influenced by the red end of the spectrum have not been available. Many responses in the 620-650 nm range of red light are thought to be mediated by the photoreceptor phytochrome. Further characteristics of phytochrome involvement include saturation by low irradiation energies and a reversion of this effect by a subsequent short exposure to far-red light (Jose and Vince-Prue, 1978). Indeed, Valadon et al. (1979) suggest that phytochrome mediates carotenogenesis in Verticillium agaricinum. Phytochrome has not as yet been isolated from fungi. No attempt was made to determine its presence in T. mentagrophytes arthrospores.

The best fit curves for suppression of arthrospore carotenogenesis by red or blue light in Fig. 22 were determined to be exponential (see Results, Section C 6). Each exponential equation was subsequently linearized by taking the natural logarithm (ln) of y. Based on the emission spectra presented in Fig. 13, we assigned maximum emission wavelengths (λ_{max}) of 460 and 650 nm to blue and red fluorescent light, respectively. If we assume a linear relationship exists between λ_{max} and the slope of the corresponding logarithmic plot of pigment content versus light intensity, then for any other light source of relatively narrow band width between 460 and 650 nm the slope of similar logarithmic plots is related to wavelength by equation 1:

$$S = 0.032\ (\lambda_{max}) - 2.14 \qquad (1)$$

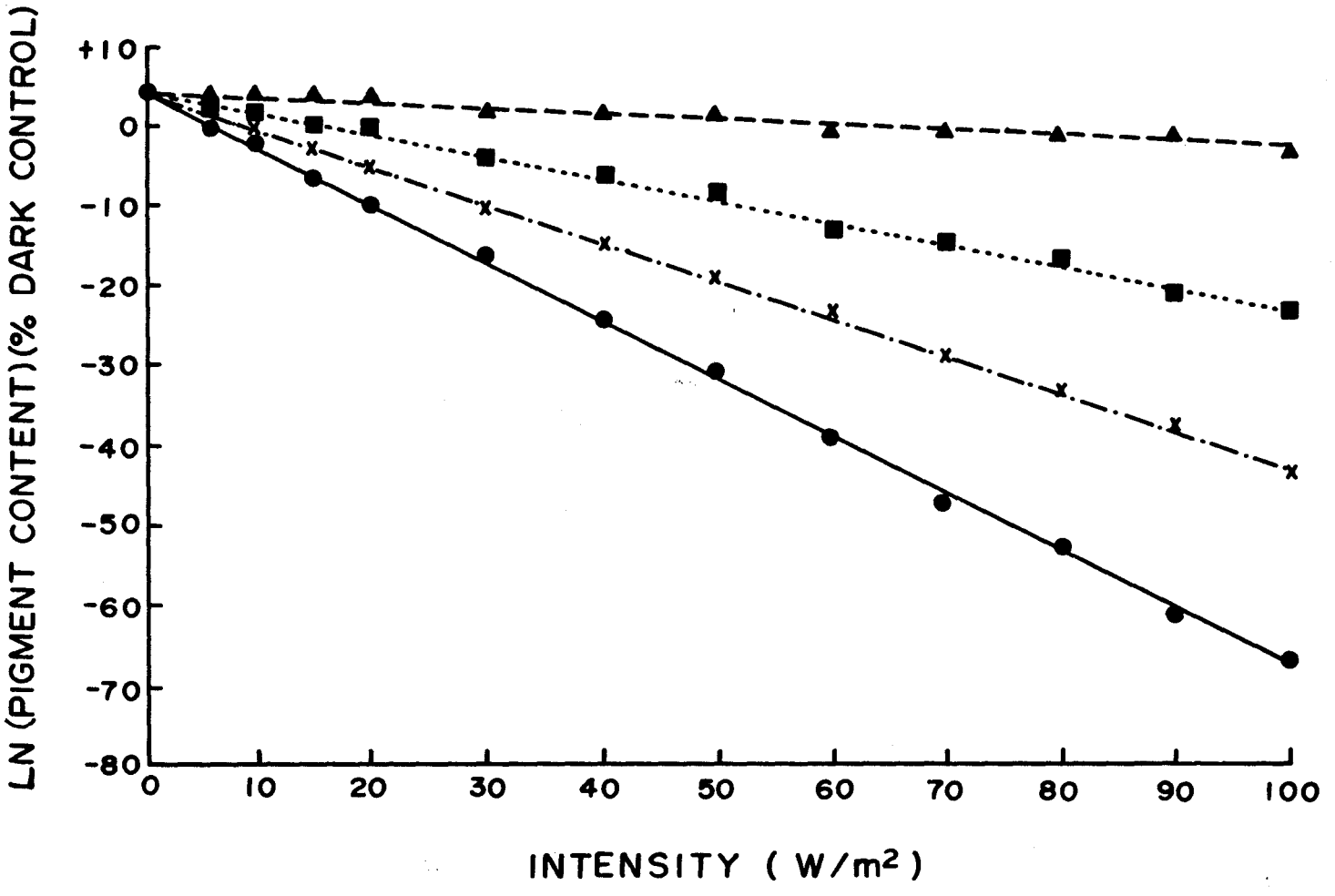
where λ_{max} is the major emission wavelength of the light source, and S

is the slope ($\frac{\ln \text{ pigment content}}{\text{intensity}}$) of the line associated with this wavelength. Equation 1 was derived by interpolating between the data points for red and blue light used in this study.

Based on the above assumptions, it should therefore be possible to determine the approximate amount of pigment content (% dark control) in arthrospores for a given light intensity at a particular wavelength. As examples, hypothetical intensity lines are projected for green and yellow fluorescent light in Fig. 36. These light sources have been assigned λ_{max} values of 525 nm and 585 nm based on emission spectra provided by GE. If arthrospores are irradiated under the specified cultural conditions using green or yellow light at intensities of 5 W/m^2, the amount of pigment content (% dark control) is determined by taking the antilogarithm (e^x) of $\frac{S}{x}$. For arthrospores continuously irradiated with green light, pigment content would theoretically amount to approx 10% of the dark control, while, for yellow light, this value would be 30%. Admittedly, the assumptions used to formulate equation 1 may not hold true, but can be tested in future studies.

The bleaching effect of visible light on the carotenoids of T. mentagrophytes was tested by using low temperature (4°C) to arrest carotenoid synthesis. The results, summarized in Figs. 28, 30, 31, and 32, strongly imply that moderate intensities of white light cause nonenzymatic photobleaching of carotenoids in T. mentagrophytes arthrospores. From these data, it is apparent that photobleaching of carotenoids in cell-free extracts and isolated pigment granules occurs much more rapidly than in whole arthrospores. Why such differences

Fig. 36. Projection of hypothetical intensity lines for yellow and green light. Lines for red (▲------, λ_{max} = 650 nm) and blue (●———, λ_{max} = 445 nm) light were derived by conversion of the exponential curves of Figs. 24B into linear equations. Based on the slope of each equation, corresponding lines were extended to intensity values of 100 W/m^2. Hypothetical lines for green (x—·—·, λ_{max} = 525 nm) and yellow (■·····, λ_{max} = 585 nm) light were calculated from S = 0.0032 (λ_{max}) - 2.14.


LN (PIGMENT CONTENT)(% DARK CONTROL)
+10
0
-10
-20
-30
-40
-50
-60
-70
-80
0
10
20
30
40
50
60
70
80
90
100
INTENSITY (W/m2)

exist, however, is not clearly understood. It is possible that the cell breakage and subsequent isolation procedures used in this study render carotenoids more easily modifiable by light. Another possibility is that the relatively thick arthrospore wall, or perhaps an intact cell membrane, is light-protective with respect to intracellular carotenoids.

The inverse relationship between blue light intensity and pigmentation of whole arthrospores incubated at 4°C (Fig. 29) further suggests that carotenoid photobleaching may account for at least part of the dose-dependent suppression of carotenogenesis by blue light observed in arthrosporulating T. mentagrophytes (Fig. 22). Due to the fact that higher temperatures are known to adversely affect the stability of carotenoids (Liaaen-Jensen and Jensen, 1971), it is possible, however, that the degree of carotenoid photobleaching at 37°C may be different.

That photobleaching of carotenoids may occur at 37°C is also implied by the finding that isolated pigment granules irradiated with visible light at 37°C have a similar, although not identical, pigmentation loss as those incubated at 4°C (Fig. 31). It is unknown in Fig. 31 whether the increased reduction of pigment in dark-incubated granules at 37°C is a result of higher temperature and/or increased activity of carotenoid degrading (altering) enzymes. Judging from the amount of pigment loss in light-exposed granules at 37°C, any increase in this type of enzymatic activity as a result of irradiation appears to be minimal.

In the present study, irradiation of cell-free extracts or isolated pigment granules in an atmosphere of reduced oxygen slightly retards pigment loss, thereby suggesting some type of oxygen involvement in carotenoid photobleaching (Figs. 30 and 32). In both types of cell-free fractions, dark controls began losing pigment by 24 h. The exact reason for this decrease is unknown. In order to more accurately assess if oxygen affects carotenoid photobleaching in these preparations, it will be necessary to use more stringent conditions of anaerobiasis, such as those described by Bramley and Davies (1975). In addition, the use of various reaction specific antioxidants or scavengers may further elucidate the nature of this pigmentation loss.

It should also be noted that photobleaching of carotenoids is caused by blue light and not red light (Fig. 33). Since carotenoids strongly absorb in the blue wavelength region, this observation is not totally unexpected and may partially explain why suppression of carotenogenesis in arthrosporulating T. mentagrophytes is due primarily to blue light (Fig. 22).

At present, the reactions involved in photobleaching of carotenoids have not been firmly established. For photochemical bleaching, the effect of visible light has been examined in solvent solutions of carotenoids (Carnevale et al., 1979; Tsukida et al., 1966) and in carotenoid-containing liposomes (Anderson and Krinsky, 1973). The loss of pigmentation under light is believed to occur by oxidative breakdown of carotenoids with the initial formation of carotenoid epoxides (Simpson et al., 1976). Further derivatives or catabolites have not

been convincingly identified. In the present study, the reactions and products of carotenoid photobleaching were not characterized. Since fairly pure preparations of isolated carotenoid-containing granules from arthrospores are available, and carotenoids can be rapidly and easily extracted from them, the future study of the effect of light on carotenoid photobleaching in these organelles appears to be feasible and may more readily depict pigment photobleaching at the cellular level rather than the synthetic systems previously used.

It is known that carotenoids serve as substrates for lipoxygenase and other "carotenoid destroying" enzymes. The majority of work concerning the enzymatically catalyzed oxidation of carotenoids has been done with the soya lipoxygenase system (Weber et al., 1974). In this system, carotenoids act as secondary substrates and appear to function as antioxidants to a primary fat substrate (Simpson et al., 1976). Phytochrome control of lipoxygenase activity has also been demonstrated (Oelze-Karrow and Mohr, 1976). To date, the only fungus in which lipoxygenase has been identified is Fusarium oxysporum (Matsuda et al., 1976). The properties of this enzyme, however, are quite different from plant enzymes. Furthermore, the ability of this enzyme to oxidize carotenoids has not been studied. Although there is some indication that carotenoids in arthrospores might be enzymatically bleached at 37°C (Fig. 31), it remains to be determined whether carotenoid oxidizing enzymes are present in T. mentagrophytes and whether they are regulated by visible light.

To further characterize the mechanism by which visible light

suppresses carotenogenesis in T. mentagrophytes, we took advantage of the observation that arthrospores accumulate carotenoids endotrophically, that is, in the absence of any exogenous carbon and nitrogen sources. This endotrophic system, which occurs in developing as well as mature arthrospores, has proven to be a valuable tool for the study of light-mediated carotenoid suppression. In contrast to agar cultures where multicellular layers are necessary for complete arthrosporulation and pigmentation, virtually single layers of cells can be used in the endotrophic system. Also, by starting with a "batch" population of arthrosporulating cells, there is less pigment variation of identical samples than in agar cultures. Therefore, smaller changes in pigmentation can be observed.

As shown in Figs. 34 and 35, endotrophic carotenoid accumulation in arthrospores is suppressed by low intensities of white light, mainly from the blue region of the spectrum. It is also dose dependent. Although not shown, higher intensities of white or blue light (approx 2 W/m^2) cause arthrospores to contain less pigment than the initial "batch" control. These data suggest that the suppressive effect of visible light on endotrophic carotenoid accumulation is similar to that observed on SDA-acetate and does not result from some type of light interaction with media components (Leach, 1971).

It should be pointed out that arthrospore formation itself is able to proceed endotrophically in phosphate buffer once the sporulation process begins (R. Emyanitoff, Ph. D. dissertation, Loyola University of Chicago, 1978). Endotrophic sporulation has been described in

a number of bacteria and has been used to study the requirements for sporulation (Vinter, 1969). The present study demonstrates some added features that can be derived from the use of an endotrophic sporulation system.

The observation that red light (1,000 lux) does not cause a decrease in endotrophic carotenoid accumulation is noteworthy (Fig. 35). As mentioned previously, long periods of irradiation with this intensity of light may be necessary to demonstrate pigment suppression in arthrosporulating T. mentagrophytes. Arthrospores transferred to phosphate buffer at day 3 subsequently have negligible amounts of endotrophic carotenoid accumulation. Therefore, a comparison of carotenoid reduction under continuous red light (1,000 lux) in SDA-acetate cultures (Fig. 22) with corresponding endotrophic cultures (day 5 of A in Fig. 35) is impossible.

The data of Fig. 34 also indicate that fairly low intensities of white or blue light cause insignificant photobleaching of carotenoids at 37°C. The highest nonbleaching intensity of either type of light cannot be determined since formaldehyde-treated cells have larger pigment losses than buffer controls after continuous irradiation with blue light (2 W/m^2 or higher) for 24 h at 4°C (Mock and Hashimoto, unpublished data).

It is apparent that these nonbleaching intensities of white or blue light suppress carotenoid accumulation in a dose-dependent manner. Therefore, it is postulated that, in addition to nonenzymatic photobleaching of carotenoids, one or more other processes must be involved

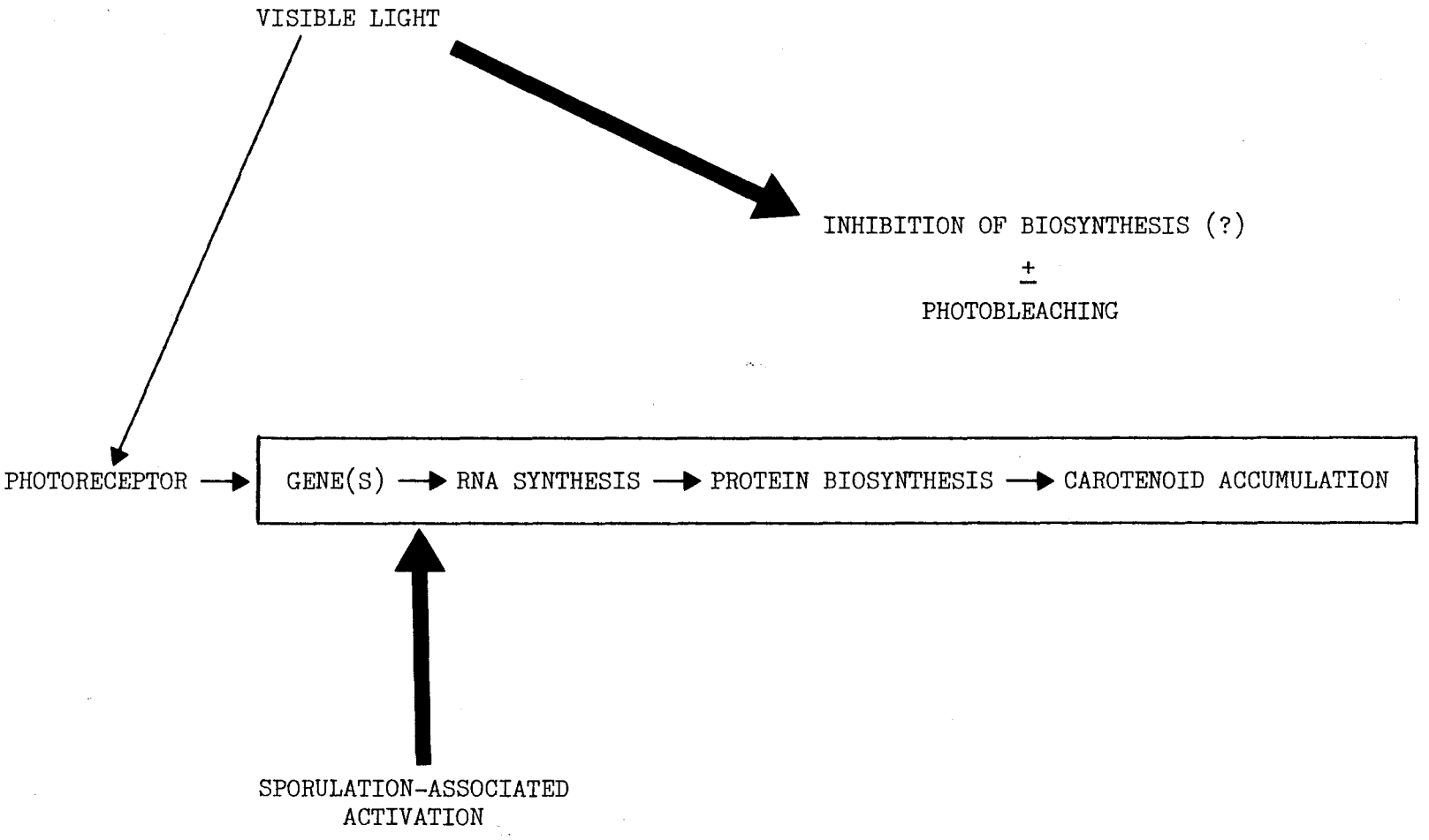
in the light-mediated suppression of carotenoid accumulation in arthrospores. Furthermore, it is likely that photochemical bleaching of carotenoids and these additional processes are concurrently suppressing carotenoid accumulation at high intensities of visible light. Three possibilities, in any combination, may explain this additional light-promoted suppression of carotenogenesis in arthrospores: First, as was originally suggested for fungi with light-mediated suppression of carotenogenesis, pigment biosynthesis is directly inhibited by visible light. Second, suppression of carotenoid accumulation may result from some indirect light effect, which, for example, could reduce the amount of carotenoid precursors. Third, carotenoid degrading enzymes may be induced or activated by light.

Further studies, such as following the fate of labeled carotenoids and their precursors under bleaching and nonbleaching intensities of blue light, may resolve the mechanisms involved in the suppression of carotenoid accumulation in arthrospores. As pointed out in Chapter I, there are certain inherent problems associated with the use of radiolabeled substrates in carotenogenesis investigations. The relatively thick arthrospore wall, which appears to confer resistance to a variety of antimycotics (Hashimoto and Blumenthal, 1978) may also preclude the use of even early carotenoid precursors, such as radiolabeled mevalonate and isopentenyl pyrophosphate. It is therefore likely that a major approach for determining photosuppression of carotenogenesis will be the identification and characterization of light-sensitive enzymes or processes in cell-free extracts of arthrospores.

The data in Fig. 15 and Table 2 indicate that, in T. mentagrophytes, carotenoids accumulate during arthrosporulation; however, these pigments are not detected in the hyphal stage. It is also clear that, in arthrosporulating T. mentagrophytes, carotenogenesis, which occurs in the dark (Fig. 15), is suppressed in a dose-dependent fashion by visible light, even at very low intensities (Figs. 21 and 22). Photobleaching of carotenoids was observed in arthrospores under carotenogenic arrest (Figs. 28 and 29) and in cell-free extracts, including isolated carotenoid-containing granules, from mature, pigmented arthrospores (Figs. 30, 31, 32, and 33). Furthermore, at nonbleaching intensities of white and blue light, the endotrophic accumulation of carotenoids is suppressed (Fig. 34), thereby suggesting that photobleaching of pigment is not solely responsible for the light-mediated decrease of carotenogenesis in T. mentagrophytes.

Taking into account the pre-existing information on carotenoid photoinduction in fungi, a model is proposed for light-mediated suppression of carotenoid accumulation in arthrosporulating T. mentagrophytes (Fig. 37). It is suggested that carotenoid biosynthesis in arthrospores bypasses the photoreceptor and photochemical reaction product which affects carotenoid genes. Instead, carotenoid accumulation appears to be invoked through sporulation-specific processes. The exact step in the classical pathway (box in Fig. 37) at which sporulation-associated activation occurs is unknown. In the above model, low intensities of visible light are postulated to suppress carotenoid accumulation in arthrospores, possibly by inhibition of

Fig. 37. Proposed model for the effect of visible light on carotenogenesis in fungi. Thick arrows indicate the regulatory system found in T. mentagrophytes arthrospores.


VISIBLE LIGHT
INHIBITION OF BIOSYNTHESIS (?)
±
PHOTOBLEACHING
PHOTORECEPTOR
GENE(S)
RNA SYNTHESIS
PROTEIN BIOSYNTHESIS
CAROTENOID ACCUMULATION
SPORULATION-ASSOCIATED
ACTIVATION

carotenoid biosynthesis. At higher intensities, both carotenoid photobleaching and the possible inhibition of pigment synthesis operate concurrently.

As mentioned earlier in the Discussion, light-mediated suppression of carotenoid accumulation in fungi with photoinducible carotenogenesis is likely to be masked under conditions of prolonged irradiation. Therefore, the pigment content of these organisms represents a balance between photoinduction and photosuppression of carotenogenesis. It is predicted that mutants which lack photoinducible carotenogenesis and synthesize these pigments constitutively in the dark should exhibit photosuppression of carotenoid accumulation. Furthermore, based on the findings that a few fungi do have a reduction of carotenogenesis under visible light (see Chapter I), it is anticipated that other carotenogenic fungi in which photoinducible carotenogenesis is normally absent will also demonstrate this photosuppressive effect, which appears to be part of a rather complex light regulatory system for pigmentation.

There have been only a few reports which indicate that visible light inhibits the germination of fungal spores. White light suppresses the germination of Monilina mali conidia (Harda, 1975) and Pseudoarachniotus marginosporus ascospores (Bragg, 1981). In more detailed studies, blue light was found to inhibit germination of uredospores from Puccinia graminis and P. recondita (Calpouzos and Chang, 1971), as well as the germination of microconidia from several dermatophytes (Buchníĉek, 1974). The mechanism of inhibition is unknown, although some type of photodynamic effect may be involved in dermatophytes.

Continuous blue light (16 W/m^2) significantly inhibits the germination of T. mentagrophytes arthrospores (Results, Section F; Table 4). This observation was not totally unexpected, since T. mentagrophytes is one of the dermatophytes whose microconidial germination is affected by blue light (Buchníček, 1974). To our knowledge, this is the first report demonstrating the inhibitory nature of blue light on arthrospore germination of dermatophytes. Of further importance is the fact that, in T. mentagrophytes, two different types of spores, microconidia and arthrospores, may be photoinhibited with respect to germination. This suggests that visible light may in some way be harmful during or after germination. Some preliminary data also indicate that arthrospore germ tube elongation may be negatively affected by blue light (Mock and Hashimoto, unpublished results).

In fungi, the role of carotenoids is not firmly established. Several main functions attributed to carotenoids include photoprotection, photoreception, protein and membrane stabilization, and the influence of secondary metabolites, such as trisporic acids for sexual reproduction and sporopollenins for cell wall stability (Krinsky, 1971). In addition, it has been suggested that carotenoids which are stored within spores may be acting as energy reserves (Mills and Cantino, 1977; Weber and Hess, 1974).

Based on the previous findings in this investigation, it is unlikely that the carotenoids in T. mentagrophytes arthrospores are functioning as classic "blue light photoreceptors," membrane stabilizers, or precursors of the secondary metabolites, trisporic acid and

sporopollenin. Furthermore, the relatively low concentration of carotenoids in isolated lipid granules contraindicates a major storage or energy reserve capacity. Since methods were available for the fairly synchronous activation and germination of arthrospores (Hashimoto and Blumenthal, 1977), we tested the effect of continuous blue light on the germinative ability of arthrospores which were physiologically manipulated to contain different amounts of carotenoids. The data in Table 4 indicate that, in some cases, arthrospores with reduced carotenoid content do have significantly lower amounts of germination when irradiated with blue light. It is unknown in this study whether carotenoids are synthesized during germination. However, the amount of increase would probably be minimal for the relatively short irradiation period used. Clearly this parameter must be further examined under light and darkness.

The results shown in Table 4 suggest a possibility that carotenoids may play a protective role during arthrospore germination under light. To date, a photoprotective function for carotenoids in fungi has been demonstrated in Sporidiobolus johnsonii (Goldstrom, 1964), Dacryopinax spathularia (Goldstrom and Lilly, 1965), Neurospora crassa (Blanc et al., 1976; Ramadan-Talib and Prebble, 1978), and Fusarium aquaeductuum (Huber and Schrott, 1980). Studies with N. crassa indicate that carotenoids within mitochondria appear to confer protection against light-inactivation of respiratory quinones (Ramadan-Talib and Prebble, 1978).

It is apparent that the methods used for preparing the different

types of arthrospores in Table 4 may alter other cellular processes in addition to reducing carotenoid accumulation. Even though the germination rates of these various kinds of arthrospores appear normal in the dark, other components besides carotenoids may be responsible for visible light-mediated inhibiton of germination. To undertake further studies, more precise methods of altering pigmentation will be necessary. Specific inhibitors for carotenogenesis, such as [4-chloro-5(dimethylamino)-2-trifluoro-m-tolyl-3-(2H)pyridotinone] (SAN 6706) and [2-(4-chlorophenylthio)-triethylamine hydrochloride] (CPTA), should be tested. In addition, the creation of pigment-deficient mutants may be helpful. In the above study, arthrospores were germinated on a relatively rich medium under moderate intensities of blue light. It is possible that more striking results, such as killing, might be attained by using a less rich, or a minimal, medium and higher intensities of blue light. The employment of such media will also be necessary to better determine the nutritional requirements of arthrospores during germination and germ tube formation under light.

There has been a tendency to seek a universal function for carotenoids in microorganisms and plants. If a single one exists, it has not yet been clearly ascertained. From a variety of studies, it has been suggested that the most plausible proposal is that carotenoids protect cells from damage caused by incidental absorption of visible light. On the basis of preliminary studies in this investigation, T. mentagrophytes may be a most suitable candidate for future studies involving carotenoid photoprotection.

SUMMARY

The effect of visible light on carotenoid accumulation of arthrosporulating Trichophyton mentagrophytes ATCC 26323 was investigated. The dermatophyte T. mentagrophytes produces several carotenoids during arthrospore formation on Sabouraud dextrose agar at 37°C. In arthrospores, essentially all of the carotenoid pigments (predominantly γ-carotene) were localized in discrete lipid granules, which became prominent only after the initiation of sporulation. These granules were surrounded by single-layered membranes. Trichophyton mentagrophytes accumulated carotenoid pigments only during sporulation. Carotenogenesis in arthrospores was not induced or stimulated by visible light. When this fungus was continuously irradiated with white fluorescent light, the resultant arthrospores contained considerably less carotenoids in comparison with dark controls. Reduction of carotenoid content in arthrospores was due primarily to blue light, although red light caused a slight decrease in pigmentation. The suppressive effect of visible light was dose dependent. Carotenoid accumulation was exponentially and inversely related to light intensity. Growth and arthrosporulation of this fungus were unaffected by light. An analysis of isolated carotenoids from irradiated arthrospores revealed that visible light caused a quantitative reduction in pigmentation.

The mechanisms underlying this visible light-mediated suppression of carotenoid accumulation were partially elucidated. Photobleaching

of carotenoids was demonstrated in whole spores in which de novo synthesis of pigment was arrested. In addition, cell-free extracts and isolated pigment granules from fully-pigmented arthrospores became photobleached when irradiated with white or blue light. Red light, however, had no effect. Oxygen appeared to enhance carotenoid photobleaching.

Rinsed arthrospores of T. mentagrophytes continued to accumulate carotenoids endotrophically when suspended in sodium phosphate buffer and incubated at 37°C. At low (nonbleaching) intensities of white or blue light, arthrospores undergoing endotrophic carotenoid accumulation had a dose dependent decrease in pigment content. Red light did not suppress endotrophic carotenogenesis.

Based on the literature and the results of this investigation, a model is presented to account for photosuppression of carotenogenesis in arthrosporulating T. mentagrophytes. It is proposed that, at low intensities of light, suppression of carotenoid accumulation possibly occurs by inhibition of pigment synthesis. At higher intensities, both photobleaching of carotenoids and a possible inhibition of pigment synthesis operate simultaneously.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology.

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