Photoregulation of carotenoid biosynthesis

in Aspergillus giganteus mut alba

A Thesis submitted by

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I would like to express my great thanks to the many people who have helped me during this time: firstly, to my supervisors Dr. P.M. Bramley and Dr. A. Mackenzie for their useful advice and sound guidance throughout my research. Secondly, I would like to thank the members of the Biochemistry Department for their help in various ways and Juba University, The Sudan, for their financial support.

Finally, I wish to express my thanks to my family for their patience and continuous support.
ABSTRACT

Aspergillus giganteus mut. alba, grown in shake culture in the light, accumulated 170 μg/g dry wt. of β-carotene after 6 days of growth. In darkness, no carotenoids were produced, although growth yields were the same as in illuminated cultures. A white light intensity of 50w/m² gave the maximum amount of β-carotene. Blue light, but not red light, induced β-carotene formation. Oxygen was required for photoinduction, but the effect of light was temperature-independent. A light induction period of 10 h was required for maximum β-carotene production and this was attained 48 h after illumination. 5-flourouracil (10⁻⁴M), actinomycin D (10⁻⁷M) and cycloheximide (10⁻⁴M) all prevent photoinduction of carotenogenesis indicating that photoregulation is at a transcriptional level.

Cell extracts from illuminated culture of A. giganteus mut. alba were able to incorporate radioactivity from [2⁻¹⁴C] mevalonic acid into carotenoids, but no such incorporation was obtained with extracts from dark grown cultures. Enzyme activities increased with culture age up to 4 days, and then decreased. A linear relationship was found between the incorporation of radioactivity into carotenoids and the amount of protein in the cell extract up to the maximum with 5 mg of protein. A rapid rate of increase with time in the incorporation of [2⁻¹⁴C] mevalonic acid into carotenoids was found up to 1 h at 37°C and 2 h at 24°C.

Oxygen was necessary for phytoene desaturation to β-carotene, the former being accumulated under anaerobic conditions. Glutathione, NADP, NAD, ATP,
K\textsuperscript{2+} and Mg\textsuperscript{2+} were found to be necessary for the incorporation of \(^2\textsuperscript{14}C\) mevalonic acid into carotenoids, while FAD was needed for lycopene and \(\beta\)-carotene formation. Neither FDN nor NADPH was required.

Coupling cell extracts of dark- and light-grown cultures of \textit{A. giganteus} with dark-grown \textit{A. giganteus} (which accumulates PPPP), and with \textit{Phycomyces blakesleeanus} C5 \textit{carB10} (-) (a phytoene accumulator) and C9 \textit{carR21} (-) (a lycopene accumulator), showed that the enzymes beyond prephytoene pyrophosphate i.e. phytoene synthase, phytoene dehydrogenase and lycopene cyclase, were totally photoinduced.
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The abbreviations used in this text are those recommended by the Biochemical Journal (1983) with the following additions:

<table>
<thead>
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<th>Abbreviation</th>
<th>Name</th>
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<tr>
<td>DMPP</td>
<td>Dimethylallyl pyrophosphate</td>
</tr>
<tr>
<td>DPA</td>
<td>Diphenylamine</td>
</tr>
<tr>
<td>FPP</td>
<td>Farnesyl pyrophosphate</td>
</tr>
<tr>
<td>GGPP</td>
<td>Geranylgeranyl pyrophosphate</td>
</tr>
<tr>
<td>HMG</td>
<td>3-hydroxy-3-methylglutaric acid</td>
</tr>
<tr>
<td>IPP</td>
<td>Isopentenyl pyrophosphate</td>
</tr>
<tr>
<td>MVA</td>
<td>Mevalonic acid</td>
</tr>
<tr>
<td>MVAP</td>
<td>5-phosphomevalonic acid</td>
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CHAPTER 1

INTRODUCTION

The carotenoid pigments are members of the terpenes, which are polymers of a basic branched chain \( \delta \)-carbon unit. The terpenes are classified by the number of \( \delta \)-units of which they are composed e.g., hemiterpene (\( C_5 \)), monoterpenes (\( C_{10} \)), sesquiterpene (\( C_{15} \)), diterpene (\( C_{20} \)), triterpene (\( C_{30} \)) and tetraterpene (\( C_{40} \)).

Carotenoids are compounds basically consisting of eight isoprenoid units joined so that the arrangement of the units is reversed at the centre of the molecule, i.e., the central methyl groups are in a 3, 8 position relative to each other whilst the remaining aromatic methyl groups are in a 1, 6 relationship. The series of conjugated double bonds constitutes the chromophoric system of the carotenoids. These characteristics are exemplified by the structure of lycopene (\( C_{40} \) \( \delta_{15} \)), (Figure 1.1), from which all other carotenoids can formally be derived by repetitive involving hydrogenation, dehydrogenation, cyclisation, insertion of oxygen in various forms, double bond migration, methyl migration, chain elongation or chain shortening. Oxygen containing carotenoids are termed xanthophylls, while the hydrocarbons are called carotenes.

1.2 Carotene nomenclature

Individual carotenoids are named by the suffix ending group which they contain. The semi-systematic names of some carotenes as proposed by
1:1 Introduction

The carotenoid pigments are members of the terpenes, which are polymers of a basic branched chain 5-carbon unit. The terpenes are classified by the number of C5 units of which they are composed e.g. hemiterpene (C₅), monoterpen (C₁₀), sesquiterpene (C₁₅), diterpene (C₂₀), triterpene (C₃₀) and tetraterpene (C₄₀).

Carotenoids are compounds basically consisting of eight isoprenoid units joined so that the arrangement of the units is reversed at the centre of the molecule. Thus the two central methyl groups are in a 1, 6 position relative to each other whilst the remaining non-terminal methyl groups are in a 1, 5 relationship. The series of conjugated double bonds constitutes the chromophoric system of the carotenoids. These characteristics are exemplified by the structure of lycopene (C₄₄H₇₄), (Figure 1:1), from which all other carotenoids can formally be derived by reactions involving hydrogenation, dehydrogenation, cyclisation, insertion of oxygen in various forms, double bond migration, methyl migration, chain elongation or chain shortening. Oxygen containing carotenoids are termed xanthophylls, while the hydrocarbons are called carotenes.

1:2 Carotene nomenclature

Individual carotenoids are named by the specific end groups which they contain. The semi-systematic names of some carotenes as proposed by
Figure 1:1 Lycopene
the Commission on Biochemical Nomenclature (1975), are listed below, together with their trival names:

<table>
<thead>
<tr>
<th>Trival name</th>
<th>Semi-systematic name</th>
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<tbody>
<tr>
<td>β-Carotene</td>
<td>β,β'-carotene</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>β,ε-carotene</td>
</tr>
<tr>
<td>γ-Carotene</td>
<td>β,γ-carotene</td>
</tr>
<tr>
<td>δ-Carotene</td>
<td>7,8,7',8'-tetrahydro-γ,γ-carotene</td>
</tr>
<tr>
<td>Lycopene</td>
<td>γ,γ-carotene</td>
</tr>
<tr>
<td>Neurosporene</td>
<td>7,8-dihydro-γ,γ-carotene</td>
</tr>
<tr>
<td>Phytoene</td>
<td>7,8,11,12,7',8',11',12'-octahydro-γ,γ-carotene</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>7,8,11,12,7',8',-hexahydro-γ,γ-carotene</td>
</tr>
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</table>

Hereafter carotenes will be referred to by their trival names.

Distribution of carotenoids

Carotenoids are found in all groups of living organisms but are synthesised only by plants and microorganisms (Rau, 1976). In animals, the carotenoid pigments are accordingly derived from dietary sources, although they may be subjected to limited enzymatic transformation in the animal's body after assimilation (Pitt, 1971). Among the biological groups which synthesise carotenoids, a primary distinction can be made between photosynthetic forms (higher plants, algae, purple and green bacteria) and non-photosynthetic forms (fungi, yeasts, other bacteria and non-photosynthetic tissues of higher plants).
Functions of carotenoids

One of the more probable possibilities for the function of carotenoids in green plants is that they serve a residual protective function towards photodegradation catalysed by chlorophyll via the production of singlet oxygen (¹⁰O₂), a potent destroyer of cell components. Carotenes have the ability to "mop up" ¹⁰O₂ forming triplet oxygen (¹⁰O₂) and triplet carotene, both of which are harmless to the cell. They also act as light-harvesting pigments. In addition, carotenoids are found in animals, algae, fungi and bacteria and several functions not associated with photosynthesis have been ascribed to, for instance, the photoprotection of membranes, reproduction in fungi, and as a precursor of vitamin A. Another suggestion is that since carotenoid production normally occurs towards the end of log-phase growth in the fungal life-cycle, it may simply represent the product of a convenient "shunt" pathway. This pathway would operate to remove excess carbon from the environment, not to serve as a carbon source, but to act as an "osmotic buffer" for the fungus. It is difficult to see how this would be important, since the majority of fungi produce carotenoid in such small amounts that only a small part of the metabolic turnover would be involved in the synthesis.
Biosynthesis of carotenoids

Early studies using a variety of possible carotenogenic precursors showed that acetate, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) and mevalonic acid (MVA) were all converted into β-carotene (Yokoyama et al., 1962; Britton, 1976b). The first specific precursor for all terpenoids is MVA (C\textsubscript{10}) which is converted to the universal C\textsubscript{5} biological isoprene precursor, isopentenyl pyrophosphate (IPP) (see Figure 1.2). Bloch et al. (1959) demonstrated, using yeast autolysates, that MVA was phosphorylated into 5-phosphomevalonic acid (MVAP) and again into 5-pyrophosphomevalonic acid, the latter being converted into IPP with the loss of C-1. Isomerisation of IPP to dimethylallyl pyrophosphate (DMAPP), followed by a series of condensation reactions results in the formation of geranyl pyrophosphate (GPP, C\textsubscript{15}), farnesyl pyrophosphate (FPP, C\textsubscript{15}) and geranylgeranyl pyrophosphate (GGPP, C\textsubscript{20}, Figure 1.2).

Radioactivity from the labelled 5-carbon compound IPP was shown to be incorporated into squalene by yeast autolysates (Bloch et al., 1959), squalene and carotenoids in Streptococcus faecium (Taylor and Davies, 1982) and lycopene by tomato fruit homogenates (Varma and Chichester, 1962) and tomato fruit plastids (Porter and Spurgeon, 1979). Davies et al. (1975) were able to develop a cell free system from seed embryos of barley grain which had been used to prepare substantial quantities of radiolabelled C\textsubscript{5}–C\textsubscript{20} intermediates of...
Figure 1:2 Biosynthesis of GGPP (Davies, 1980)

The enzyme geranylgeranyl pyrophosphate synthase has been identified as a product from \( \text{HPR} \) (1975), with a soluble enzyme fraction prepared from immature Achiotus macrocarpa seeds and a white mutant of \( \text{HPR} \). The enzyme geranylgeranyl pyrophosphate synthase has been isolated from carrot and has been identified by these authors. The enzyme has been purified from yeast and liver samples and from human sources.

The biosynthesis of \( \text{HPR} \) is represented in Figure 1:6. It has been shown that 20-hydroxycholesterol can be converted into cholesterol by cells of \( \text{HPR} \) and 20-hydroxycholesterol can be incorporated into the sterol pool. Moreover, \( \text{HPR} \) was also shown to promote the biosynthesis of \( \text{HPR} \) from \( \text{HPR} \), which is grown in...
terpenoid biosynthesis from MVA. GGPP has been identified as a product from [2-14C] MVA with a soluble enzyme fraction prepared from endosperm homogenates from immature Echinocystis macrocarpa seeds (Oster and West, 1968) and a white mutant of Phycomyces blakesleeanus (Sandmann et al., 1980). The enzyme geranylgeranyl pyrophosphate synthetase which catalyses the consecutive chain elongation of C5 + C10 + C15 + C20 has been isolated from carrot root and pig liver (Nandi and Porter, 1964), pig liver (Holloway and Połjác, 1967), Micrococcus lysodeikticus (Allen et al., 1967). Other enzymes in the pathway have also been isolated. Prenyltransferases specific for the synthesis of FPP have been purified from yeast (Eberhardt and Rilling, 1975) and from chicken liver (Reed and Rilling, 1975).

The dimerisation of FPP via presqualene pyrophosphate (PSPP) results in the formation of squalene, the C30 acyclic precursor for sterols (Epstein and Rilling, 1970) (Figure 1:3).

1:5:2 Biosynthesis of phytoene

The proposed pathways for the synthesis of lycopersene and phytoene from GGPP are represented in Figure 1:4. It has been shown that radioactivity from [14C] GGPP was incorporated into phytoene by cell-free systems prepared form P. blakesleeanus (Lee and Chichester, 1969). Altman et al., (1972) showed that PPPP could be produced from [3H] GGPP by cell-free extracts from a Mycobacterium sp. grown in
Figure 1:3  Biosynthesis of squalene

\[
\begin{align*}
&\text{PretiwafPM}^* \text{PyTcpXosghAle} \\
&\text{NADPH}
\end{align*}
\]
Figure 1: Formation and possible conversion of prephytoene.

Pyrophosphate (Dayk, 1980)

Cyclopropylcarbonyl cation can be stabilized by:
(a) gain of $H_2$ from NADPH
(b) stereospecific loss of pro-$R H^+$
(c) pro-$S H^+$

GGPP

Lycopersene

All-trans phytoene

15-Cis phytoene
Furthermore, the $[^3]P$ PPPP thus synthesised could be converted by the same system into $[^3]H$ phytoene. A similar experiment using extracts from a phytoene accumulating mutant of *P. blakesleeanus* (Lee et al., 1972) revealed that on incubation with $[^1]P$ GGPP a radioactive bound intermediate (PPPP) was produced, which was then converted into $[^1]C$ phytoene. Radioactive $[^1]C$ PPPP was also seen to accumulate when purified yeast squalene synthetase or a system from an acetone powder of tomato fruit plastids were incubated with $[^1]C$ GGPP in the absence of NADPH (Qureshi et al., 1972, 1973; Barnes et al., 1973). However, in the presence of NADPH, $[^1]C$ lycopersene was detected. Both $[^1]C$ PPPP and $[^1]C$ lycopersene appeared to be converted into phytoene. Lycopersene also accumulated when a particulate fraction of *Neurospora crassa* mycelium was incubated with $[^1]C$ GGPP in the presence of NADPH (Grob et al., 1961). Therefore, it was thought possible that lycopersene may be a precursor for phytoene synthesis and would yield phytoene by losing two hydrogen atoms. However, there is now general acceptance that PPPP and not lycopersene is the intermediate for phytoene formation (Davies, 1975, 1980; Goodwin, 1980). There are now numerous reports using a variety of enzyme systems for synthesising phytoene and other carotenoids, in which radiolabelled lycopersene has not been found. Formation of phytoene from $[2^{-1}C]$ MVA, $[^1]C$ GGPP and $[^1]C$ PPPP has been demonstrated using enzymes prepared from tomato plastids (Jungalwala and Porter, 1967, Shah et al., 1968; Porter and Spurgeon, 1979), homogenates of the white mutant of *P. blakesleeanus* (Sandmann et al., 1980) and *P. blakesleeanus* CS carBlO(−) (Bramley and Davies, 1975).
The formation of radioactive lycopersene from $[^{14}\text{C}]$ GGPP by yeast squalene synthetase is thought to result from lack of substrate specificity by this enzyme (Davies and Taylor, 1976) and semi-purified phytoene synthetase from tomato fruit plastids no longer synthesised lycopersene (Porter et al., 1980).

1:5:3 Desaturation of phytoene

The sequential desaturation of phytoene to lycopene involves a series of didehydrogenations alternatively to the left and right of the central phytoene chromophore to produce in succession, phytofluene, $\gamma$-carotene, neurosporene and lycopene (Figure 1:5). In certain photosynthetic bacteria, however, $\gamma$-carotene is replaced by its asymmetrical isomer, 7,8,11,12-tetrahydrolycopene (Davies, 1970, Schmidt and Liaaen-Jensen, 1973), while in some other bacteria (Malhotra et al., 1970 a,b; Weeks, 1971) and fungi (Davies and Rees, 1973; Davies et al., 1974) both tetrahydrolycopene isomers are present. Each desaturation step involves the trans elimination of hydrogen.

The desaturation steps from phytoene to lycopene have been demonstrated with various cell-free systems. Kushwaha et al., (1970) showed that enzyme systems from tomato fruit incorporated radioactivity from $[^{14}\text{C}]$ phytoene into phytofluene, $\gamma$-carotene, neurosporene and lycopene. An enzyme preparation from spinach also demonstrated conversion of $[^{14}\text{C}]$ phytoene to phytofluene and lycopene.
Figure 1.5  Desaturation of phytoene

Phytoene

Phytofluene

\(\gamma\) - Carotene

Neurosporene

Lycopene
Using an enzyme system from a red tomato fruit plastid acetone powder, Qureshi et al., (1974) demonstrated the incorporation of radioactivity from $[^{14}C]$ phytofluene into $\xi$-carotene and from $[^{14}C]$ $\xi$-carotene into neurosporene, lycopene and cyclic carotenes. The last step of the normal desaturation pathway, neurosporene into lycopene, has been demonstrated using $[^{14}C]$ neurosporene with cell-free preparation from *Halobacterium cutirubrum* (Kushwaha et al., 1976) and *P. blakesleeanus* (Bramley et al., 1977). It has been shown that radioactivity is incorporated into more desaturated carotenoids when each of $[^{14}C]$ phytoene, $[^{14}C]$ phytofluene, $[^{14}C]$ $\xi$-carotene and $[^{14}C]$ neurosporene was incubated with the enzyme system from *H. cutirubrum* (Kushwaha et al., 1976).

**Figure 1.6**

Figure 1.6 shows the possible pathway of cyclisation of neurosporene and lycopene into the different carotene derivatives. Hill and Rogers (1969), using bean leaf chloroplasts and tomato plastids, demonstrated the incorporation of radioactivity in $\beta$-carotene from $[^{14}C]$ lycopene. The conversion of radiolabelled lycopene into $\beta$-carotene via $\gamma$-carotene has been demonstrated with cell-free systems of tomato (Kushwaha et al., 1970; Papastephanou et al., 1973), spinach (Kushwaha et al., 1969) and into $\beta$, $\gamma$- and $\delta$-carotene by isolated tomato plastids or spinach chloroplasts (Wells et al., 1964). Although these studies demonstrated that lycopene could be converted into $\beta$-carotene, they did not necessarily eliminate the possibility of cyclisation of neurosporene into $\beta$-zeacarotene either as an
Figure 1.6  Cyclisation of neurosporene and lycopene

nc 28 -

Neurosporene → α-Carotene

Lycopene → Υ-Carotene

α-Carotene → Ε-Carotene

ε-Carotene → n-Carotene

neurosporene has been found to occur before complete desaturation of precursors (Williams et al., 1969). Further evidence is provided by the exposure for ten minutes on a carotene, through lycopene or neurosporene, with the subsequent aqueous phase reaction products to be isomeric to those found in vivo. These results lead to the conclusion that precursors of neurosporene and carotenes are consistent with the hypothesis that both precursors are isomeric to neurosporene and carotenes. The relatively high amounts of retinol and 11-cis retinal are consistent with these suggestions that both precursors of neurosporene and carotenes are isomeric to those found in vivo. Neurosporene has been used to label the inclusions in the isomerisation of the retinoids, and cyclisation in carotene biosynthesis giving...
alternative or accompanying pathway. Indeed, β-zeacarotene was found in cultures of *P. blakesleeanus* inhibited by diphenylamine (DPA), an inhibitor of desaturation activity, thus indicating that cyclisation may occur before complete desaturation of carotenoids (Williams et al., 1965). β-zeacarotene has also been found in normal cultures of *N. crassa* (Davies, 1973) and various mutant strains of *Ustilago violacea* (Williams et al., 1984). Further evidence for the existence of the two pathways to γ-carotene, through either lycopene or β-zeacarotene, was demonstrated with cell-free systems from *P. blakesleeanus* (Bramley and Davies, 1976). The radioactivity incorporated into β-carotene from [2-14C] MVA by this system was effectively diluted out by the addition of unlabelled γ-carotene and neurosporene but not so effectively by either lycopene or β-zeacarotene. The relatively small amounts of radioactivity trapped in lycopene and β-zeacarotene are consistent with the view that both these carotenes are intermediates on different alternative biosynthetic routes. When this system was incubated with [14C] neurosporene equal amounts of radioactivity were trapped in lycopene and β-zeacarotene, suggesting that both pathways may be of equal physiological importance (Bramley et al., 1977).

1.6 Stereochemistry of carotenogenesis

1.6.1 Stereochemical aspects of phytoene synthesis

Stereochemical studies have been used to investigate the mechanism of dehydrogenation and cyclisation in carotene biosynthesis giving
greater insight into precise reaction steps, the intermediates involved, and the nature of the enzymes.

Mevalonic acid (Figure 1:7) has three prochiral centres. A prochiral centre has two identical substituents (hydrogen in the case of C-2, C-4 and C-5 of MVA). If one of the two chemically indistinguishable hydrogens at such centres is replaced by deuterium or tritium, for example, then a chiral centre is established. The hydrogen which when replaced by another substituent results in S chirality is known as the pro-S hydrogen. In the same way when the pro-R hydrogen is replaced by another substituent R chirality results, the hydrogens are designated $H_r$ and $H_s$. In the biosynthetic sequence leading to carotenoids there are several reactions in which a carotenogenic system has the choice of removing either the pro-R or pro-S hydrogen from each of these three centres. Chemically the two hydrogens at each centre are indistinguishable but enzymatically they are likely to retain their spatial individuality when attached to the asymmetric enzyme surface. Cornforth, Popjack and their colleagues synthesised the six species of MVA stereospecifically labelled with their deuterium or tritium and tested them in appropriate biochemical systems. The first experiments were carried out with [(4R)-4-$^2$H$_1$] and [(4S)-4-$^2$H$_1$] MVA which were incubated separately with a liver enzyme to yield FPP. In the case of [(4S)-4-$^2$H$_1$] MVA the farnesol obtained contained no deuterium whilst in the case of [(4R)-4-$^2$H$_1$] MVA a trideuterated species of farnesol was obtained. This meant that the same hydrogen (pro-S) was eliminated in the reaction leading to FPP formation. The same results were obtained when
Figure 1:7 Mevalonic acid

The formation of the tricyclic compound, mevalonate, leads to the production of mevalonic acid. This isomer is converted to isopentenyl pyrophosphate (IPP) which is then converted to farnesyl pyrophosphate (FPP). FPP is converted to geranylgeranyl pyrophosphate (GGPP) which is then converted to squalene (SQ). SQ is converted to cholesterol (CH). SQ is also converted to farnesyl pyrophosphate (FPP) which is then converted to geranylgeranyl pyrophosphate (GGPP) which is then converted to squalene (SQ). SQ is then converted to cholesterol (CH).

1.5.3 Geometric isomers

Although 16 cis-phytoene is the predominant isomer to accumulate in many microorganisms where desaturation has been induced, it appears that it is the all-trans isomer that is desaturated. 'Native' phytoene (containing 25% all-trans isomer) is much better than pure 16 cis-phytoene at diluting out radioactivity being incorporated from
[2-\textsuperscript{14}C, \((4R)-4-\textsuperscript{3}H,\)] and \([2-\textsuperscript{14}C, \,(4S)-4-\textsuperscript{3}H,\)] MVA were used. Similarly, when phytoene is formed only the pro-R hydrogens from C-4 are retained but not the pro-S hydrogens. Two phytoene isomers are found to accumulate in Nature, predominantly the 15-cis isomer although all trans-phytoene is the major form found in some non-photosynthetic bacteria. The question is whether both are formed biosynthetically as primary products or whether only one is formed from PPPP and then isomerised into the other. Figure 1:4 shows the possibility of their formation from PPPP. Loss of the pro-R hydrogen would yield all-trans-phytoene and the pro-S hydrogen, cis-phytoene. The carbon atoms involved arise originally from C-5 of MVA and by using [2-\textsuperscript{14}C \,(5R)-5-\textsuperscript{3}H,\] MVA it was shown that in tomato slices (Williams et al., 1967) formation of cis-phytoene resulted in the loss of the 5-pro-S hydrogens from C-15 and C-15'. This was confirmed by incubating [4-\textsuperscript{14}C \,(1S)-1-\textsuperscript{3}H,\] GGPP with a preparation from \textit{P. blakesleeanus}. No tritium was retained in the resulting cis-phytoene (Gregonis and Rilling, 1974). However, with a preparation from \textit{Mycobacterium sp}, the all-trans-phytoene was produced with the 1-pro-S hydrogen (tritium) retained.

1:6:2 Geometric isomers

Although 15 cis-phytoene is the predominant isomer to accumulate in many microorganisms where desaturation has been impaired, it appears that it is the all-trans isomer that is desaturated. 'Natural' phytoene (containing 2% all-trans isomer) is much better than pure 15-cis phytoene at diluting out radioactivity being incorporated from
[2-^{14}C] MVA into β-carotene by cell free systems from P. blakesleeanus (Bramley, 1973).

There is no doubt that either all-trans- or 15-cis-phytoene can be synthesised by different systems as the primary product of dimerisation of GGPP and that cis-phytoene can be converted into all-trans-phytoene can be converted into all-trans-phytoene. There is also support for the view that cis-trans isomerisation can take place at the level of phytofluene and Χ-carotene. Although cis-phytoene is converted into cis-phytofluene, there is little evidence for the conversion of cis-phytofluene into cis-5-carotene (Goodwin, 1980). However, the recent demonstration that the polyenes found in tangerine tomatoes are 15-mono cis-phytoene, 15,9'-dicis-phytofluene, 9,9'-dicis-Χ-carotene, 9,7',9'-tricis-neurosporene (proneurosporene) and 7,9,7',9'-tetracis-lycopene (prolycopene) (Clough and Pattenden, 1979) has stimulated reassessment of the accepted biosynthetic pathway of these compounds (Figure 1:8).

1:6:3 Stereochemistry of desaturation

In each step of desaturation one hydrogen is lost from carbons originating from the C-2 and C-5 of MVA. When [2-^{14}C,(S)-5-^{3}H_{1}] MVA and [2-^{14}C,5-^{3}H_{1}] MVA were incubated with tomato slices it became clear that the hydrogens lost were those originating from the 5-pro-R hydrogens of MVA (Williams et al., 1967a). A similar experiment with [2-^{14}C, (2R)-2-^{3}H_{1}] MVA or [2-^{14}C,(2S)-2-^{3}H_{1}] MVA was inconclusive. It was suggested that the prenyl transferases
Figure 1:8 Biosynthesis of cis carotenes (Porter and Spurgeon, 1979)

- 15-cis Phytoene → 15-cis Phytofluene → trans-Phytofluene
- cis-5-Carotene ← trans-5-Carotene
- Proneurosporene ← Neurosporene
- Prolycopene ← Lycopene

This effect was overcome by synthesizing stereospecifically labelled GDP using an E. coli strain. The resulting 4'-C-14 GDP, when incubated with tomato aleurone, showed that the precursor of VFA were eliminated during desaturation.
caused randomisation of label between the 2S and 2R positions (Goad, 1970). This effect was overcome by synthesising stereospecifically labelled GGPP using an *Echinocystis macrocarpa* system from 2S- and 2R-tritiated [2-\textsuperscript{14}C] MVA substrates (West et al., 1979). The resulting [\textsuperscript{14}C,\textsuperscript{3}H] GGPP, when incubated with tomato slices, showed that the pro-2S hydrogens of MVA were eliminated during desaturation.

1:6:4 Stereochemistry of cyclisation

Since both neurosporene and 5-carotene are substrates for cyclases, and 7,8,11,12,tetrahydro-5-\textsuperscript{y}-carotene is detected in DPA-inhibited cultures of *P. blakesleeanus* (Davies and Rees, 1973), it would appear that these cyclases have specificity for desaturated half-carotene molecules or perhaps only molecules with unsaturated 7,8-carbon bonds.

Although it was suggested that the \textsuperscript{e}-ring was isomerised from the \textsuperscript{b}-ring (Porter and Lincoln, 1950), inheritance studies with tomatoes suggest that both were formed independently from a common precursor (Tomes, 1967). From experiments with stereospecifically labelled [\textsuperscript{14}C, \textsuperscript{3}H] MVA, it was concluded that carotenoid cyclisation involved the formation of an active cyclic intermediate (Figure 1:9) stabilised by loss of a proton from either C\textsubscript{a} to form an \textsuperscript{e}-ring or from C\textsubscript{b} to form a \textsuperscript{b}-ring (Williams et al., 1967,a,b).

The PG1 strain of *Scenedesmus obliquus* when grown heterotrophically in the dark accumulates \textsuperscript{z}-carotene. Britton et al., (1977) harvested
Figure 1.9 Mechanism of carotene cyclisation

carotene (e.g., β-carotene, lutein, and canthaxanthin) after a period of autotrophic growth. Atom spectroscopic analysis demonstrated that each bicyclic carotene contained two molecules of deuterium at C2 and C2'. Deuterium incorporation into neoxanthin extracted from
Flavonol (1971) and 

position with 2H. Incorporation is revealed by proton n.m.r. analysis (Keller et al., 1978). This suggests that the proton attack proposed by Villani et al. (1975) is stereospecific for the carotene at the C-1,3 double bond of the acyclic precursor.

Colletor requirements

As yet, no carotenes have been purified. Therefore the reactions they describe may not be specific; they require only an essential factor or factors. The essential factor requirements for the different phases in the carotenoid pathway have been reviewed by Stanley (1963). Although the lack of pure systems has limited the accurate determination of the carotenoids necessary for the preparation of the precursors such as β-carotene (e.g., Thalassiosira pseudonana, Repa et al., 1974), spinach, lettuce, and others (Blau, 1973, tomato fruit,

Kowalczyk et al., 1977, R. grass, N. - California and New, 1956, 


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such cells, resuspended them in D₂O and extracted the bicyclic carotenoids (α-, β-carotene, lutein and zeaxanthin) after a period of autotrophic growth. Mass spectroscopic analysis demonstrated that each bicyclic carotenoid contained two molecules of deuterium at C₂ and C₂'. Deuterium incorporated into zeaxanthin extracted from Flavobacterium R1519 was also found to be located at the C₂ and C₂', position with 2S, 2S conformation as revealed by proton n.m.r. analysis (McDermott et al., 1973). This suggests that the proton attack, proposed by Williams et al. (1967b) is stereospecific for the re-re face at the C-1,2 double bond of the acyclic precursor.

1:7 Cofactor requirements

As yet, no carotenogenic enzyme has been purified. Therefore the reactions they catalyse and any cofactors they require can only be surmised from experiments with cell-free systems. The cofactor requirements for the different steps in the carotenoid pathway have been reviewed by Bramley (1985) and, as stated, the lack of pure enzymes has limited the accurate elucidation of the cofactor requirements.

Numerous cell-free systems are capable of forming phytoene from precursors such as MVA (e.g. Phaseolus vulgaris, Buggy et al., 1974, spinach, Grumbach, 1980, Zea mays, Berry et al., 1972, tomato fruit, Maudinas et al., 1977, N. crassa, Mitzka - Schnabel and Rau, 1981, P. blakesleeanus, De la Concha et al., 1983, Corynebacterium poinsettiae, Swift and Milborrow, 1981, H. cutirubrum, Kushwaha et al., 1976,

Obviously ATP and Mg++ (or Mn++) are required for the first steps up to IPP formation. However, ATP has been shown to stimulate some cell-free systems when either IPP (tomato, Maudinas et al., 1977; P. blakesleeanus, Clarke et al., 1981) or GGPP (Aphanocapsa, Clarke et al., 1982a) is used as substrate. Mn++ is an absolute requirement for cell-free systems from pea (Graebe, 1968), S. faecium (Taylor and Davies, 1982) and tomato (Maudinas et al., 1977). Other systems have no such requirement. Both FAD and NAD(P) stimulate phytoene formation in cell-free systems from Aphanocapsa (Clarke et al., 1982a), Flavobacterium (Brown et al., 1975) and S. faecium (Taylor and Davies, 1982). Phytoene synthesis is stimulated by NAD(P)H and FAD in systems from tomato (Maudinas et al., 1977), P. blakesleeanus (Yokoyama et al., 1962) and Staphylococcus aureus (Suzue, 1962). Stimulation is also effected by the addition of sulphhydrol reagents to cell-free preparations from Capsicum annum (Camara and Brangeon, 1981a,b), P. blakesleeanus (Lee and Chichester, 1969), S. faecium (Taylor and Davies, 1982) and tomato (Shah et al., 1968). In contrast, phytoene formation is inhibited by NAD in P. blakesleeanus (Yokoyama et al., 1962) and NADPH in tomato (Maudinas et al., 1977).
The cofactor requirements for the phytoene to lycopene sequence are not known with any certainty. Studies with extracts from spinach (Subbarayan et al., 1970), tomato (Kushwaha et al., 1970) and H. cutirubrum (Kushwaha et al. 1976) suggest that the conversion of phytoene to phytofluene (whether the cis or trans isomers) requires NADP, while the subsequent desaturation steps to lycopene are dependent upon FAD. Little is known about the number and properties of the cyclisation enzymes. The formation of α-, β-, δ-, and γ-carotenes in tomato cell-free systems requires FAD (Kushwaha et al., 1969) which also stimulates β-carotene formation by cell extracts from P. blakesleeanus (Clarke et al., 1981).

Site of carotenoid biosynthesis

In higher plants and algae carotenoid pigments are normally formed in the plastids, which might suggest that plastids are the site of carotenoid biosynthesis. Intact spinach chloroplasts have been shown to synthesise carotenoids from CO₂ via the Calvin cycle intermediates pyruvate and acetate (Grumbach, 1980). MVA has been converted into carotenoids using extracts from pea chloroplasts (Charlton et al., 1967; Buggy et al., 1969). A partially purified phytoene complex (MW 200,000), able to convert IPP to phytoene, has been isolated from tomato fruit plastids (Maudinas et al., 1975, 1977). Further purification resulted in loss of all activity with respect to phytoene biosynthesis, although enzyme activity for the synthesis of both GGPP and PPPP from IPP was retained in a MW 40,000 protein (Islam et al., 1977; Porter and Spurgeon, 1979). However, a more recent report has
shown that this protein was not pure but contaminated with numerous proteins including IPP isomerase and prenyl transferase (Spurgeon et al., 1984).

Fractionated cell extracts from chloroplasts of C. annuum incubated with [1-\(^{14}\)C] IPP showed that phytoene formation took place in the stroma, while desaturation and cyclisation reactions took place in membrane fractions (Camara and Monéger, 1982; Camara et al., 1982). In Narcissus chromoplasts, phytoene formation was catalysed by peripheral membrane proteins which easily dissociated while \(\beta\)-carotene biosynthesis was catalysed by integral membrane proteins of the inner chromoplast membrane (Kreuz et al., 1982). These results agree with similar studies using spinach chloroplasts where prenyl transferase was found to be located in the stroma (Block et al., 1981; Lütke-Brinkhaus et al., 1983); the enzymes for lycopene formation from phytoene (Lütke-Brinkhaus et al., 1983) and the conversion of zeaxanthin to violaxanthin (Costes et al., 1979) were located within the chloroplast envelope. Carotenogenic enzymes were not detected in the thylakoids (Lütke-Brinkhaus et al., 1983). The enzymes involved in conversion of phytoene to \(\beta\)-carotene are coded by nuclear genes and therefore be synthesised outside the chloroplast and transported there at some stage during organelle formation (Kirk and Tilney-Basset, 1967).

Cytosolic proteins prepared from H. cutirubrum were able to convert labelled MVA and IPP into \(\beta\)-carotene (Kushwaha et al., 1976). Phytoene synthetase was also found in the cytosolic fractions in Mycobacterium.
sp. (Gregonis and Rilling, 1974) and *Flavobacterium* R1560 (Britton et al., 1980), later enzymes were known to be membrane bound in *Flavobacterium*. In contrast, the steps from MVA to prenyl pyrophosphates are catalysed by a membrane-bound multienzyme complex in *M. luteus* (Evans and Prebble, 1980, 1981). The failure of membrane preparations from *Aphanocapsa* to use MVA or IPP as precursors for carotene synthesis *in vitro* suggests that these compounds are metabolized by soluble enzymes (Clarke et al., 1982a).

In fungi carotenoids are located either in a single organelle, e.g. mitochondria (Neupert and Ludwig, 1971) or in combinations of organelle fractions (Riley and Bramley, 1976; Mitzka-Schnabel and Rau, 1980). However, subcellular fractionation studies have shown that the sites of carotenoid biosynthesis and storage are different. For example, conversion of GGPP into carotenoids by cell-free preparations from *N. crassa* takes place maximally with membrane fractions of the endoplasmic reticulum (Mitzka-Schnabel and Rau, 1981) although the majority of carotenoids are located in spherosomes (Mitzka-Schnabel and Rau, 1980). Phytoene biosynthesis in *P. blakesleeanus* is catalysed by peripheral membrane proteins which are easily dislodged during preparation of cell extracts (Bramley and Taylor, 1985). However, the later steps of β-carotene production take place within the membranes and possibly in oil droplets (Riley and Bramley, 1982).
Photoregulation of carotenogenesis

Seedlings of higher plants grown in the dark have some capacity for carotenoid biosynthesis but show a light-dependent stimulation of carotenoid production during the development of the young plant. In investigations of photoregulation in angiosperm seedlings one has to consider that the complete transformation of proplastids or etioplasts to photosynthetically-active chloroplasts, including the synthesis of chlorophyll, is completely light-dependent. As a consequence, the stimulation of carotenoid synthesis is only a part of photomorphogenetic transformations and therefore not entirely independent of them (Rau, 1985). It is a well-documented phenomenon, though, that light grown plants contain higher levels of carotenoids than dark-grown plants (Britton, 1976). In many plants the ripening of fruits is accompanied by a transformation of chloroplast into chromoplasts and by a concomitant massive synthesis of carotenoids which are very often non-chloroplast pigments. Photoregulation of these processes has been reported only in tomato and paprika (Thomas and Jen, 1975; Raymundo et al., 1976; Simpson and Lee, 1977).

As a generalisation, algae form intact chloroplasts with the usual plastid carotenoids when grown in the dark. Therefore normal chloroplast development and carotenoid production in algae is not light-dependent, although variations in the carotenoid patterns have been reported for species on illumination (Rau, 1985). However, a
number of exceptions to this rule have been reported. Although dark-grown wild type strains of Euglena gracilis are able to synthesise all the carotenoids that are present in light-grown cultures (Fong and Schiff, 1979), they require light for chlorophyll synthesis, development of functioning carotenoid and increased levels of carotenoids. Also bleached mutants of Euglena lack the xanthophylls found in the wild type and showed photostimulation of carotenogenesis (Dolphin, 1970). Data obtained from experiments with Euglena treated with herbicide SAN 9789, a compound that inhibits the synthesis of carotenoids, resulting in accumulation of phytoene, indicate that the synthesis of chlorophyll and carotenoids are strongly correlated. Photoregulation has also been detected with mutant strains of Chlorella. In a chlorophyll-free yellow mutant, carotenoid biosynthesis was enhanced by blue light (Dresbach and Kowallik, 1974). A chemically induced mutant strain of Scenedesmus obliquus has been examined by Britton et al., (1977). It appears normal in the light, synthesising appreciable amounts of chlorophyll and cyclic carotenoids. However, in dark-grown cultures mainly acyclic carotenoids accumulated and only trace amounts of chlorophyll were present. During a subsequent period of illumination cyclic carotenes and xanthophylls appeared at the expense of the accumulated precursors. The authors concluded that the presence of xanthophylls was important for the development of normal chloroplast structures. Photocontrol of carotenogenesis has only been reported in bacterial species of three genera, Myxococcus, Mycobacterium and Flavobacterium (see Weeks et al., 1973. However, photoregulation has been reported for a wide number of different fungal species (Rau, 1976). In
certain species e.g. *P. blakesleeanus* (Garton et al., 1951, Sandmann and Hilgenberg, 1978) and *Rhodotorula rubra* (Nakayama et al., 1954) carotenoid levels are increased in light over existing dark-grown levels whilst in other species (Table 1) photoregulation is absolute since no or only trace amounts of carotenoids are synthesised in the dark.

In those species studied in detail the indications are that a classical photoinduction mechanism is involved, consisting of a primary photochemical reaction followed by subsequent de novo synthesis of the carotenogenic enzymes (Rau, 1976). The main evidence for this is that the addition of protein synthesis inhibitors prior to illumination completely blocks the synthesis of carotenoids whilst the inhibitory effect was progressively reduced when added at time intervals after illumination e.g. *N. crassa* (Harding and Mitchell, 1968) and Mitzka-Schnabel and Rau, 1981) *M. marinum* (Batra and Storms, 1968).

Carotenoid production is believed to occur in three stages: an initial photochemical reaction which requires light and is temperature-independent, followed by a lag period in which the carotenogenic enzymes are synthesised and a final stage in which the carotenoid pigments are produced. The latter two stages are light-independent but temperature-dependent.
Table 1 - Species of fungi which carotenogenesis is strictly photoinduced (Rau, 1976)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. giganteus mut. alba</td>
<td>Zurzycka, (1963)</td>
</tr>
<tr>
<td>Cephalosporium diospyri</td>
<td>Codner and Patt, (1959)</td>
</tr>
<tr>
<td>Dacryospinax spathularia</td>
<td>Goldstrohm and Lilly, (1965)</td>
</tr>
<tr>
<td>Fusarium aqueductuum</td>
<td>Rau and Theimer, (1970)</td>
</tr>
<tr>
<td>F. coerulim</td>
<td>Rau, (1975)</td>
</tr>
<tr>
<td>F. oxysporum</td>
<td>Carlile, (1956)</td>
</tr>
<tr>
<td>Neurospora crassa</td>
<td>Zalokar (1954); Harding and Mitchell, (1968)</td>
</tr>
<tr>
<td>M. sitophylla</td>
<td>Ishi and Akayi, (1948)</td>
</tr>
<tr>
<td>Pyronema confluentes</td>
<td>Carlile and Friend, (1956)</td>
</tr>
<tr>
<td>Sphaerobolus stellatus</td>
<td>Friederichsen and Engel, (1951)</td>
</tr>
<tr>
<td>Syzygites megalocarpus</td>
<td>Wenger and Lilly, (1966)</td>
</tr>
<tr>
<td>Verticillium agaricinum</td>
<td>Valadon and Nummerly, (1971)</td>
</tr>
</tbody>
</table>
The primary chemical reaction has generally been shown to be independent of temperature (Zalokar, 1954; Rau, 1962; Rilling, 1962; Mathews, 1963; Valadon, 1971; Seviour and Codner, 1973; Harding, 1974; Tada and Shiroishi, 1982). In several cases a "memory" of the light treatment has been found. To demonstrate this effect, the organism is irradiated at low temperature and then maintained at the low temperature in the dark. No carotenoids are found. However, sometime later, increasing temperature results in carotenoid production. This effect has been demonstrated in Flavobacterium dehydrogenans (Weeks et al., 1973). Mycobacterium sp. (Rilling, 1962), Cephalosporium diospyri (Seviour and Codner, 1973), F. aquaeductuum (Rau, 1962), N. crassa (Zalokar, 1955) and R. minuta (Tada and Shiroishi, 1982).

Several investigators conclude that oxygen is required for the primary chemical reaction (Rilling, 1962 and 1964; Howes et al., 1969; Rau, 1969; Seviour and Codner, 1973), although there has been some disagreement concerning the precise role of oxygen in this reaction. Rau (1969) has proposed that oxygen acts as an electron acceptor which keeps the photoreceptor in the proper oxidation state, whilst other investigators (Rilling, 1964; Howes et al., 1969 and Batra, 1971) have proposed that oxygen is directly involved in the light reaction.

When dark-grown mycelia of F. aquaeductuum and N. crassa are exposed to as little as one minute of light, substantial amounts of carotenoids are synthesised (Schrott, 1980). However higher doses of
light are required for maximum production of carotenoids in *N. crassa* (Rau and Rau-Hund, 1977; Schrott, 1980), *F. aquaeductuum* (Schrott, 1980) and *V. agaricinum* (Valadon and Mummery, 1971). A biphasic response to light saturation has been reported for *F. aquaeductuum* (Theimer and Rau, 1972) and *N. crassa* (Rau, 1967).

The exact chemical nature of the photoreceptor has not yet been determined. A common approach to this problem is the determination of the action spectrum of the response to light. The relative effectiveness of different wavelengths in producing the photoreponse is determined, and is assumed to be the action spectrum of the photoreceptor compound. Zalokar (1955) was the first to determine an action spectrum for carotenogenesis in the blue spectral region (between 400 and 500nm) for non-conidiating cultures of *N. crassa*. Prevention of conidiation is important because conidial carotenoid production is not light dependent in this species. More detailed action spectra for carotenogenesis in *F. aquaeductuum* (Rau, 1967) and in a *Mycobacterium* sp. (Howes and Batra, 1970) showed a maximum at 370-380nm and three peaks between 400 and 500nm. Wavelengths longer than 520nm were ineffective in inducing carotenogenesis. On the bases of action spectra studies, different molecules have been proposed to act in different species. For example a porphyrin-type photoreceptor has been suggested for *M. marinum* (Batra and Rilling, 1964), a flavin- or carotenoid-type for *N. crassa* (Zalokar, 1955; De Fabo et al., 1976), *F. aquaeductuum* (Rau, 1967) and *Mycobacterium* sp. (Batra and Rilling, 1964) and a flavin-porphyrin complex for *E. dehydrogenans* (Weeks et al., 1973).
However, flavin-deficient mutants of *N. crassa* have reduced sensitivity to light induction of carotenogenesis compared to wild types (Paietta and Sargent, 1974) strongly suggesting that flavin is involved in photoreception. Later experiments by the same authors (1983) cast some doubt on this interpretation since addition to such mutants of flavin analogs, which have red-shifted absorption failed to elicit photoinduction of carotenogenesis by light at 540nm, although photosuppression and phase shifting of circadian conidiation still occurred.

Since the early steps of carotenogenesis are still photoregulated in mutants of *P. blakesleeanus* (Bergman, 1973) and *N. crassa* (Goldie and Subden, 1973) lacking β-carotene, it seems unlikely that coloured carotenoids are photoreceptors. Valadon et al., (1982) suggested that there may be two photoreceptor systems, different from carotenoids or flavins in *F. agaricinum*, one operating in the ultraviolet (UV-A) and the other for the red, far red region, most likely phytochrome. Here red light induction of carotenoid synthesis is reversed by far-red.

The fluence response of light-induced carotenogenesis in *N. crassa* (Harding, 1974; Schrott, 1980a, 1981), and *F. oxysporium* (Schrott, 1980b) also raises the possibility of two separate photoreceptors. However, there is conflicting evidence for this proposition. The observations that in *P. blakesleeanus* the high fluence response is selectively blocked by transcription and translation inhibitors (Jayaram et al., 1979) or mutations in the pic region (Cerdá-Olmedo, quoted in Lipson, 1980) support the two-receptor hypothesis. On the
other hand, both fluence responses were eliminated in *N. crassa* treated with cycloheximide (Harding and Mitchell 1968) and in mad mutants of *P. blakesleeana* (Jayaram et al., 1979). An alternative explanation for the biphasic fluence response curve may result from the rapid utilisation of precursors causing saturation at low fluence whereas synthesis of more precursors during continuous radiation, over extended time periods produces additional carotenes. Normally carotenogenesis is induced by blue light in *Fusarium*, but when dyes such as methylene blue and toluidine blue are added, red light is effective. These dyes are known to mediate photooxidation whilst carotenoids can not, thereby supporting flavin as the endogenous photoreceptor for this response.

In conclusion, although in most cases blue light is responsible for photoregulation of carotenogenesis, as yet there is no clear evidence for the precise identity of the photoreceptor.

1.9.3 Dark reactions and mechanisms of regulation

Immediately after the light treatment, there is a lag period before carotenoid production begins. The lag period is about 4 h in *M. marinum* (Batra, 1967) 2 h in *R. minuta* (Tada and Shiroishi, 1982) and 1 h in *Mycobacterium* sp. (Rilling, 1962), *N. crassa* (Rau et al., 1968) and *F. aquaeductuum* (Rau, 1967). In *N. crassa* (Harding, 1974) and *F. aquaeductuum* (Rau, 1962), the length of the lag period for total carotenoid production is increased to about 6 h if low incubation temperatures (6-10°C) are used. *N. crassa* shows an unusual
temperature effect in that more carotenoid pigment accumulated at 6°C than at higher temperatures (Harding, 1974). Numerous investigators have demonstrated that protein synthesis is required during the lag period following illumination in order for carotenoid production to occur. Cycloheximide, an inhibitor of protein synthesis in eukaryotes (Pestka, 1977), if added immediately after the light treatment, blocks carotenoid biosynthesis in F. crassa (Harding, 1974, Harding and Mitchell, 1968; Rau et al., 1968), F. aquaeductuum (Bindl et al., 1970) and V. agaricinum (Mummery and Valadon, 1973). Chloramphenicol, an inhibitor of protein synthesis in prokaryotes (Pestka, 1977), inhibits carotenogenesis in Mycobacterium sp. (Howes and Batra, 1970; Rilling, 1962; 1964), M. marinum (Batra, 1967; Batra et al., 1971) and F. dehydrogenans (Weeks et al., 1973). Inhibition of carotenogenesis is reduced by delaying the addition of these compounds after light treatment. Thus, it has been proposed that in these investigations the effect of illumination is to induce the de novo synthesis of one or more enzymes in the carotenoid biosynthetic pathway not present in the dark-grown cultures.

The precise mechanism in which the light treatment causes induction of protein synthesis is unknown. The control mechanism could involve regulation of either transcription or translation. It has been proposed that the light reaction involves the inactivation of a repressor or the production of an inducer (Rilling, 1964; Batra, 1971). According to this model, control would be at transcriptional level, directly regulating gene expression. Inhibition of photocarotenogenesis by proflavine in Mycobacterium sp. (Batra and
Storms, 1968), by actinomycin D in F. dehydrogenans (Weeks et al., 1973) and N. crassa (Subden and Bobowski, 1973), and by distamycin A in F. aquaeductuum (Rau, 1976) has been cited as support for this proposal. All these compounds have been shown to inhibit RNA synthesis in various organisms (Kersten and Kersten, 1974).

Direct evidence that light increases mRNA formation has been obtained with F. aquaeductuum (Schrott and Rau, 1977) and N. crassa (Mitzka-Schnabel et al., 1984) where the amount of poly(A)-RNA was shown to increase in illuminated mycelia. In addition, fluorographs showed that some in vitro translated polypeptides were increased in response to photoregulation (Mitzka-Schnabel et al., 1984). However, neither the new mRNAs nor polypeptides induced by light have been shown to be connected with carotenogenic enzymes.

Evidence that the photoinduction of carotenogenesis may be regulated at the translation level has also been obtained in V. agaricinum (Valadon et al., 1975). In V. agaricinum 80S ribosomes from light-treated cultures were more active in the polymerisation of phenylalanine [using poly(U) as mRNA] than ribosomes from dark-control cultures. Thus, it was proposed that the effect of the light treatment was at the level of the ribosome. It is therefore important to determine whether this light effect is specific to photoinduced carotenogenesis or to some other photoreponse.

A number of chemicals have been shown to mimic photoinduction of carotenogenesis. For example addition of p-chloromercuribenzoate

- 51 -
(PCMB) or p-hydroxymercuribenzoate (PHMB) to dark-grown \textit{F. aquaeductuum} mycelium has been shown to induce carotenoid production (Rau, 1967; Theimer and Rau, 1969 and 1972). However, the site of action of these compounds is different from that of light, since the effects of light and PHMB are additive (Theimer and Rau, 1972). Furthermore, addition of PHMB or PCMB to mutants of \textit{Fusarium} which produce carotenoids in the dark and show no light effect still increased the synthesis of carotenoids (Theimer and Rau, 1969). Addition of antimycin A, an inhibitor of electron transport, to dark-grown cultures of \textit{M. marinum} has been shown to induce carotenogenesis (Batra, 1967; 1971; Batra and Storms, 1968; Batra et al., 1969; Batra et al., 1971). However, using structural analogs of antimycin A, it was shown that induction of carotenogenesis and inhibition of electron transport require different functional groups of the antimycin A molecule (Batra et al., 1971).

The induction of carotenogenesis by PCMB and PHMB in \textit{F. aquaeductuum} and antimycin A in \textit{M. marinum} can be blocked by inhibitors of RNA synthesis (Rau, 1967; Batra, 1967, 1971; Batra et al., 1969; Batra et al., 1971). Thus, although the initial sites of action of the chemicals and light may be different, presumably subsequent events share a common mechanism. PCMB and PHMB are known to inactivate SH groups and hence could be inactivating a repressor.

There are other examples where low molecular weight compounds have been shown to stimulate carotenogenesis. Trisporic acid sex hormones have been shown to be natural stimulators of \(\beta\)-carotene synthesis in
Blakeslea trispora (Thomas and Goodwin, 1967; Thomas et al., 1967; Sutter and Rafelson, 1968).

1:9:4 Carotenogenic enzymes regulated by light

The pattern of carotenoids synthesised by a particular species is characteristic to that species and undoubtedly genetically determined. However, this pattern, as well as the quantities of the various carotenoids is greatly influenced by external conditions such as light, culture conditions and age of culture. For instance, E. dehydrogenans when incubated after photoinduction in an optimal growth medium, synthesises essentially one carotenoid, decaprenoxanthin, but, under conditions of nutritional imbalance which somehow reduces biosynthetic processes, precursor carotenoids accumulated (Weeks et al., 1973). Different quantities of carotenoids have been reported to be present in N. crassa by different investigators (Rau, 1976) which undoubtedly has something to do with strain and media differences.

As a first analysis, to determine which carotenogenic enzymes are photoinduced, the in vivo patterns and quantities of the various carotenoids and their intermediates are compared in dark- and light-grown cultures. This may be examined by similar comparisons using the appropriate cell-free extracts. Although such studies point to the first photoregulated step, it does not necessarily mean that all subsequent steps are also light regulated. In addition, it is also very difficult to interpret the data from complete cell-free
systems with respect to determining the precise activities of the individual enzymes by solely monitoring radioactive incorporation into the various carotenoid intermediates. Unfortunately it has proved extremely difficult to quantitate individual enzymes since some enzymes, often membrane bound, have been difficult to isolate and also their respective substrates are generally not commercially available and, because of their hydrophobicity, are inaccessible in the in vitro reaction mixture. In those organisms showing increased levels of carotenoid biosynthesis in light, compared to dark-grown cultures, there is a general increase in the level of all carotenoids. Therefore overall carotenoid synthesising activities are increased although the individual enzymes responsible for such increases remain unknown. However, those organisms showing strict photoregulation have proved more useful for such studies. For example, in E. aquaeductuum, after a lag period of 30 minutes following photoinduction, a sequential increase in carotenoids occurred in the order phytoene, \( \gamma \)-carotene, neurosporene, \( \gamma \)-carotene, torulene, neurosporoxanthin and lycopene. With the exception of lycopene, this sequence followed a decreasing degree of saturation. Addition of cycloheximide at various times after illumination resulted in a differential inhibition of the synthesis of the various carotenoids the pattern of which closely resembled the time sequence of increase of the carotenoids following photoinduction. The authors concluded that the synthesis of all these carotenogenic enzymes is photoinduced and these enzymes are synthesised sequentially after photoinduction (Bindl et al., 1970; Rau, 1976).
In *N. crassa* phytoene accumulated in the dark but phytoflueene, \( \beta \)-carotene, neurosporene, lycopene, \( \gamma \)-carotene and \( \beta \)-carotene accumulated in the light (Zalokar, 1954). Similar results were obtained with cell-extracts from light- and dark-grown cultures (Harding et al., 1969). This suggests that the carotenogenic enzymes after phytoene are photoinduced. However, later studies using cell-free systems from this fungus have shown that phytoene synthesising activity is also enhanced several-fold on illumination of dark-grown wild-type and albino mutant mycelia (Spurgeon et al., 1979; Mitzka-Schnabel and Rau, 1981). Even earlier enzymes, GGPP and PPPP formation in *Mycobacterium* sp. (Johnson et al., 1974) and HMG-CoA reductase activity in *R. minuta* (Tada and Shiroishi, 1982) have been shown to increase after illumination, and in the case of PPPP synthetase, to be fully photoinducible.

1.10 Genetic analysis

Much work on the genetics of carotenoid biosynthesis has been done on *P. blakesleeanus*. Four genes have been identified to affect carotenogenesis; \( \text{carB} \) and \( \text{carR} \) are structural genes that code for the desaturation and cyclising enzymes, respectively (Dotaki et al., 1973), gene \( \text{carS} \) controls \( \beta \)-carotene feedback inhibition (Murillo and Cerdá-Olmedo, 1976) and gene \( \text{carA} \) codes for a protein that has transfer activity (Murillo et al., 1981). Mapping experiments have shown that gene \( \text{carB} \) is closely linked to \( \text{carRA} \), a bifunctional gene specifying both lycopene cyclase and the substrate transfer protein (Roncero and Cerdá-Olmedo, 1982). The regulatory gene \( \text{carS} \) lies on
the same chromosome but is not close to the structural genes. This situation is reminiscent of an operon. Two mutants, S119 and S144, have been reported to be insensitive to retinol and 2-(4-chlorophenyl)triethylamine (GPTA), stimulation and carry mutants in separate genes. One of these is probably identical to carRA but the other is a new gene termed carE (Roncero and Cerdá-Olmedo, 1982). Strain S106, a carS mutant, is not stimulated by retinol and it was proposed that this carried an additional mutation (car102) that affected retinol stimulation (Murillo and Cerdá-Olmedo, 1976).

Six independent mutants of Phycomyces disturbed in the biosynthesis of β-carotene were isolated after treatment of wild type with the mutagen 4-nitroquinoline-1-oxide (Revuelta and Eslava, 1983). Complementation analysis revealed that all these mutants carried allelic mutations in a new gene called carC. CarC mutants are defective in photocarotenogenesis. It is suggested that the carC gene product exerts a positive regulatory function in the biosynthesis of carotenoids in Phycomyces, possibly through the expression of the carS gene. There appears to be only one desaturase (carB) and one cyclase (carR). These enzymes are membrane bound (reviewed by Bramley, 1985) and may be organised into a multienzyme complex, as suggested by qualitative genetic complementation studies with Phycomyces heterokaryons (De La Guardia et al., 1971). This multienzyme complex is believed to be composed of four desaturases (copies of carR gene product) arranged sequentially. The first cyclase may be in a position in the complex such that it can receive either neurosporene or lycopene.
Mutants of the fungus *N. crassa* defective in carotene biosynthesis were found to be of four types; those without carotenoids; with phytoene only; accumulating phytoene and partially desaturated carotenoids and those defective in carboxylated carotenoids.

Studies with white mutants have revealed three genes, al-1, al-2 and al-3. al-1 and al-2 are closely linked whilst al-3 lies on a different chromosome (Subden and Goldie, 1973). The al-1 mutant accumulated phytoene or, if leaky, a number of partially desaturated carotenoids, suggesting that the gene al-1 codes for desaturase (Subden and Goldie, 1973). al-2 and al-3 mutants were completely devoid of carotenoids (Kushwaha et al., 1978). Cell free enzyme preparations from the al-3 mutant were able to incorporate radioactivity from \(^{14}\text{C}\) GGPP into phytoene but unable to incorporate radioactivity from \(^{14}\text{C}\) into GGPP as the reverse is true of enzyme preparations from the al-2 mutants (Harding and Turner, 1981). Since enzyme preparations from the al-3 mutant were able to incorporate radioactivity from \(^{14}\text{C}\) IPP into FPP it was postulated that gene al-3 coded for a prenyl transferase specific for the synthesis of GGPP whilst gene al-2 coded for phytoene synthetase itself. A yellow mutant of *N. crassa* (ylo-1) synthesised carotenoids but not the carboxylated carotenoid, neurosporaxanthin. Complementation studies with these mutants showed that the gene responsible for neurosporaxanthin synthesis, ylo-1, is not linked to the al-1, al-2 genes (reviewed by Cerdá-Olmedo and Torres-Martinez, 1979).
The patterns of carotenes accumulated by white and coloured strains of *V. violacea* have been interpreted as resulting from lesions in three separate genes, each coding for different dehydrogenases, phytoene dehydrogenase (VB, phytoene → neurosporene), neurosporene dehydrogenase (β, neurosporene → lycopene) and cis-β-zeacarotene dehydrogenase (double mutant, AB278 a-1, β-zeacarotene → γ-carotene). The cyclase enzyme is coded for by the O/P gene complex (Well et al., 1984). A number of mutant strains for carotenogenesis, including a superproducer, have been isolated for Gibberella fujikuroi.

1.11 Other lipids

Relatively little work has been reported on the analysis of other lipids in fungi. *P. blakesleeanus* was found to contain 26–28 g free lipids per 100 g dry matter (Chenouda, 1970) which were composed of phospholipids, sterols (mainly ergosterol), diglyceride, triglycerides, wax, free fatty acid and carotenes. Phosphatidylserine, sphingomyelin, phosphatidylcholine, phosphatidyethanolamine, phosphatic acid, and cardiolipin were identified in the phospholipid fraction.

Analysis of fatty acid composition of the total lipids of dormant spores, mycelia and stage 1 sporangiophores of *P. blakesleeanus* indicated the presence of unsaturated fatty acids (oleic, linoleic and γ-linoleic acids) (Marouf and Malhotra, 1976). The percentage of unsaturated fatty acids amongst the total lipids was higher in the spores than in the mycelia or stage 1 sporangiophores. Of the
sterols ergosterol, lanosterol, 24-methylene-21, 25-dihydrolanosterol
and episterol have been isolated from *P. blakesleeanus* (Goulston and
Mercer, 1975) and labelled L-methionine-[methyl-\textsuperscript{14}C] was incorporated
into some of these sterols when supplied in vivo. The freely
extractable sterols of spores of *Linderina pennispora, Spicaria
elegans, Penicillium claviforme, A. niger U.nuda U.maydis, Puccinia
graminis and P. striiformis* were examined by Weete et al., (1973).
Each species contained at least 3-5 detectable sterol components.
When present, ergosterol was generally the most abundant sterol
produced by an individual species (Weete and Lactoler, 1973).

Tada et al., (1982) studied the effect of light on the production of
ergosterol and phytoene in *R. minuta*. Although phytoene production
was stimulated by light, the ergosterol level remained the same in
both light and dark conditions. There appears to be no other reports
on the effect of light on the quantity and composition of lipids.

1:12 Carotenogenesis and photoresponses in *A. giganteus* *mut. alba*

The fungus *Aspergillus* is noted for its unusually large
conidial structures which are markedly influenced by environmental
factors. Initial experiments examined the effect of such factors,
including light, in conidiophore growth (Gard ner, 1949; 1955). Light
(wavelengths below 520nm) stimulated conidiophore elongation and
pigments isolated from the tall conidiophores showed characteristic
carotenoid absorption spectra. The correlation between the action
spectrum of the photoresponse and the absorption spectrum of the
carotenoid led to the suggestion that β-carotene was acting as the photoreceptor for this response. Later it was shown that both conidiophore growth and carotenogenesis were photoinduced (Trinci and Banbury, 1969). An A. giganteus mut. alba lacking melanin was discovered which visibly demonstrated that carotenoids were only present in the mycelium and conidia in light (Zurzycka, 1963a,b). Analysis of the carotenoids showed that β-carotene was the major carotenoid (over 80%) although other minor carotenoids were present (van Eijk et al., 1979). Colony growth is inhibited by high irradiation and, using a series of mutants with varying amounts of carotenoids (Zurzycka, 1967), the degree of photoprotection was shown to increase with increasing carotenoid levels (Lazarski, 1971, 1972, 1975).

Other light-regulated phenomena include decrease of the carbon/nitrogen ratio (Fiema, 1972), increase of hydroxyproline content in proteins (Fiema, 1979) and increase of chitin accumulation (Fiema, 1981). Nitrogen uptake was unaffected (Fiema, 1974). Finally, Zurzycka et al. (1983), have demonstrated a good correlation between the levels of cAMP and cAMP phosphodiesterase and photostimulated conidiophore growth. They concluded that cAMP and cAMP phosphodiesterase may be involved in this particular photoresponse.
1:13 Aims of the project

Although *N. crassa* is an excellent organism from the point of view of genetics, the photoinducibility of carotenogenesis and its amenability to modern recombinant DNA techniques (reviewed by Bennett and Lasuri, 1985), the cell-free preparations for carotenoid synthesis available so far are rather poor, particularly after phytoene, and so offer only limited opportunities for enzyme studies (Mitzka-Schnabel and Rau, 1981). The opposite is true of *L. blakesleeanus*.

Obviously, it would be advantageous to find an organism which combines the best features of both of *Neurospora* and *Phycomyces*. From the experimental data available so far, *Aspergillus giganteus* mut. *alba* appears to have many of these qualities. Carotenogenesis is totally photoinduced and it is likely that the genetic and molecular biological techniques available for *A. nidulans* could be directly applied to *A. giganteus*. In addition, initial experiments (Bramley and Mackenzie, 1982; unpublished) indicated that active cell-free systems could be prepared from this organism. Therefore a research programme was designed to look in detail at various aspects of photocarotenogenesis in *A. giganteus*.

The main objectives were:

1. Optimisation of growth conditions for maximum carotenoid synthesis;
2. Investigation of the light requirements and its relation to carotenoid formation;

3. Development and optimisation of an active cell-free system;

4. Measurement of the different carotenogenic enzymes to determine which are photoinduced;

5. Analysis of other lipids with respect to photoinduction.
CHAPTER 2

MATERIALS AND METHODS
2:1 Materials

2:1:1 Solvents

All solvents were of the highest purity available and were supplied by B.D.H. Ltd., Poole, Dorset.

2:1:2 Chemicals

General laboratory chemicals were purchased from B.D.H. or Sigma and were of analytical grade whenever possible. Cofactors and glutathione were obtained from Sigma Chem. Co., Poole, Dorset.

Thin layer plates, both plastic sheets pre-coated with silica gel 60F254, 20×20 cm, 0.2 mm layer thickness and aluminium sheets pre-coated with aluminium oxide 60F254, 20×20 cm, 0.2 mm layer thickness, were supplied by E. Merck Ltd, Darmstadt, W. Germany.

Neutral agar, yeast extract, malt extract and mycological peptone were supplied by Oxoid Ltd., Basingstoke, Hampshire.

Neutral alumina for column chromatography was purchased from Woelm Pharama GmbH Eshwege, W. Germany.

Enzymic colourimetric test kit. (Cat. No. 124958) was purchased from Boehringer Mannheim, BCL, Lewes, Sussex, BN7 1LG.
Cycloheximide, actinomycin D and 5-fluorouracil were purchased from Sigma.

2:1:3 Radiochemicals

R-[2-14C] Mevalonic acid lactone (51mCi/mmol) and [1-14C] isopentenyl pyrophosphate (55 mCi/mmol) were supplied by Amersham International, Amersham. The mevalonic acid lactone was converted to the sodium salt prior to use by the addition of sodium hydroxide.

2:1:4 Organisms

A. giganteus mut. alba was obtained as strain 101.64 from the Centraal Bureau voor Schimmel Cultures, The Netherlands. P. blakesleeanus C5 carB10(-) and C9 carR21(-) were supplied by Professor E. Cerdá-Olmedo, Department of Genetics, University of Seville, Spain. A white mutant of A. giganteus alba, designated E5, was produced by U.V. mutagenesis and isolated in this laboratory.

2:2 Methods

2:2:1 Growth and maintenance conditions

2:2:1:1 A. giganteus mut. alba and E5 mut+
A. giganteus mut. alba and E5 were maintained on 2% agar slopes of Wickerham's MYPG medium (Wickerham, 1951) and stored at 4°C. MYPG medium contained:

- Malt extract: 3.0g
- Yeast extract: 3.0g
- D-glucose: 10.0g
- Peptone: 5.0g per litre distilled water

A basic synthetic medium contained:

- D-glucose: 25.0g
- KH₂PO₄: 1.5g
- MgSO₄.7H₂O: 0.5g
- NH₄NO₃: 1.5g
- (NH₄)₂SO₄: 1.5g
- Leucine: 0.5g
- Asparagine: 0.5g
- Vitamin B₁: 0.25g per litre distilled water

Liquid cultures of A. giganteus were grown in MYPG or the basic synthetic medium on an orbital shaker (160 rpm) at 25°C. Inocula were prepared by adding water (10 ml) to each agar culture and shaking. Aliquots (2 ml) of the spore suspension were then added to the medium (100, 200, 300, 400, 500 ml) in a 2 litre wide based flask. Aseptic techniques were used throughout. Cultures were grown in light or dark as described in the relevant results section. Light was prevented from reaching the cultures by wrapping the flasks in two layers of black plastic. In certain experiments (see section 3.6)
cultures were initially grown in the dark for 4 days, then filtered through muslin, washed twice with sterile 0.05M potassium phosphate buffer pH7 and suspended in the same buffer (300 ml) containing 2% glucose and 2% ammonium sulphate. This procedure was carried out in the dark. The cultures were then placed in the light for different lengths of time as required and returned to darkness for up to 3 days (see Figure 3:3, 3:4). Finally, the cells were harvested through muslin. In some experiments the resuspended cultures were maintained under anaerobic conditions by continuous flushing with nitrogen. Inhibitors of RNA and protein synthesis were added to the medium as sterile concentrates prior to illumination.

2:2:1:2 P. blakesleeanus strains C5 carB1Q(-) and C9 carR21(-)

P. blakesleeanus strains C5 carB10(-) and C9 carR21(-) were maintained on nutrient 2% agar slopes and stored at 4°C. The nutrient agar medium contained, per litre distilled water:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-asparagine</td>
<td>2.0g</td>
</tr>
<tr>
<td>D-glucose</td>
<td>20.0g</td>
</tr>
<tr>
<td>nutrient agar</td>
<td>20.0g</td>
</tr>
<tr>
<td>50x - concentrate</td>
<td>20.0ml</td>
</tr>
</tbody>
</table>

The 50x - concentrate contained, per 100ml distilled water:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH2PO4</td>
<td>2.5g</td>
</tr>
<tr>
<td>MgSO4</td>
<td>2.5g</td>
</tr>
<tr>
<td>14% CaCl2 solution</td>
<td>1.0ml</td>
</tr>
<tr>
<td>thiamine hydrochloride</td>
<td>10.0mg</td>
</tr>
<tr>
<td>trace element solution</td>
<td>0.5ml</td>
</tr>
</tbody>
</table>
The trace element solution contained, per 100 ml distilled water:

- citric acid (monohydrate) 2.0g
- Fe(NO₃)₃·9H₂O 1.5g
- ZnSO₄·7H₂O 1.5g
- MnSO₄·H₂O 0.3g
- CuSO₄·5H₂O 0.05g
- NaMoO₄·2H₂O 0.05g

The liquid cultures were grown in light for 3 days in the following medium:

- D-glucose 25.0g
- L-asparagine 1.25g
- L-leucine 1.25g
- MgSO₄·7H₂O 0.5g
- KH₂PO₄ 1.5g
- yeast extract 0.5g
- thiamine-HCl 0.25g

per litre distilled water

2:2:2 Light sources and illumination procedures

White light was provided by fluorescent lamps (type F30T12/W/W/RG/GTE, Sylvanian Ltd., Charlestown, Shipley, West Yorkshire, BD17 7SN) with intensities of 12-64 W/m², as measured at the surface of the cultures. Blue (1.6 W/m²) and red (11.5 W/m²) illuminations were achieved by placing filters of transmittance ranges 400-520nm and 510-750nm, respectively, in front of the white light lamps.
Precautions when extraction/analysing carotenoids

Throughout the extracting procedures, the solutions containing carotenoids were shielded from light with black cloth whenever possible and stored in an ice bath because carotenoids are both light and heat sensitive. Carotenoid solutions stored for prolonged periods were gassed with nitrogen and kept at -20°C.

Extraction of carotenoids

The mycelia were filtered through muslin, washed thoroughly with distilled water, squeezed to hand dryness and placed in a beaker containing acetone. Samples could be stored at -70°C prior to acetone treatment. The acetone mixture was macerated thoroughly using a Silverson blender and filtered through a sintered glass funnel under vacuum. The extraction and filtration was repeated several times using acetone (x2) and diethyl ether (x3) until no further colour was extracted and the mycelium appeared fine and powdery. The acetone and ether extracts were combined and partitioned with an equal volume of distilled water in a separating funnel (500ml).

The epiphase layer, containing the lipids, was transferred to a round bottomed flask, dried over anhydrous sodium sulphate for an hour and then filtered through a sintered glass funnel. The solvent was then evaporated almost to dryness using a rotary evaporator and finally evaporated to dryness with a stream of nitrogen. The carotenoids
were re-dissolved in light petrol (b.p. 40-60°C) and the absorption spectrum from 250-550nm recorded.

2:2:5 Identification and quantification of carotenoids

Carotenoids were purified by column chromatography, using a Brockmann grade III alumina column. Samples were applied in a minimum volume of light petrol, using a Pasteur pipette, and the column developed with light petrol containing increasing concentrations of diethyl ether. The carotenoid fractions were collected and the absorption spectra recorded from 250-550nm e.g. β-carotene eluted with 0.5% diethyl ether in light petrol and identified by its spectral maxima at 425, 450, 477nm, and quantified using an \( E_{1\text{cm}}^{1%} \) of 2500 (Davies, 1976).

2:2:6 Preparation of cell-free extracts

Washed, hand-dried mycelia were frozen at -70°C for 3 h and then lyophilised under reduced pressure (Alpha Christ) for 4 days. The freeze-dried mycelium was rubbed through a 40 mesh sieve onto aluminium foil and weighed. The fine powder was poured into a centrifuge tube (50ml) and mixed with 0.4MTris-HCL buffer pH 8.0 (1:8 w:v). The resulting paste was centrifuged for 30 min. at 10,000 xg and the supernatant (S₁₀) then used as the cell-free extract. Lyophilised mycelia could be stored at -70°C under desiccation for up to 4 weeks. Subcellular fractions were obtained by centrifugation of the S₁₀ at 105,000 xg for 1 h to obtain the
supernatant \((S_{10s})\) and pellet \((P_{10s})\) factions. The latter was resuspended in an equal volume of buffer used originally for the cell-free extract.

2.2.7 Incubation mixture and conditions

Incubations were performed in Thunberg tubes. The standard incubation mixture contained:

\[
\begin{align*}
\text{ATP} & \quad 10.0 \ \mu \text{mol} \\
\text{NAD} & \quad 1.0 \ \mu \text{mol} \\
\text{NADP} & \quad 1.0 \ \mu \text{mol} \\
\text{FAD} & \quad 1.0 \ \mu \text{mol} \\
\text{glutathione} & \quad 10.0 \ \mu \text{mol} \\
\text{MnCl}_2 \cdot 4\text{H}_2\text{O} & \quad 2.0 \ \mu \text{mol} \\
\text{MgCl}_2 \cdot 6\text{H}_2\text{O} & \quad 3.0 \ \mu \text{mol} \\
\text{R-[2-\text{\textsuperscript{14}C}] Mevalonic acid} & \quad 0.25 \ \mu \text{C}_4 \\
\text{cell-free extract} & \quad 200.0 \ \mu \text{l} \\
\text{Total volume} & \quad 500.0 \ \mu \text{l}
\end{align*}
\]

Any variations to the basic incubation mixture are described in the relevant results section.

Incubations were typically carried out for 2 h at either 20°C or 35°C, in darkness and aerobically. When necessary, anaerobic conditions were achieved by successively flushing the incubation tubes with nitrogen followed by evacuation.
Analysis of the carotenoids in the incubation mixtures

The extraction and purification procedures were adapted from those of Bramley and Davies (1974). Methanol (3 ml) was added to each incubation mixture and the mixture agitated on a rotary mixer (10 sec). Light petrol (b.p. 60-80°C, 9 ml) and a solution (100 μl) of carrier terpenoids containing phytoene (25 μg), lycopene (20 μg), β-carotene (25 μg), and squalene (20 μg) were added. The tube was shaken vigorously (40 sec) and the contents washed into a separating funnel (100 ml) that contained saturated sodium chloride (30 ml) and light petrol (10 ml).

The aqueous hypophase was discarded and the epiphase (containing the terpenoids) was delivered into a tube containing anhydrous sodium sulphate (ca. 2 g) and stored in darkness for at least 1 h. The mixture was filtered through a sintered funnel into a round-bottomed flask (100 ml) and evaporated to small volume under reduced pressure. Finally the solution and washings were transferred into a tapered, graduated tube. An aliquot (1%) was removed for radioassay, and the remaining sample (ca. 100 μl) was applied as a band to silica gel plates (5x20 cm) together with a diethyl ether wash.

The plates were developed with 15% toluene/light petrol (b.p. 60-80°C). A band containing lycopene (Rf 0.18) was removed for radioassay. The band containing β-carotene, phytoene and squalene (Rf 0.5-0.6) was also scraped off into a plugged filter funnel. The pigments were eluted into a tapered tube by the immediate addition of
diethyl ether (ca. 10 ml) and then the solvent was evaporated to a small amount (100 µl) under a stream of nitrogen.

The sample was applied onto an alumina plate (5x20 cm) which was developed with 3% toluene in light petrol (b.p. 60-80°C). Phytoene (Rf 0.65) was located by viewing under U.V. light. The yellow band of β-carotene (Rf 0.4) together with phytoene were scraped off separately for radioassay. Squalene (Rf 0.75) was located on the plate by exposure to iodine vapour and was then radioassayed.

2.2.9 Extraction and separation of ['^14C]-labelled prenylpyrophosphates

The cell-free incubation produced prenylpyrophosphates which were water soluble and not extracted with the other terpenoids. These compounds were initially partitioned against diethyl ether (see 2.2.8), followed by addition of c. HCl to the aqueous fraction (200 µl per 1.5 ml). The mixture was incubated in a shaking water bath for 30 min. at 37°C. The hydrolysis was stopped by addition of 0.25 M EDTA (equal vol. to c. HCl) and then adjusted to pH 8.0 with c. NH₄OH (about 2 vol. to c. HCl). The basic aqueous fraction was then extracted three times with diethyl ether (% vol.) and the combined diethyl ether extract was left over anhydrous sodium sulphate for 1 h. This fraction was then filtered and concentrated for consequent application to TLC. The sample (100 µl) was applied to a silica gel plate which was treated with 5% paraffin in petrol (60-80°C).
The reversed-phase thin layer was then developed in methanol water (80:20) saturated with paraffin. The \(^{14}\text{C}\) geraniol (Rf 0.6), farnesol (Rf 0.5), geranylgeraniol (Rf 0.36) and prephytoene alcohol (Rf 0.17) were located by use of a spark chamber (2:2:11). These bands were then scraped off and radioassayed.

2:2:10 Radioassay

This was achieved by liquid scintillation counting with a Beckman LS 7500 counter and printer (Beckman Instruments, California, USA), using external standard ratios to correct for quenching.

Samples to be assayed from TLC plates were scraped into vial inserts (Sterilin Ltd., Feltham, Surrey) and immersed in scintillation fluid (3 ml) consisting of 2,5-diphenyloxazole (PPO) in toluene (5\%v) and then radioassayed.

2:2:11 Detection of radioactivity on thin layer plates

Radioactivity from \(^{14}\text{C}\) on TLC plates of a density greater than 1000 dpm/cm\(^2\) was detected with a spark chamber (Birchover Instruments, Letchworth, Herts.).

2:2:12 Spectrophotometric analysis of carotenoids

Absorption spectra from 250-550 nm were recorded using a Beckman 24 spectrophotometer using light petrol (b.p. 40-60°C) as the solvent.
2:2:13 Determination of total lipids

For the extraction of total lipids the mycelial samples were extracted with acetone and diethyl ether as for carotenoids (section 2:2:4) except a final extraction with methanol was also carried out. The organic phase was evaporated to dryness and the lipids weighed.

2:2:14 Qualitative determination of different lipids

The lipid sample and markers were applied to silica gel plates and developed with petroleum (b.p. 60-80°C): diethyl ether:acetic acid (90:10:1). The plate was then placed in a tank containing iodine vapour and the different lipids were detected: sterol (Rf 0.06), fatty acids (Rf 0.2), glycerides (Rf 0.32).

2:2:15 Quantitative determination of different lipids

Glycerides and glycerol were assayed with the Boehringer enzymic triglycerides assay kit, (cat. no. 124958) after saponification with ethanolic KOH (Eggstein, 1966)

Fatty acids were determined by using the method of Falholt et al., (1973). Phosphate buffer, pH 6.4, (1 ml) and chloroform:heptane: methanol solution (6 ml; 49:49:2) were added to the lipid sample (50 μl) in a test tube with a polyethylene cap. The mixture was shaken for 10 min and centrifuged (4000 x g for 10 min). The buffer was removed carefully by suction and the lower organic phase (5 ml)
was shaken with a solution containing 0.05 M Cu(NO₃)₂ and 0.1 M triethanolamine, pH 8.1 (2 ml) for 5 min. After centrifugation (4000 × g for 5 min), the upper phase (3 ml) was transferred to a test tube containing a solution (prepared from 10 ml of 14 g/l 1,5-diphenyl carbazide in ethanol, and 0.1 ml of 1 M triethanolamine) and mixed carefully. After 15 min the colour was measured at 550 nm. A standard curve for palmitic acid (0, 0.2, 0.4, 0.8, 1.0 mmol) was prepared in the same way.

Sterols were determined using the method of Schoenheimer and Sperry (1934), the sterols being precipitated as their digitonides and then measured spectrophotometrically. The lipid sample was dissolved in ethanol (1 ml) and boiled. A hot solution of digitonin in ethanol (1 ml) was added and the mixture boiled gently for 1 min. After cooling, one drop of 30% aqueous aluminium chloride was added and allowed to stand for 5 min. The precipitated sterol digitonides were separated by centrifugation and the supernatant liquid decanted. The precipitate was then washed successively with a mixture of ethanol:acetone (1:1), acetone:diethyl ether (1:1) and diethyl ether:ethanol (1:1) to remove adsorbed carotenoids from the digitonides. When washed and dried, the digitonides were dissolved in glacial acetic acid (3 ml) and colour reagent (acetic acid:c. sulphuric acid, 19:1; 6 ml) was added. The colour was allowed to develop in the dark at 25°C for 30 min and the absorbance measured at 620 nm. A standard curve was prepared using ergosterol (0, 1, 2, 3, 4, 6, 8 and 10 mg).
Phospholipids were measured by placing the lipid samples in a 15 ml glass-stoppered centrifuge tube and evaporated with nitrogen at 30°C to ca. 0.3 ml. Acetone (5 ml) and 10% MgCl₂·6H₂O in methanol (0.1 ml) were added and then cooled on ice for 1 h. The precipitated phosphatides were centrifuged at 2500×g for 5 min. and the supernatant acetone removed by suction. The precipitate was then washed twice by suspension in cold acetone (1 ml) cooling on ice and centrifuging as above. Excess solvent was removed by drying under nitrogen and the precipitated phosphatides dried in a desiccator over KOH. The dried residue was weighed.

2:2:16 Other determinations

Mycelial dry weights were determined on lyophilised material.

Protein determination were carried out using the Polin, Lowry method (Lowry et al., 1951).

All the results presented in this thesis are average values obtained from duplicate experiments.
As discussed in section 2.1, photoregulation of carotenoid biosynthesis has been demonstrated in higher plants, algae, fungi, and microorganisms and bacteria (Harding and Naraghi, 1980; Ben-24; 1985 and 1987). Also in the case of fungi, this phenomenon is usually mediated by blue light and causes an increase in carotenogenesis in species which form significant quantities of carotenoids in the dark (e.g. A. bicaudatus, Horemans et al., 1981; and Aspergillus and Eilson, 1975). Also photoreduction occurs in organisms which produce negligible amounts of carotenoids in darkness (e.g. A. craven, Zalokar, 1958; Ben and Ben-Nooh, 1977; Schrott, 1960). A. giganteus belongs to the latter category. It forms pigment only in the 1ight. Gardner, 1949; Zalokar, 1958; Trinci et al., 1967). Since then a great deal of work has been reported concerning the mechanism of photoregulation in Aspergillus.

CHAPTER 3

THE PHOTOREGULATION OF CAROTENOID BIOSYNTHESIS IN A. GIGANTEUS

In the present study, the suitability of A. giganteus as a model system for detailed investigations of the photoregulation of carotenogenesis has been assessed by establishing the spectral type and intensity of light required for carotenoid formation, the kinetics of carotenogenesis and the metabolic level at which photoregulation occurs.

3.1 Optimisation of growth conditions

In the first instance, various parameters, such as values of nutrient and medium type, were altered to determine conditions for maximum
As discussed in section 1.9 photoregulation of carotenoids has been demonstrated in higher plants, algae, fungi and non-photosynthetic bacteria (Harding and Shropshire, 1980; Rau, 1983 and 1985). Also in the case of fungi this phenomenon is usually mediated by blue light and causes either an increase in carotenogenesis in species which form significant quantities of carotenoids in the dark (e.g. P. blakesleeanus, Garton et al., 1951 and Sandmann and Hilgenberg, 1978), or else photoinduction occurs in organisms which produce nil or negligible amounts of carotenoids in darkness (e.g. N. crassa, Zalokar, 1954; Rau and Rau-Hund, 1977; Schrott, 1980). A. giganteus belongs to the latter category, as it forms \( \beta \)-carotene only in the light (Gardner, 1949; Zurzycka, 1963; Trinci et al., 1984). Since these early studies, however, no investigations have been reported concerning the mechanism of photoregulation in Aspergillus.

In the present study the suitability of A. giganteus as a model system for detailed investigations of the photoregulation of carotenogenesis has been assessed by establishing the spectral type and intensity of light required for carotene formation, the kinetics of carotenogenesis and the metabolic level at which photoregulation occurs.

3:1 Optimisation of growth conditions

In the first instance various parameters (such as volume of medium and medium type) were altered to determine conditions for maximum
carotenoid production. As shown in Table 3.1 A. giganteus produced more β-carotene when grown in Wickerham's MYPG medium than in a basic synthetic medium. This could be because the MYPG medium is richer as a source of carbon and nitrogen necessary for carotenoid production. Comparing different volumes, 300 ml was the best for both media and it is assumed that this gave the best aeration condition for carotenoid biosynthesis.

3:2 Growth and carotenoid content of Aspergillus

Using 300 ml Wickerham's MYPG medium in 2 litre flasks and the shake cultures stated in Section (2:2:1:1), the fungus had a lag phase of 24 h, followed by a trophophase of 4 days, finally reaching idiophase 5 days after inoculation. The growth patterns and cell yields were virtually the same in light- and dark-grown cultures (Figure 3:1). In darkness, however, no carotenoids were detected, whereas in the light, the β-carotene content increased throughout growth, to reach 167 μg/g dry wt. at 5 days. No other carotenoids were found at any age of culture.

3:3 The effect of light on β-carotene biosynthesis

Growth of A. giganteus mut. alba under continuous white light of different intensities demonstrated that β-carotene formation increased linearly between intensities of 10 and 50 W/m² (3740-18700 lux, Figure 3.2). In order to ascertain which spectral type of light was required for carotenogenesis, cultures were continuously
Table 3.1  The amounts of β-carotene produced by \textit{A. giganteus} mut. alba grown in different volumes of Wickerham's WYPG and a basic synthetic media in 2 litre conical flasks

<table>
<thead>
<tr>
<th>Volume of the medium (ml)</th>
<th>β-carotene (μg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wickerham's WYPG medium</td>
</tr>
<tr>
<td>100</td>
<td>56</td>
</tr>
<tr>
<td>200</td>
<td>108</td>
</tr>
<tr>
<td>300</td>
<td>168</td>
</tr>
<tr>
<td>400</td>
<td>102</td>
</tr>
<tr>
<td>500</td>
<td>95</td>
</tr>
</tbody>
</table>
Figure 3:1 Growth and β-carotene formation of *A. giganteus* cultured in light and dark

Light grown cultures were illuminated with white light at an intensity of 50 W/m². Fungal growth in light ○ and dark ●, β-carotene in light △ and dark ▲.
Cultures were exposed to white light of different intensities for 7 days, and then analysed for their β-carotene contents.

Figure 3.2 The effect of white light intensity on β-carotene formation

In this experiment, the cultures were exposed differently as follows: (1) illumination at 25°C and illumination at 4°C and (2) illumination at 25°C under anaerobic conditions, and (a) dark-grown cultures as control.
illuminated with white, blue and red light for 7 days (Table 3:2).
Both white and blue light caused β-carotene biosynthesis, in contrast to the culture grown in red light, which contained no carotenes.

3:4 The properties of photoregulation of carotenogenesis

Experiments designed to establish the duration of light exposure required for maximum β-carotene production and the rate of carotenogenesis subsequent to light exposure, were carried out using starvation medium with mycelia which had been grown for 4 days in darkness of Wickerham's medium (see 2:2:1:1). At a white light intensity of 50 W/m², the β-carotene content reached 100% of that found in cells under continuous light after 10 h of illumination. Longer periods of light had no increasing effect on carotenogenesis (Figure 3:3). This saturation effect at ca. 10 h was confirmed in a separate experiment which showed that the rate of β-carotene formation was both maximal (3.9 µg/g dry weight) and approximately linear between 12 and 48 h after prior exposure to 12 h of white light (Figure 3:4).

3:5 Determination of the nature of carotenogenic reactions

In this experiment the different cultures were treated differently on exposure to white light; (1) illumination at 25°C, (2) illumination at 4°C and (3) illumination at 25°C under anaerobic conditions, and (4) dark-grown culture as control.
Table 3:2 The effect of red, blue and white light on β-carotene biosynthesis by *A. giganteus* mut. *alba*

<table>
<thead>
<tr>
<th>Light source</th>
<th>Intensity (W/m²)</th>
<th>Wavelength (nm)</th>
<th>β-carotene (µg/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>50.0</td>
<td>370 - 700</td>
<td>159</td>
</tr>
<tr>
<td>Blue</td>
<td>1.8</td>
<td>400 - 520</td>
<td>35</td>
</tr>
<tr>
<td>Red</td>
<td>11.5</td>
<td>600 - 780</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. - not detected (<0.1 µg/g dry wt).
Figure 3.3  The effect of light exposure as a variable in the reaction of BC among different conditions. The graph shows the decrease in BC content (%) over time (hr) with increasing exposure time. The initial BC content is 100%, and it decreases to almost 0% after 30 hr of exposure.
4-day old dark-grown cultures were illuminated with white light (50 W/m²) for periods of 0-30 hrs, on a starvation medium and then returned to darkness for 4 days prior to carotenoid analyses.
Figure 3.3 The effect of light exposure on β-carotene formation

The diagram shows the relationship between the duration of light exposure (hours) and the β-carotene content (% control) of a sample. As the duration of light exposure increases, the β-carotene content decreases, indicating a decrease in β-carotene formation under light exposure.
Figure 3:4 Dependence of β-carotene biosynthesis on the duration of exposure to light

Cultures were grown in darkness for 4 days, transferred to a starvation medium and illuminated for periods of 2 (○), 10 (★), 60 (▲), 240 (▲) and 720 (□) min. After these periods, the cultures were returned to darkness for up to 72 hrs and finally analysed for their β-carotene contents.
Photoregulation of \( \beta \)-carotene biosynthesis was dependent upon oxygen, but temperature independent (Table 3:3). Thus only mycelia in the light and under aerobic conditions synthesised \( \beta \)-carotene.

3.6 Effect of transcription and translation inhibitors on \( \beta \)-carotene biosynthesis

Two inhibitors of transcription (5-fluorouracil: \( 10^{-5}, 10^{-4} \) and \( 10^{-3} \) M and actinomycin D; \( 10^{-6}, 10^{-7} \) and \( 10^{-6} \) M) and one of translation (cycloheximide: \( 10^{-6}, 10^{-4} \) and \( 10^{-2} \) M) were added separately to different cultures before exposing the cultures to light. All three compounds prevented photoinduction of carotenogenesis in Aspergillus (Table 3:4). The growth patterns and cell yields were unaffected.

3.7 Discussion

The total dependence of *A. giganteus* mut. alba on light for carotene biosynthesis (Figure 3:1) confirms earlier reports using static cultures (Zurzycka, 1963; Trinci, 1969) and establishes this fungus as one of a small group known to exhibit strict photoregulation. I was unable to detect any carotenoids other than \( \beta \)-carotene, in contrast to an earlier report in which 6 other carotenoids were identified in this species (van Eijk et al., 1979). The reason for this anomaly is unknown, especially as the same strain and culture medium was used in both studies. No other carotenoids were detected during the photoinduction experiment (Figures 3:3 and 3:4), whereas the intermediates between phytoene and \( \beta \)-carotene appear sequentially
Table 3:3  The effect of temperature and aeration on the photoinduction of β-carotene formation by *Aspergillus*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>β-carotene (µg/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Illumination</strong></td>
<td><strong>Temp.°C</strong></td>
</tr>
<tr>
<td>Dark</td>
<td>25</td>
</tr>
<tr>
<td>Light</td>
<td>25</td>
</tr>
<tr>
<td>Light</td>
<td>4</td>
</tr>
<tr>
<td>Light</td>
<td>25</td>
</tr>
</tbody>
</table>

n.d. - not detected (<0.1 µg/g dry wt). White light intensity was 50 W/m².
Table 3:4 The effect of inhibitors of transcription and translation on the photoinduction of \( \beta \)-carotene formation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>( \beta )-carotene (µg/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>159</td>
</tr>
<tr>
<td>10(^{-8})M</td>
<td>8</td>
</tr>
<tr>
<td>10(^{-7})M</td>
<td>n.d.</td>
</tr>
<tr>
<td>10(^{-6})M</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

(a) Cycloheximide

<table>
<thead>
<tr>
<th>Concentration</th>
<th>( \beta )-carotene (µg/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>168</td>
</tr>
<tr>
<td>10(^{-8})M</td>
<td>19</td>
</tr>
<tr>
<td>10(^{-7})M</td>
<td>n.d.</td>
</tr>
<tr>
<td>10(^{-6})M</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

(b) 5-Fluorouracil

<table>
<thead>
<tr>
<th>Concentration</th>
<th>( \beta )-carotene (µg/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>171</td>
</tr>
<tr>
<td>10(^{-8})M</td>
<td>0.5</td>
</tr>
<tr>
<td>10(^{-7})M</td>
<td>n.d.</td>
</tr>
<tr>
<td>10(^{-6})M</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

(c) Actinomycin D

<table>
<thead>
<tr>
<th>Concentration</th>
<th>( \beta )-carotene (µg/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>159</td>
</tr>
<tr>
<td>10(^{-8})M</td>
<td>8</td>
</tr>
<tr>
<td>10(^{-7})M</td>
<td>n.d.</td>
</tr>
<tr>
<td>10(^{-6})M</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. - not detected (<0.1 µg/g dry wt)

This suggests that in *A. giganteus* the kinetics of the overall pathway are strongly in favour of the formation of the end product β-carotene. Clearly, the absence of carotenoids in dark-grown cultures has no effect on cell yield (Figure 3:1), and they may only be necessary in illuminated cells to minimise photosensitisation (Lazarski, 1971).

Some general properties of the light induction phenomenon in *A. giganteus* are similar to those of other fungal species. A blue light response (Table 3:1) is common in several fungi (Rau, 1976; 1985), although red light is also effective in *Verticillium* (Osman and Valadon, 1978; Schrott et al., 1981), but not in *Aspergillus* (Zurzycka, 1963) nor in *Fusarium* (Bindl et al., 1970). In addition, the dependence on oxygen, but not temperature for the initial photochemical event has been demonstrated with several fungi, e.g. *F. aquaeductuum* (Rau, 1980) and *R. minuta* (Tada and Shiroishi, 1982). The white light used in these experiments contained 30% blue light, i.e. 15 W/m². Therefore a comparison of the levels of β-carotene produced with white light and blue light (Table 3:1), shows values of 10.6 and 19.4 μg/g dry wt/W/m², respectively, suggesting that the white light may be partially inhibitory. This can not be due to far red light, as the white lamps do not emit in this region of the spectrum.
A biphasic response to saturation by light was not found with *A. giganteus* (Figure 3:2), but has been reported for *Fusarium* (Theimer and Rau, 1972) and *N. crassa* (Schrott, 1980). Other aspects of pigment formation and dosage of light required for carotenogenesis are also different in *A. giganteus* to other fungi. Exposure to white light (50 W/m²) for approximately 2 h is required in order to initiate carotenoid formation (Figure 3:3); this is a considerably longer period than that required by either *F. aquaeductuum* (Rau, 1980) or *N. crassa* (Bindl et al., 1970). However, the formation of β-carotene is proportional to the duration of light exposure between 2 and 8 h (Figure 3:3), and the final amount of β-carotene produced is the same as that in continuously illuminated cultures. In other fungi, the levels of carotenoids synthesised in such experiments are significantly lower than those in control cultures, for example in *N. crassa* (Rau and Rau-Hund, 1977), *F. aquaeductuum* (Schrott, 1980) and *V. agaricinum* (Valadon and Mummery, 1971). The rate of synthesis after illumination is also affected by the duration of light exposure (Figure 3:4), with a 12 h light period producing 100% of the β-carotene in the control, light-grown cultures after 48 h in darkness. The lag period is approximately 8 h. This response to illumination differs to *Fusarium* (Rau, 1967), *Neurospora* (Rau et al., 1968) and *Rhodotorula* (Tada and Shiroishi, 1982), where lag periods are proceeded by relatively short periods of carotenoid synthesis which do not lead to carotenoid levels comparable with control cultures. In the case of *Verticillium* carotenogenesis only occurs during illumination (Osman and Valadon, 1978). Therefore β-carotene biosynthesis in *Aspergillus* shows the classical features of an...
induction mechanism, but differs in detail from other fungi which have been studied, since the cells accumulate the same quantity of β-carotene under continuous illumination (Figure 3:1) as they do after a light pulse of 10 h (Figure 3:3).

The effects of the inhibitors used to prevent photoinduction (Table 3:4) demonstrate that regulation occurs at the transcriptional level, as has been found for Fusarium (Rau, 1980). In contrast, inhibitors of transcription are only partly effective in Verticillium (Valadon and Mummery, 1969). Evidence to support the synthesis of poly(A)RNA following illumination has been found with Neurospora (Schrott and Rau, 1977), and recently specific proteins from the in vitro translation of light-specific mRNAs has been identified, although their biological functions are as yet, unknown (Mitzka-Schnabel et al., 1984). Also, cAMP levels are thought to regulate carotene gene expression in Neurospora (Kritsky et al., 1982). Precisely which carotenogenic enzymes are photoinducible in Aspergillus cannot be deduced from the above investigation. In contrast to other fungi such as Neurospora (Zalokar, 1954), no phytoene accumulates in dark-grown cultures, indicating that phytoene synthetase and/or prephytoene pyrophosphate synthetase are photoregulated. Both of these enzymes are totally photoinduced in Mycobacterium (Rilling, 1964; Johnson et al., 1979). However, developing an active cell extract from this strictly photoregulated fungus gives conclusive evidence as to which carotenogenic enzymes are photoinduced.
Carotenoid biosynthesis in a number of non-photosynthetic microorganisms has been shown to be photoregulated (Bau, 1985). On the basis of these studies it has been proposed that light induces the de novo synthesis of carotogenic enzymes. The most direct approach for establishing if this is correct, and to determine which enzymes are photoinducible, is to compare in situ activities of cell extracts from light- and dark-grown cultures. In the previous chapter it has been demonstrated that carotenoid biosynthesis in *A. giganteus* var. *alba* is photoregulated at the transcriptional level. In order to ascertain which enzymes are photoinduced it is necessary to develop a cell-free system of *A. giganteus* which exhibits light-induced xanthophyll-like activities in light-grown cultures. In addition, some of the properties of the enzymes associated with carotenoid biosynthesis were studied.

**CHAPTER 4**

**DEVELOPMENT OF A CAROTENOGENIC CELL-FREE SYSTEM FROM *A. GIGANTEUS***

### 4.1 Effect of protein concentration on incorporation of [2-C] NVA into carotenoids

This experiment was designed to investigate the effect of protein concentration in the cell extract on the incorporation of [2-C] NVA into carotenoids of *A. giganteus*. A cell-free extract from light-grown cultures converted [2-C] NVA into phytoene, lycopene, β-carotene and echinenone (Table 4.1, Figure 4.1). Incorporation of radioactivity from [2-C] NVA into phytoene, lycopene and β-carotene increased linearly with the increase in the protein concentration in the extract up to 5 mg of protein (Figure 4.1A). The specific enzyme activity of the extract (typically ca. 1700 cpm/mg protein)
Carotenoid biosynthesis in a number of non-photosynthetic microorganisms has been shown to be photoregulated (Rau, 1985). On the basis of these studies it has been proposed that light induces the de novo synthesis of carotenogenic enzymes. The most direct approach for establishing if this is correct, and in determining which enzymes are photoinducible, is to compare in vitro activities of cell extracts from light- and dark-grown cultures. In the previous chapter it has been demonstrated that carotene biosynthesis in \textit{A. giganteus} mut. alba is photoregulated by blue light at the transcriptional level. In order to ascertain which enzymes are photoinduced it is necessary to develop a cell-free system of \textit{A. giganteus} which exhibits high carotenogenic activities in light-grown cultures. In addition, some of the properties of the enzymes associated with carotene biosynthesis have been investigated.

4:1 Effect of protein concentration on incorporation of \([2-^{14}C]\) MVA into carotenoids

This experiment was designed to investigate the effect of protein concentration in the cell extract on the incorporation of \([2-^{14}C]\) MVA into carotenoids of \textit{A. giganteus}. A cell-free extract from light-grown cultures converted \([2-^{14}C]\) MVA into phytoene, lycopene, \(\beta\)-carotene and squalene (Table 4.1, Figure 4.1). Incorporation of radioactivity from \([2-^{14}C]\) MVA into phytoene, lycopene and \(\beta\)-carotene increased linearly with the increase in the protein concentration to the maximum with 5 mg of protein (Figure 4.1A). The specific enzyme activity of the extract (Typically ca. 1700 dpm into \(\beta\)-carotene/mg)
Table 4.1 The effect of oxygen on carotenogenesis by cell extracts of *A. giganteus*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Incorporation (dpm/mg protein)*</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+O₂</td>
<td>-O₂</td>
</tr>
<tr>
<td>Total terpenoids</td>
<td>33917</td>
<td>33143</td>
</tr>
<tr>
<td>Phytoene</td>
<td>2130</td>
<td>6330</td>
</tr>
<tr>
<td>Lycopene</td>
<td>1009</td>
<td>260</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>1659</td>
<td>165</td>
</tr>
<tr>
<td>Squalene</td>
<td>4210</td>
<td>4122</td>
</tr>
</tbody>
</table>

*From 0.25μCi R-[2-14C] MVA. Protein concentration 4.2 mg/incubation (0.5 ml)
Figure 4:1 The formation of Phytoene (△) lycopene (○) and β-carotene (●) by cell extracts of A. giganteus with respect to protein (A) and time (B).

All incubations were at 35°C and contained 0.25 μCi R-[2-14C] MVA. The protein content in B was 4.9 mg/incubation.
protein) is considerably higher than those reported for other photoregulated fungi, i.e. *N. crassa* (170 dpm into coloured carotenoids/mg protein) (Kitzka-Schnabel and Rau, 1981) and *P. blakesleeanus* (444 dpm into β-carotene/mg protein; De la Concha et al., 1983).

A typical time course for carotene production at 35°C is shown in Figure 4:1B. As can be seen, there is an initial rise in the incorporation of radioactivity into phytoene, lycopene, and β-carotene, reading a maximum after one hour. The same maximum was read after 2 h when incubated at the lower temperature of 24°C.

**4:2 Effect of age of cultures on incorporation of [2-14C] MVA into carotenoids**

Cell extracts were prepared from light-grown, 48, 72, 88, 120 and 144 h-old mycelia and then incubated with [2-14C] MVA. Extracts prepared from different ages of mycelia exhibited significantly different specific activities when incubated aerobically with [2-14C] MVA (Figure 4:2). Maximum incorporation of radioactivity was found with cultures grown for 88 h in vitro preparations of older cultures (5 days) containing only some 5% of the maximum activities.

A similar age-related phenomenon has been reported for the C9 carR21(−) (lycopene-accumulating) strain of *Phycomyces* (Bramley and Davies, 1975), although the in vitro formation of phytoene and lycopene in this fungus has two peaks of activity, one at late
Cell extracts of *Aspergillus* prepared from different ages of culture, as estimated by the incorporation of R-[2-\(^{14}\)C] MVA (0.25 μCi) into phytoene (▲), lycopene (○) and β-carotene (△). The growth curve (---0---) is shown.
exponential and another at late stationary phase (Bramley and Davies, 1975).

4:3 Effect of oxygen on incorporation of [2-¹⁴C] MVA into carotenoids by cell extracts from A. giganteus

Although the incorporation of [2-¹⁴C] MVA into both total terpenoid and squalene is virtually unaffected by in vitro anaerobiosis, the pattern of carotene formation is noticeably altered (Table 4:1). The presence of oxygen enhances the conversion of phytoene into lycopene and β-carotene. The same effect has been noted in Phycomyces (Bramley and Davies, 1975) and in Aphanocapsa (Clarke et al., 1982), but the formation of unsaturated C₅₀ carotenoids by a cell extract of S. faccium shows no such requirement for oxygen (Taylor and Davies, 1982), and phytoene biosynthesis is not increased under anaerobic conditions in a cell free preparation of Flavobacterium.

4:4 Cofactor requirements

In order to determine cofactor requirements, it was first of all necessary to remove the endogenous cofactors. This was accomplished by passing the S₁₀ fraction through a Sephadex G25 column (1.5 cm cross section x 8 cm height), pre-equilibrated with 0.4 M Tris-HCl buffer, pH 8.0 at a flow rate of 0.3 ml/min at 4°C. The protein fraction was then used for incubations with either [2-¹⁴C] MVA or [1-¹⁴C] IPP and various cofactor combinations (Table 4:2).
Table 4:2 Cofactor requirements for carotenoid biosynthesis from [2-\textsuperscript{14}C] MVA and [1-\textsuperscript{14}C] IPP in cell extracts of \textit{A. giganteus}.

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Phytoene</th>
<th>Lycopene</th>
<th>0-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>None</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>- GSH</td>
<td>0.0</td>
<td>0.9</td>
<td>2.2</td>
</tr>
<tr>
<td>- FAD</td>
<td>78.7</td>
<td>13.9</td>
<td>1.8</td>
</tr>
<tr>
<td>- NADP</td>
<td>0.5</td>
<td>3.2</td>
<td>1.6</td>
</tr>
<tr>
<td>- NAD</td>
<td>0.9</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>- ATP</td>
<td>0.0</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>- Mn\textsuperscript{2+}</td>
<td>2.8</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>- Mg\textsuperscript{2+}</td>
<td>0.6</td>
<td>0.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Phytoene</th>
<th>Lycopene</th>
<th>0-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>- ATP</td>
<td>56.8</td>
<td>58.9</td>
<td>53.9</td>
</tr>
<tr>
<td>- Mn\textsuperscript{2+}</td>
<td>80.5</td>
<td>89.4</td>
<td>80.5</td>
</tr>
<tr>
<td>- Mg\textsuperscript{2+}</td>
<td>0.0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\* Incorporations from 0.25\textmu Ci R-[2-\textsuperscript{14}C] MVA were 4703, 2875 and 4201 dpm into phytoene, lycopene and \(\beta\)-carotene, respectively. Protein concentration, 0.8 mg/incubation (0.5 ml).

\*\* Incorporations from 0.25\textmu Ci [1-\textsuperscript{14}C] IPP were 93551, 4464 and 10911 dpm into phytoene, lycopene and \(\beta\)-carotene, respectively. Protein concentration 0.8 mg/incubation (0.5 ml).
In common with many other carotenogenic systems (reviewed by Bramley, 1985) the conversion of MVA or IPP into carotene necessitates the presence of ATP, a divalent metal ion and GSH (Table 4:2). However, the metal ion requirement differs with the two substrates: with MVA both Mn$^{2+}$ and Mg$^{2+}$ are essential, but only Mg$^{2+}$ is required when IPP is the substrate. Presumably the phosphorylation reaction from MVA to IPP requires Mn$^{2+}$, and the subsequent isomerisation and prenylation steps are dependent upon Mg$^{2+}$. Although ATP is not required as a phosphate donor for the conversion of IPP into carotenes, it clearly stimulates these steps in vitro (Table 4:2). Similar results with cell extracts of Aphanocapsa (Clarke et al., 1982), Streptococcus (Taylor and Davies, 1982) tomato fruits (Maudinas et al., 1975, 1977) and Phycomyces (Clarke et al., 1981) have been reported, but the mechanism of activation is unknown. It has been suggested that this apparent stimulation is a secondary effect, whereby the ATP drives a parallel pathway necessary for terpenoid biosynthesis (Taylor and Davies, 1982), or else it is an allosteric effect (Porter and Spurgeon, 1979). Elucidation of this phenomenon must await studies on the purified enzyme.

4.5 Subcellular localisation of the carotenogenic enzymes

The *Aspergillus* extracts were fractionated into cytosolic (S$_{10S}$) and microsomal (P$_{10S}$) fractions which were then assayed for their carotenogenic activities using either [2-14C] MVA or [14C] phytoene. As shown in Table 4:3 only phytoene and squalene were synthesised from [2-14C] MVA by the S$_{10S}$ fraction. Reconstitution of the crude
Table 4:3 Subcellular distribution of carotenogenic enzymes in *A. giganteus*

<table>
<thead>
<tr>
<th>Compound</th>
<th>From R-[2-14C] MVA*</th>
<th>From [14C] Phytoeneb</th>
<th>Incorporation (dpm/mg protein)</th>
</tr>
</thead>
</table>
|               | $S_{10}$   | $S_{10}$+P<sub>10</sub> &nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&nbsp;&n

* - From 0.25nCi R-[2-14C] MVA. Protein concentrations: $S_{10}$, 4.7; $S_{10s}$, 3; $P_{10s}$, 2.7 $S_{10s}+P_{10s}$, 3.7 mg/incubation (0.5 ml)

b - 40296 dpm [14C] phytoene, obtained from *Phycomyces* C5 carB10(-). Protein concentrations: $S_{10s}$, 3.3; $P_{10s}$, 2.5 mg/incubation.

c - 36601 dpm [14C] squalene also present from C5 carB10(-).
extract, by mixing S_{10S} with P_{10S} restored full carotenogenic activities, indicating that lycopene and \( \beta \)-carotene biosynthesis is catalysed by microsomal enzymes. This was confirmed by the use of \[^{14}C\] phytoene as substrate, prepared in situ from \[^{2-14}C\] MVA using a coupled assay with the C5 \textit{carBil}(-) (phytoene-accumulating) strain of \textit{Phycomyces}. Under these incubation conditions the P_{10S} fraction converted some 38% of the \[^{14}C\] phytoene into lycopene and \( \beta \)-carotene, whereas the cytosol did not metabolise this substrate (Table 4.3). The membrane-bound nature of phytoene-metabolising enzymes has been found in all organisms studied to date, e.g., \textit{Phycomyces} (Bramley and Taylor, 1985), \textit{Neurospora} (Mitzka-Schnabel and Rau, 1981), \textit{Capsicum} (Camara et al., 1982) and \textit{Narcissus} (Kreuz et al., 1982), with the exception of the halophile \textit{H. cutirubrum} (Kushwaha et al., 1976), but the location of "phytoene synthetase" may be either as a peripheral membrane protein, which is readily dislodged during the preparation of a cell extract (Bramley and Taylor, 1985; Kreuz et al., 1982) or genuinely soluble, cytosolic enzyme (Camara, 1984). It is possible that the high osmolarity buffer used in the present study has removed phytoene synthetase from its loose association with a membrane during the extraction procedure.

4.6 Carotenes synthesised by cell-extracts from dark- and light-grown \textit{Aspergillus} cultures.
A comparison of the incorporation of \( [2^{-14}C] \) MVA into the terpenoids of cell extracts from light- and dark-grown cultures showed major differences. Total incorporation dropped some 80% in the dark-grown extract; there was no synthesis of carotenes and squalene formation fell to 21% of the light-grown values (Table 4:4). In order to eliminate the possibility of an inhibitor being present in the dark-grown cell, a mixture of cell extracts from light- and dark-grown cultures was incubated with \( [2^{-14}C] \) MVA. The specific enzyme activities in this case were approximately 50% of those in the light-grown extract (Table 4:4). These data show that the loss of activity in the dark-grown extract is not due to the presence of an inhibitor, and therefore must be a result of the absence of the appropriate enzymes. The precise number and type of enzymes which are only present in light-grown cells cannot be defined from these results, since MVA is not an immediate precursor of phytoene and the other carotenes. However, in vitro synthesis of squalene, albeit at a significantly reduced level, indicates that enzymes up to FPP formation are present, probably in reduced amounts. Therefore the protein(s) which are only present in illuminated cultures of Aspergillus must be one or all of those catalysing the conversions of FPP to \( \beta \)-carotene.
Table 4: Carotene and squalene formation by cell extracts of light- and dark-grown cultures of *A. giganteus*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Incorporation (dpm/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light-grown</td>
</tr>
<tr>
<td>Total terpenoids</td>
<td>38341</td>
</tr>
<tr>
<td>Phytoene</td>
<td>1664</td>
</tr>
<tr>
<td>Lycopene</td>
<td>921</td>
</tr>
<tr>
<td>β-carotene</td>
<td>1712</td>
</tr>
<tr>
<td>Squalene</td>
<td>3344</td>
</tr>
</tbody>
</table>

* From 0.25 μCi R-2-14C MVA, protein concentrations: light-grown, 4.1; dark-grown, 4.6; mixture of light- and dark-grown, 4.3 mg/incubation (±5 ml)
CHAPTER 5

PHOTOREGULATION OF CAROTENOGENIC ENZYMES

...
As discussed earlier in Section 4.6 (Table 4:4) cell-extracts from dark-grown *Aspergillus* failed to synthesise either phytoene, lycopene or 8-carotene from [2-^14^C] MVA and the level of squalene was 21% of that produced by extracts from light-grown cultures. This raises the possibility that one or more of the terpenoid enzymes is photoinduced or photoregulated.

5:1 Time course of appearance of carotenogenic enzyme activities during illumination

Cell extracts were prepared from cultures grown initially in the dark for four days, transferred to the light for different periods of time and then assayed for carotenogenic enzyme activities using [2-^14^C]-MVA. The kinetics of the appearance of phytoene synthetase, phytoene dehydrogenase and lycopene cyclase are shown in Figure 5:1A. As can be seen after a lag period of approximately twelve hours all three enzymes began to appear reaching the maximum at 36 h. This time course agrees with the *in vivo* synthesis of 8-carotene (Figure 3:4). These enzymes are absent in cell extracts from dark-grown cultures over the same time periods (Figure 5:1B). Since all three enzyme activities appeared simultaneously rather than sequentially, it seems likely that the carotenogenic enzymes are co-induced. Alternatively, only phytoene synthetase may be induced, the other enzymes already being present. Although the enzymes for squalene synthesis were present in the dark-grown cultures, light increased squalene synthesis following a similar time course pattern as the carotenogenic enzymes.
Cell extracts were prepared from cultures grown initially in the dark for 4 days, transferred to the light for different periods of time (A), or left in darkness throughout (B) and then assayed for carotenogenic enzyme activities using R-(2-14C) MVA (0.25µCi) (phytoene O, lycopene ●, β-carotene Δ, squalene ▲). Protein concentration: Time 0-4.7, 6-4.5; 12-4.8; 18-4.7; 24-4.3; 30-4.5; 36-4.6; 42-4.8 (A); 0-4.5; 12-4.8; 24-4.8; 36-4.7; 42-4.8 mg/incubation.
Figure 5:1

Radioactivity in carotenoids (dpm x 10^3)

Radioactivity (dpm x 10^3) in squalene

Illumination regime (h)

Although there is little doubt that the first enzymes in the sequence are pheophytin synthetase, it only appears after illumination. It requires confirmation for the other enzymes, pheophytin diphosphate and lycopene cyclase. Ideally, each enzyme should be assayed individually for each substrate and reconstituted activities that fulfill the requirements for pheophytin synthetase, pheophytin for pheophytin diphosphate and lycopene cyclase.
Examination of pre-phytoene intermediates synthesised by cell extracts from dark-grown A. giganteus

So far, only carotenoids have been analysed but, in terms of the carotenoid pathway, FPP is the branch point on the terpenoid pathway for carotenoid synthesis and, therefore, the enzymes, GGPP synthetase and PPPP synthetase, may be the site of photoregulation rather than phytoene synthetase. This was investigated by analysing the prenyl pyrophosphates produced by cell extracts from dark-grown Aspergillus cultures with [2-\(^{14}\)C] MVA (Table 5:1). Synthesis of PPPP clearly shows that phytoene synthetase is a different enzyme from PPPP synthetase and thereby the latter is present in the dark-grown cultures. Therefore, phytoene synthetase is strictly photoinduced whilst PPPP synthetase is constitutive in the dark. The latter enzyme may still increase in the light but such a possibility required further investigation.

Enzyme analysis using [\(^{14}\)C]-labelled precursors

Although there is little doubt that the first enzyme in the sequence, phytoene synthetase, is only present after illumination, it still requires confirmation for the other enzymes, phytoene dehydrogenase and lycopene cyclase. Ideally, each enzyme should be assayed individually. For such a purpose, radiolabelled substrates (i.e. PPPP for phytoene synthetase, phytoene for phytoene dehydrogenase and lycopene for lycopene cyclase) are required. Since such substrates were not available commercially, radiolabelled phytoene and lycopene
Table 5:1  Prenyl pyrophosphates produced by cell extract from four days old dark-grown A. giganteus

<table>
<thead>
<tr>
<th>Prenyl pyrophosphates</th>
<th>Incorporation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPPP</td>
<td>7872</td>
</tr>
<tr>
<td>GGPP</td>
<td>4405</td>
</tr>
<tr>
<td>FPP</td>
<td>1220</td>
</tr>
<tr>
<td>GPP</td>
<td>3764</td>
</tr>
</tbody>
</table>

* - From 0.25 μCi R-[2-¹⁴C] MVA; Protein concentration 4.5 mg/incubation (0.5 ml)
were prepared by incubating cell-free extracts from *Phycomyces*
mutant C5carR10(-) and C9carR21(-) (Bramley and Davies, 1975),
respectively with [2-14C] MVA and extracting the appropriate carotene
required. For example, the purified phytoene (from C5) was dissolved
in acetone (8ml), and phytoene solution (2ml) was mixed with 0.8 ml
incubation mixture (containing 20 μl Tween60) and the acetone then
removed under N2. Finally, cell-free extract (0.2 ml) from either
dark- or light-grown *Aspergillus* was added, incubated for 2 h at
35°C, and then analysed for incorporation into the different
carotenoid fractions (Table 5;2). Although there appears to have
been experimental loss of radioactivity, particularly with "light-
grown" cell extracts, phytoene was clearly converted to lycopene and
β-carotene by cell extracts from light-grown cultures. Therefore, it
appears that phytoene dehydrogenase is also photoinduced. Similar
experiments with radiolabelled lycopene (from C9), however, were
unsuccessful, possibly because of greater difficulties in solubilising
lycopene in aqueous solution.

5:4 Enzyme analysis using a coupled system

An alternative approach to preparing specific labelled precursors is
the use of a coupled system. Cell-free preparations of *Aphanocapsa*,
strain 6714, use MVA and IPP very poorly (Clarke et al., 1982).
However, Sandmann and Bramley (1985) were able to study β-carotene
and xanthophyll formation by thylakoid preparations from the
cyanobacterium, *Aphanocapsa* by coupling to appropriate cell extracts
Table 5:2  Conversion of [14C] phytoene into other carotenoids by cell extracts from light- and dark-grown A. giganteus

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Dpm/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>light</td>
</tr>
<tr>
<td>Total terpenoids</td>
<td>10438</td>
</tr>
<tr>
<td>Phytoene</td>
<td>3125</td>
</tr>
<tr>
<td>Lycopene</td>
<td>412</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>890</td>
</tr>
</tbody>
</table>

Phytoene 75 μM (sp. act. 0.4 mCi/mmmole).

Cell extracts from suspensions cultures, grown initially in the dark for 4 days, transferred to the light for different time periods, or left in the darkness throughout, were mixed with extracts from dark-grown cultures previously incubated with [14C] KFA. At the time of coupling, excess unlabelled KFA was added (500 μg) in small volumes (5 μl) to the incubation mixture so that only the association of the labelled products produced by the "dark" extract (in this case, prenyl pyrophosphates) would be recognized. Thus, procedure is typical of pulse-chase experiments used to follow precursor/product relationships. After the coupling incubation, the prenyl alcohols and carotenoids were isolated and assayed for radioactivity. The results are shown in Figure 5.3 and will be discussed further with...
from F. blakesleeanus mutants. Simultaneous incubation of Aphanocapsa thylakoids and Phycomyces (carS) cell-free preparations resulted in the synthesis of β-cryptoxanthin and other xanthophylls characteristic of the cyanobacterium.

In another series of experiments, they studied the formation of β-carotene and its intermediates from [14C] phytoene by Aphanocapsa thylakoids by coupling to cell-extracts of Phycomyces (C5 strain) which had previously been incubated with [2-14C] MVA. By using this method it was possible to follow precursor/product relationships with time (Bramley and Sandmann, 1985). A similar approach was adopted to study the individual carotenogenic enzymes in Aspergillus by initially incubating [2-14C] MVA with cell-free extracts from appropriate sources blocked at specific precursors.

Cell extracts from Aspergillus cultures, grown initially in the dark for 4 days, transferred to the light for different time periods, or left in the darkness throughout, were coupled with extracts from dark-grown cultures previously incubated with [14C] MVA. At the time of coupling, excess unlabelled MVA was added (700 μg) in small volumes (5 μl) to the incubation mixture so that only the metabolite of the labelled products produced by the "dark" extract (in this case, prenyl pyrophosphates) would be recognised. This procedure is typical of pulse/chase experiments used to follow precursor/product relationships. After the coupled incubation, the prenyl alcohols and carotenes were isolated and monitored for radioactivity. The results are shown in Figure 5.2 and confirm those discussed earlier with
Position of privilege appearing in privilege designation...

may include exercise of any combination of privileges...

with complete exercise of...

Privilege G.9, 9.3 and 9.4.

Figure 2:

Call necessary from your position, go to the right for...

and exercise as near the front of...

and exercise from your position, advantage to...

importation via M-15A-1.5 X45 (0.55 MCI) or any other...

by placing G.10 beneath G.10.

- 117 -
Estimation of phytoene synthetase, phytoene dehydrogenase and lycopene cyclase, in cell extracts of *Aspergillus*, using coupled enzyme systems.

Figure 5:2 Cell extracts from *Aspergillus*, grown in the dark for 4 days transferred to the light for different periods of time (A) or left in darkness throughout (B), were coupled with extracts from dark-grown *Aspergillus*, previously incubated with R-[2-\(^{14}\text{C}\)] MVA (0.25 \(\mu\text{Ci}\)) (prenyl alcohol \(\text{x}\), phytoene \(\text{O}\), lycopene \(\bullet\), \(\beta\)-carotene \(\Delta\), squalene \(\triangle\)).
Figure 11: Graph showing the relationship between X and Y variables. The x-axis represents time in days, and the y-axis represents a specific measurement. The data points indicate a trend that requires further analysis.
Figure 5:4 Cell extracts from Aspergillus cultures grown in the dark for 4 days transferred to the light for different periods of time (A) or left in darkness throughout (B), were coupled to extracts from P. blakesleeanus C9 carR21(-) previously incubated with R-[2-14C] (0.25µCi) phytoene 0, lycopene 0, β-carotene Δ)
Radioactivity (dpm x 10^3) in terpenoids

Radioactivity (dpm x 10^3) in prenyl alcohols
Figure 5:3  Cell extracts from Aspergillus cultures grown in the dark for 4 days transferred to the light for different periods of time (A) or left in darkness throughout (B), were coupled with extracts from P. blakesleeanus C5 carB10(-) previously incubated with R-[2-\textsuperscript{14}C] MVA (0.25 \textmu ci) (phytoene 0, lycopene 0, \beta-carotene \Delta).
Figure 5:4 Cell extracts from *Aspergillus* cultures grown in the dark for 4 days transferred to the light for different periods of time (A) or left in darkness throughout (B), were coupled to extracts from *P. blakesleeanus* C9 carR21(-) previously incubated with R-[2-¹⁴C] (0.25µCi) phytoene 0, lycopene ●, β-carotene △)
single incubation of extracts from either light- or dark-grown *Aspergillus* cultures with [2-\(^{14}\)C] MVA (Figure 5:1). \(\beta\)-carotene, lycopene and phytoene were only synthesised by extracts from light-grown cultures, appearing after 12 h illumination and reaching a maximum at 36 h. The carotenes were produced at the expense of the prenyl pyrophosphate precursors. Clearly, phytoene synthetase is photoinducible. As before, squalene synthesis was stimulated in cultures grown in the light.

To examine phytoene dehydrogenase directly, extracts from light- or dark grown *Aspergillus* were coupled with an extract from *carB10*\(^{-}\) mutant of *P. blakesleeanus*. This mutant is blocked in phytoene dehydrogenase and the cell extract only metabolises [2-\(^{14}\)C] MVA as far as phytoene (De la Guardia *et al.*, 1971). As seen in Figure 5:3, phytoene was only converted to lycopene and \(\beta\)-carotene by extracts from light-grown cultures. This suggests that phytoene dehydrogenase is also photoinduced and follows the same timing of induction as phytoene synthetase.

Finally, lycopene cyclase was examined following a similar procedure, except in this case the *Aspergillus* extracts were coupled to a different *Phycomyces* mutant, *carR21*\(^{-}\), which accumulated lycopene (De la Guardia *et al.*, 1971). As before, lycopene cyclase activity was only present in extracts from light-grown *Aspergillus* and followed an identical time course production as that observed for the other carotenogenic enzymes (Figure 5:4). Summarising, all three enzymes, phytoene synthetase, phytoene dehydrogenase and lycopene
cyclase, appear to be coinduced by light, responding in a similar fashion to certain bacterial enzyme systems which function genomically as oparon. As far as is known, this is the first time these particular carotenogenic enzymes have been examined in detail and shown to be specifically photoregulated.

5:5 Characterisation of albino mutants of \textit{A. giganteus}

A number of albino mutants of \textit{A. giganteus} have been isolated after U.V. irradiation of wild-type cultures. Carotenoid analyses showed that these mutants lacked phytoene and therefore were either blocked at some stage prior to phytoene synthesis or in the photoregulatory mechanism and consequently unable to synthesise carotenoids in the light. Cell extracts prepared from such mutants were incubated with [2-\textsuperscript{14}C] MVA and analysed for prenyl pyrophosphates. The results for only one of these mutants (E5) is presented since all the mutants behaved similarly (Table 5:3). Unlike the wild-type extracts, light had no effect on the patterns of incorporation of [2-\textsuperscript{14}C] MVA into the various intermediates. More significantly, both cell extracts were unable to synthesise PPPP, accumulating GGPP. It would appear that these are structural gene mutants blocked at PPPP synthetase rather than at some photoregulatory function. Unexpectedly, light appeared to have no effect on squalene synthesis.

However, the "dark" levels of squalene are much higher compared to extracts from dark-grown wild type cultures. It may be that blockage of PPPP synthetase has resulted in diversion of inter-
Prenyl pyrophosphates and squalene produced by cell extracts from light- and dark-grown *A. giganteus* mutant E5

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incorporation (Dpm/mg protein)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
</tr>
<tr>
<td>GGPP</td>
<td>13371</td>
</tr>
<tr>
<td>FPP</td>
<td>696</td>
</tr>
<tr>
<td>GPP</td>
<td>1205</td>
</tr>
<tr>
<td>Squalene</td>
<td>6090</td>
</tr>
</tbody>
</table>

* - From 0.25 μCi R-(2-¹⁴C) MVA. Protein concentration 4.7 mg/incubation
mediates into the squalene pathway, overriding the normal light effect. Interestingly, the cell extracts of the E5 mutant (accumulates GGPP) could be used to investigate the effects of light on the levels of PPPP synthetase activity by coupling it with extracts from light- and dark-grown *Aspergillus*. Various soluble enzymes in the terpenoid pathway have been shown to be photoregulated and are discussed in Chapter 7.
CHAPTER 6

THE EFFECT OF LIGHT ON OTHER LIPIDS OF

A. GIGANTEUS
During the studies on the effect of light on carotenoid biosynthesis, measurements of total lipids were routinely carried out and used as a yardstick for experimental efficiency and reproducibility. It soon became obvious that in addition to the carotenoids, other lipids were also increased in cultures grown in the light. A set of representative results are shown in Table 6:

Light grown cultures (in white light) contained seven-fold more lipid than those grown in the dark. Further, this photostimulation appeared to be blue- rather than red-light dependent. This in vivo effect is paralleled by the earlier reported observation with cell-free systems (Table 4:4) where it was shown that five times as much [2-14C] MVA was incorporated into total terpenoids by extracts from light-grown compared to dark-grown Aspergillus cultures. In this instance squalene was measured and also increased five-fold in light. Therefore light, and particularly blue-light, not only stimulated the biosynthesis of carotenoids, but also other lipids as well, of which squalene was a specific example.

This led to the qualitative and quantitative analysis of the major groups of lipids in the light- and dark-grown cultures of Aspergillus (Table 6:2). Interestingly, all the classes of lipids were increased in the light, from three-fold in the case of phospholipids up to twelve-fold for glycerol. Also, compared to carotenoids which are only minor lipid components (less than 1%), these other lipids constitute approximately one quarter of the dry weight in light-grown
Table 6:1 Effect of light and dark on total lipid biosynthesis by *A. giganteus*

<table>
<thead>
<tr>
<th>Light Source</th>
<th>Total lipid (mg/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White (370-700nm, 50W/m²)</td>
<td>243.7</td>
</tr>
<tr>
<td>Dark</td>
<td>36.7</td>
</tr>
<tr>
<td>Blue (400-520nm, 1.8W/m²)</td>
<td>73.2</td>
</tr>
<tr>
<td>Red (600-780nm, 11.5 W/m²)</td>
<td>41.5</td>
</tr>
</tbody>
</table>

Carotenoids: 0.16

Total lipids: 283.7
Table 6:2 Quantitative analysis of the different lipids produced by light- and dark-grown *A. giganteus* cultures

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Light mg/g dry wt.</th>
<th>Dark mg/g dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerides</td>
<td>107.6</td>
<td>18.6</td>
</tr>
<tr>
<td>Fatty Acids</td>
<td>82.0</td>
<td>9.8</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>21.4</td>
<td>6.7</td>
</tr>
<tr>
<td>Glycerol</td>
<td>13.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Sterols</td>
<td>2.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>0.16</td>
<td>n.d.</td>
</tr>
<tr>
<td><strong>Total lipids</strong></td>
<td><strong>243.7</strong></td>
<td><strong>36.7</strong></td>
</tr>
</tbody>
</table>
cultures. Similar quantities of these lipids, most being in the glyceride and fatty acid fraction, have been observed in *P. blakesleeanus* (Chencuda, 1970). Apart from the observation that light showed no effect on sterols in *R. minuta* (Tada et al., 1982), no other experiments on the effect of light on lipid biosynthesis have been reported.
Aspergillus niger is not able, in shake culture, grown equally well in the light and dark. This organism exhibits strict photoregulation of carotenoid biosynthesis, similar to \( E. \) aerophilum (Gay, 1974), but unlike \( E. \) niger which produces small amounts of phytomene in the dark (Malokr, 1925; Hardin et al., 1968), and \( E. \) flavus which produces small amounts of phytomene in the dark (Bergen et al., 1975; Sandmann and Hilgendorf, 1975) where light enhances the coloured carotenoid content of the cells. It has been shown to be a blue-light effect, characteristic of other fungi (reviewed by Berg, 1985).

The kinetics of photoregulation in Aspergillus follows two stages: a first "light-phase" during which light and oxygen but not temperature-independent, followed by a second light-independent, temperature-dependent phase during which carotenoid biosynthesis occurs (the dark reaction). This is similar to other fungi except for \( V. \) arvalis which, only accumulates carotenoids during illumination, but differs from \( E. \) aerophilum and \( E. \) niger in that these fungi have greater intensities and periods of light required for maximal carotenoid production. The lag period between the two phases is also longer.

Two important questions can be asked concerning the photoregulation of carotenoid biosynthesis: (i) Which enzymes are photoinactivable, and (ii) at what metabolite level does photoregulation occur?

Initial in vivo studies e.g. with \( V. \) arvalis Zelitch, 1954, suggested that the carotenogenic enzymes were
Aspergillus giganteus mut. alba, in shake culture, grows equally well in the light and dark. This organism exhibits strict photoregulation of carotenogenesis, similar to F. aquaeductuum (Rau, 1976), but unlike N. crassa which produces small amounts of phytoene in the dark (Zalokar, 1955; Harding et al., 1969) and P. blakesleeanus (Bergman et al., 1973; Sandmann and Hilgenberg, 1978) where light enhances the coloured carotenoid content of the cells. It has been shown to be a blue-light effect, characteristic of other fungi (reviewed by Rau, 1985).

The kinetics of photoregulation in Aspergillus follows two stages; a first "light phase" which required light and oxygen but is temperature-independent, followed by a second light-independent, temperature-sensitive stage during which carotenoid biosynthesis occurs (the dark reactions). This is similar to other fungi except for V. agaricinum which only accumulates carotenoids during illumination, but differs from F. aquaeductuum and N. crassa in that greater intensities and periods of light are required for maximum carotenoid production. The lag period between the two phases is also longer.

Two important questions can be asked concerning the photoregulation of carotenoid biosynthesis: (i) which enzymes are photoinducible, and (ii) at what metabolic level does photoregulation occur?

Initial in vivo studies e.g. with N. crassa (Zalokar, 1954), suggested that the carotenogenic enzymes were
coordinate induction by light. However, in order to demonstrate unequivocally which biosynthetic enzyme activities change during illumination, it is necessary to use cell-free preparations from dark- and light-grown cultures with suitable substrates. Relatively few in vitro systems are available from photoregulated fungi, but some useful information has been obtained with *N. crassa* and *P. blakesleeanus*. A cell-free system has been developed for *A. giganteus* which is more efficient than those for *Neurospora* and *Phycomyces* in its conversion of (2-¹⁴C) MVA to β-carotene. In addition, cell extracts from illuminated cultures are able to incorporate radioactivity from (2-¹⁴C) MVA into carotenoids but no such incorporation occurs with extracts from dark-grown cultures.

To determine which specific enzymes are photoregulated, ¹⁴C-labelled MVA, PPPP, phytoene and lycopene (provided by coupled systems) were incubated with cell extracts of light- and dark-grown *A. giganteus*. The enzymes catalysing the conversions from PPPP to β-carotene (phytoene synthetase, phytoene dehydrogenase and lycopene cyclase) are totally absent in dark-grown mycelia and are photoinduced upon illumination. In the same organism, IPP isomerase is stimulated 2.5-fold, but prenyl transferase activity is virtually identical under both conditions (Sandmann et al., unpublished). Other enzymes are photoregulated in other fungal species. For example, using (1-¹⁴C)-IPP with an in vitro system from *N. crassa*, it has been shown that the steps to phytoene are stimulated in light-grown cultures compared with dark-grown controls (Spurgeon et al., 1979). These results have been independently confirmed with (2-¹⁴C) MVA as the substrate.
It has been shown that HMG-CoA reductase is photoregulated (2-fold increase in activity upon illumination) using a cell-free system from R. minuta. The enzymes which convert phytoene into coloured carotenoids are not photoinducible in this yeast (Tada and Shiroishi, 1982). The results of studies with cell-free systems from P. blakesleeanus are more ambiguous. A comparison of enzymic activities from dark- and light-grown cultures of P. blakesleeanus 1+, incubated with [2-\(^{14}\)C] MVA, showed that the latter contained twice the activity of the dark-grown preparation, and these data were taken as an explanation of the increased levels of \(\beta\)-carotene in vivo following illumination (Sandmann and Hilgenberg, 1978). However, studies with wild-type Phycomyces (NRRL 1555), revealed no differences in the specific activities of the enzymes from MVA to \(\beta\)-carotene in light- and dark-grown cultures, despite a 4-fold increase in \(\beta\)-carotene levels in vivo on illumination (Orejas et al., unpublished). The reason for this anomaly between the two studies has yet to be explained experimentally, and may be due to the different wild-type strains employed. Light- and dark-grown cultures of wild type and the SG22 strains of G. fujikuroi showed photoregulation, but in vitro activities were the same (Nelki et al., 1987). However on the basis of in vitro data, phytoene synthetase is strictly photoregulated in mutant strain SG43 but not so in the wild type.
As well as the carotenoids, photoregulation of the synthesis of the other lipids in *A. giganteus* provides an excellent model for studying regulation of lipid biosynthesis in general. As yet, the lipids have only been studied as major lipid classes and it would be interesting to carry out a more detailed analysis of the individual lipid species within such classes. Also, it would be necessary to examine the capability of the *in vitro* system to synthesise these various lipids and, if necessary, improve it. This should then make it possible to identify those enzymes which are photoregulated.

*In vivo* studies with inhibitors of transcription and translation (reviewed by Rau, 1983) have generally led to the conclusion that regulation by light affects transcription e.g. *F. aquaeductuum* (Rau, 1980). Inhibitors also prevent photocarotenogenesis in *A. giganteus* and therefore it seems likely that transcriptional control also operates in this organism. Evidence for the involvement of *de novo* mRNA synthesis has also been obtained from studies with *Fusarium* (Schrott and Rau, 1975; 1977). More recently, the Munich group have extracted mRNAs from light- and dark-grown mycelia of *Neurospora* and *Fusarium* and compared their translational capacities *in vitro*. The levels of poly(A)-RNA were higher in illuminated mycelia, and differences in the *in vitro* translated polypeptides were also detected by 2-d electrophoresis (Mitzka *et al.*, 1984).

The derepression of carotenoid gene transcription in *N. crassa* is controlled by the intracellular level of cAMP. A series of experiments with dark- and light-grown cultures, and mutants deleted
In adenylate cyclase or phosphodiesterase have demonstrated that light causes a decrease in cAMP levels, which in turn alleviates carotenoid gene repression. An additional controlling mechanism is thought to be the activity of NAD kinase i.e., the cellular levels of NADP. In submerged, vegetative mycelia, light causes the photo-oxidation of NADH (an inhibitor of NAD kinase), whereas in maturing conidia, activation of NAD kinase occurs. These mechanisms work in parallel with the cAMP-mediated control of gene expression (Kritsky, 1985). Cyclic AMP may also play a regulatory role in A. giganteus (Zurzycka et al., 1983).

Obviously many intriguing questions remain to be answered. In order to understand the molecular mechanisms associated with the photoregulation of carotenoid formation it is necessary to isolate and characterise the structural regulatory gene products.

Two parallel experimental approaches should be pursued in order to achieve these goals. In order to estimate the levels of carotenogenic enzymes, and any changes which occur as a result of regulatory processes, it is necessary to develop in vitro assays which accurately reflect the physiological activities of these enzymes. None of the cell-free systems synthesise the same levels of carotenoids as those found in vivo; in all cases and especially in Neurospora there is a significant accumulation of phytoene in vitro, which is not found in whole cells. Consequently, comparison of in vivo carotenoid synthesis with in vitro activities may be misleading and should be viewed with some caution until the cell-free activities mirror physiological levels. Purification of the enzymes themselves
would be most valuable for enzymic studies, production of antibodies and for the isolation of carotenoid genes. The large numbers of structural and regulatory mutants obtained from fungi, have been valuable in establishing steps of the carotenoid biosynthetic pathway and in establishing the complexity of the regulatory processes within individual organisms (e.g., *Phycomyces*) as well as showing that no single, common regulatory mechanism exists amongst carotenogenic organisms. It is important in this connection to isolate appropriate carotenogenic mutants of *A. giganteus* for studies on the regulation of carotenogenesis by *in vitro* preparations and identification of carotenogenic genes by complementation. The ultimate aim is the isolation and characterisation of the carotenoid genes. Since purified enzymes and their antibodies are not yet available, gene isolation must use transformation techniques with suitable vectors. With the notable exception of studies by Marrs and co-workers with *Rhodopseudomonas* *capsulata* (Marrs, 1982) such experiments have yet to be reported. However, the increasing availability of transformation systems and vectors for filamentous fungi such as *Neurospora*, *Aspergillus* and *Penicillium* (reviewed by Bennett and Lasure, 1985), now makes it feasible to isolate carotenoid genes from fungi, and *A. giganteus* in particular. Once isolated, the regulatory sequences of the gene can be identified by *in vitro* mutagenesis, whilst the genes themselves can be used as probes for monitoring changes in mRNA levels following treatment of the cells with light. This approach will also provide absolute proof of transcriptional control of carotenoid biosynthesis by light.
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