



THE TERPENOID BIOSYNTHESIS OF TERPENOIDS  
IN  
GIBBERELLA FUJIKUROI

by

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## Abstract

The aim of this work was to study the terpenoid biosynthesis and its regulation in the fungus Gibberella fujikuroi. This was facilitated by the use of a number of mutants of G.fujikuroi produced using UV radiation or N-methyl-N'-nitrosoguanidine and selected mainly by mutation in colour and photoregulation (Avalos et al., 1985).

A separation procedure for the carotenoids was devised and up to 9 carotenoids were isolated. Their levels were measured in wild type G.fujikuroi and 4 of the mutants in both light and dark grown conditions. From this a carotenogenic pathway for G.fujikuroi was proposed and the carotenoid levels were shown to be photoregulated. These levels were also affected by the nature of the growth medium used.

A method for the separation of 5 of the gibberellins (GA<sub>3</sub>, GA<sub>4</sub>, GA<sub>7</sub>, GA<sub>13</sub> and GA<sub>14</sub>) was developed using a reverse phase, Spherisorb S50DS HPLC column. This methodology was used to determine time courses for gibberellin production in the wild type and 2 mutant strains. The results showed that the rate of GA production was increased in the mutants, compared to the wild type. Also light and dark grown gibberellin levels were measured in 6 mutants and 2 wild type strains, along with the effects of different media. It was shown, as in carotenogenesis, gibberellin levels were photoinduced and that the levels of photoinduction were increased to differing degrees in the mutants compared to the wild types. The overall gibberellin levels were also shown to be affected by the nature of the growth medium.

A cell-free system for G.fujikuroi was developed for the

incorporation of [2-<sup>14</sup>C]-mevalonic acid into the terpenoids, which gave the possibility of developing systems for studying individual enzymes in terpenoid biosynthesis. A coupled system was developed for assaying phytoene synthetase using the cell-free system of a non-carotenoid producing mutant to produce substrate for phytoene synthetase coupled to extracts of a high-phytoene producing mutant. This assay was then used in attempts to purify phytoene synthetase.



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## Abbreviations

IPP	Isopentenyl pyrophosphate
GA	Gibberellin (see Fig 1.9)
GGPP	Geranyl geranyl pyrophosphate
FPP	Farnesyl pyrophosphate
MVA	Mevalonic acid
MVAP	Mevalonic acid 5-phosphate
MVAPP	Mevalonic acid 5-pyrophosphate
Acetyl CoA	Acetyl coenzyme A
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
ATP	Adenosine triphosphate
DMAPP	Dimethylallyl pyrophosphate
GPP	Geranyl pyrophosphate
PSPP	Presqualene pyrophosphate
PPPP	Prephytoene pyrophosphate
PLPP	Prelycopersene pyrophosphate
DPA	Diphenylamine
cAMP	Adenosine 3',5'-monophosphate(cyclic)
SAM	S-adenosylmethionine
HPLC	High performance liquid chromatography
TLC	Thin layer chromatography
Et <sub>2</sub> O	Diethyl ether
HOAc	Acetic acid
EtOAc	Ethyl acetate
MeOH	Methanol
FAD	Flavin adenine dinucleotide
pNPP	<u>para</u> -Nitrophenol phosphate
pNP	<u>para</u> -Nitrophenol
nBuOH	Butan-1-ol
PEG	Polyethylene glycol
Al <sub>2</sub> O <sub>3</sub>	Alumina oxide
CHAPS	3-((3-cholamidopropyl)-dimethyl-amino)-1-propanesulphonate
DTT	Dithiothreitol
CHCl <sub>3</sub>	Chloroform
Tris	Tris(hydroxymethyl)aminomethane
cDNA	Complementary deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
Carotenoids	(see Fig 1.6)
HMG-CoA	Hydroxy-methyl glutaryl coenzyme A

CHAPTER I  
INTRODUCTION

"We shall not cease from exploration  
And the end of all our exploring  
Will be to arrive where we started  
And know the place for the first time"

T.S.Elliot



## 1.1 Terpenoids

The terpenoids, also known as the polyprenyls or isoprenoids are a group of natural products that are derived biosynthetically from the 5-carbon compound isopentenyl pyrophosphate (IPP).

The major classes of terpenoids, Table 1.1:

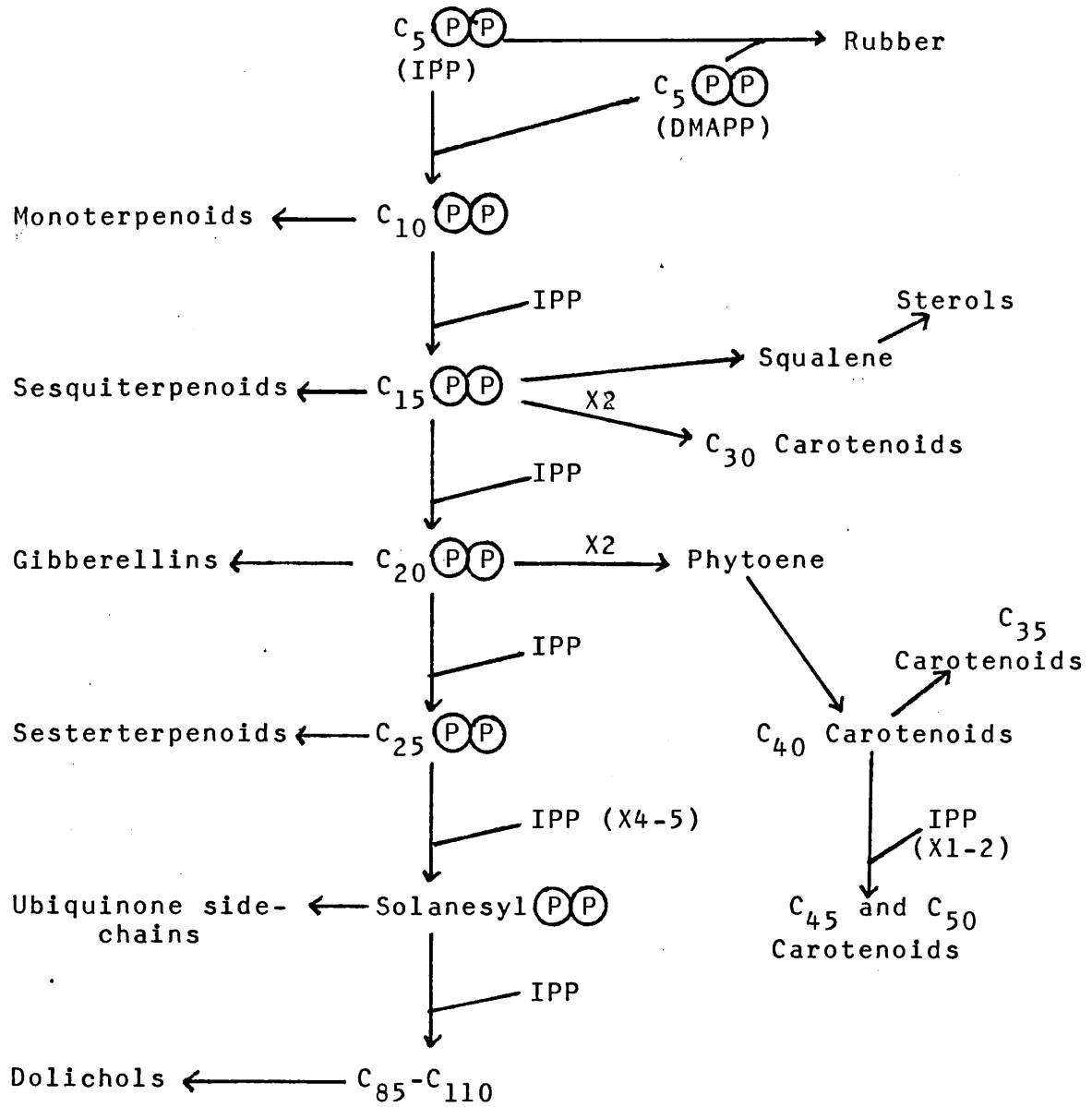
Class	Number of Carbons in Parent Compound
Monoterpenes	C <sub>10</sub>
Sesquiterpenes	C <sub>15</sub>
Diterpenes	C <sub>20</sub>
Sesterterpenes	C <sub>25</sub>
Triterpenes	C <sub>30</sub>
Tetraterpenes	C <sub>40</sub>
Polyterpenes	(C <sub>5</sub> ) <sub>n</sub>

These classes are further subdivided based on structural features e.g. monoterpenes can be divided into acyclic, monocyclic and bicyclic compounds.

The biosynthetic routes of the terpenoids are illustrated in Fig 1.1, on the whole the sterols, gibberellins (GAs) and carotenoids will be considered in these studies.

Isoprenes may be joined in a regular or irregular manner i.e. head-to-tail or tail-to-tail respectively. The triterpenes (steroids), tetraterpenes (carotenoids) and diterpenes (GAs) have a unique structural arrangement in which regularly formed terpene units are joined tail-to-tail to produce a symmetrical precursor, 2 FPPs for the steroids,

Fig 1.1 Biosynthetic Routes of Common Terpenoids



2 GGPPs for the carotenoids and a terpene unit is cyclized  
Forming GAs

Further variation in terpenoid structure is achieved by addition of functional groups, cyclisation, rearrangement, selective removal of carbon atoms and other modifications.

### 1.1.1 Early Terpenoid Biosynthesis

Mevalonic acid (MVA) is generally regarded as the first committed substrate in the terpenoid pathway since it can only be utilized by the one enzyme MVA kinase. This in turn is derived from acetyl-CoA (reviewed by Porter and Spurgeon, 1983). Acetoacetyl-CoA thiolase (acetyl-CoA acetyl transferase; EC 2.3.1.9) catalyzes the condensation of 2 molecules of acetyl-CoA to form acetoacetyl-CoA. The occurrence of the enzyme was first postulated by Lynen and coworkers (1953). It was shown to be reversible (Stern et al., 1953) favouring the formation of acetyl-CoA and is reviewed by Porter and Spurgeon (1983).

$\beta$ -Hydroxy- $\beta$ -methylglutaryl-CoA synthase (EC 4.1.3.5) catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to form hydroxy-methylglutaryl-CoA (HMG-CoA) first discovered in yeast (Ferguson and Rudney, 1959); it was later shown that the thioester linkage of acetoacetyl-CoA remains intact during the condensation (Stewart and Rudney, 1966).

$\beta$ -Hydroxy- $\beta$ -methylglutaryl-CoA reductase (mevalonate:NADP<sup>+</sup> oxidoreductase acetylating CoA; EC 1.1.1.34) catalyzes the reduction of D-HMG-CoA by reduced nicotinamide adenine dinucleotide (NADPH) to form MVA. This step has been investigated in detail especially with respect to regulation, reviewed by Dugan and Porter (1976).

The formation of IPP is ubiquitous in nature e.g. bacteria

(Ohnoki et al., 1962 and Siddiqi and Rodwell, 1967), yeast (deWaard et al., 1959 and Rilling and Bloch, 1959), higher plants (Anderson et al., 1960) and mammals (Popjak et al., 1959) and it is formed from MVA via 2 consecutive phosphorylations at position 5 followed by a decarboxylation and a dehydration of the tertiary alcohol group requiring one mole of adenosine triphosphate (ATP).

In the first reaction mevalonate kinase (ATP:mevalonate-5-phosphotransferase; EC 2.7.1.36) catalyzes the phosphorylation of R-mevalonate to R-5-phosphomevalonate. Phosphomevalonate kinase (ATP:5-phosphomevalonate phosphotransferase; EC 2.7.4.2) then catalyzes a further phosphorylation to produce 3R-5-pyrophosphomevalonate. Finally pyrophosphomevalonate decarboxylase (ATP:5-pyrophosphomevalonate carboxy-lyase (dehydrating); EC 4.1.1.33) catalyzes the decarboxylation and dehydration of 5-pyrophosphomevalonate to produce IPP (Fig 1.2).

#### 1.1.2 IPP Isomerase and Prenyl Transferases

IPP Isomerase, the enzyme that equilibrates IPP and dimethylallyl pyrophosphate (DMAPP), reviewed by Holloway (1972), was first discovered in Lynen's laboratory (Arganoff et al., 1959) and requires a divalent cation. The reaction yields 87% DMAPP and 13% IPP at equilibrium (Porter, 1969).

DMAPP then acts as an allylating agent, condensing with a second molecule of IPP to yield geranyl pyrophosphate (GPP) which, retaining the allyl group can repeat the condensation to yield farnesyl pyrophosphate (FPP) and then geranyl geranyl pyrophosphate (GGPP), (Fig 1.3); these sequential allylations are catalyzed by the prenyl transferases (prenylpyrophosphate synthetases; EC 2.5.1.1).

Fig 1.2 Biosynthesis of Isopentenyl Pyrophosphate from Acetyl CoA

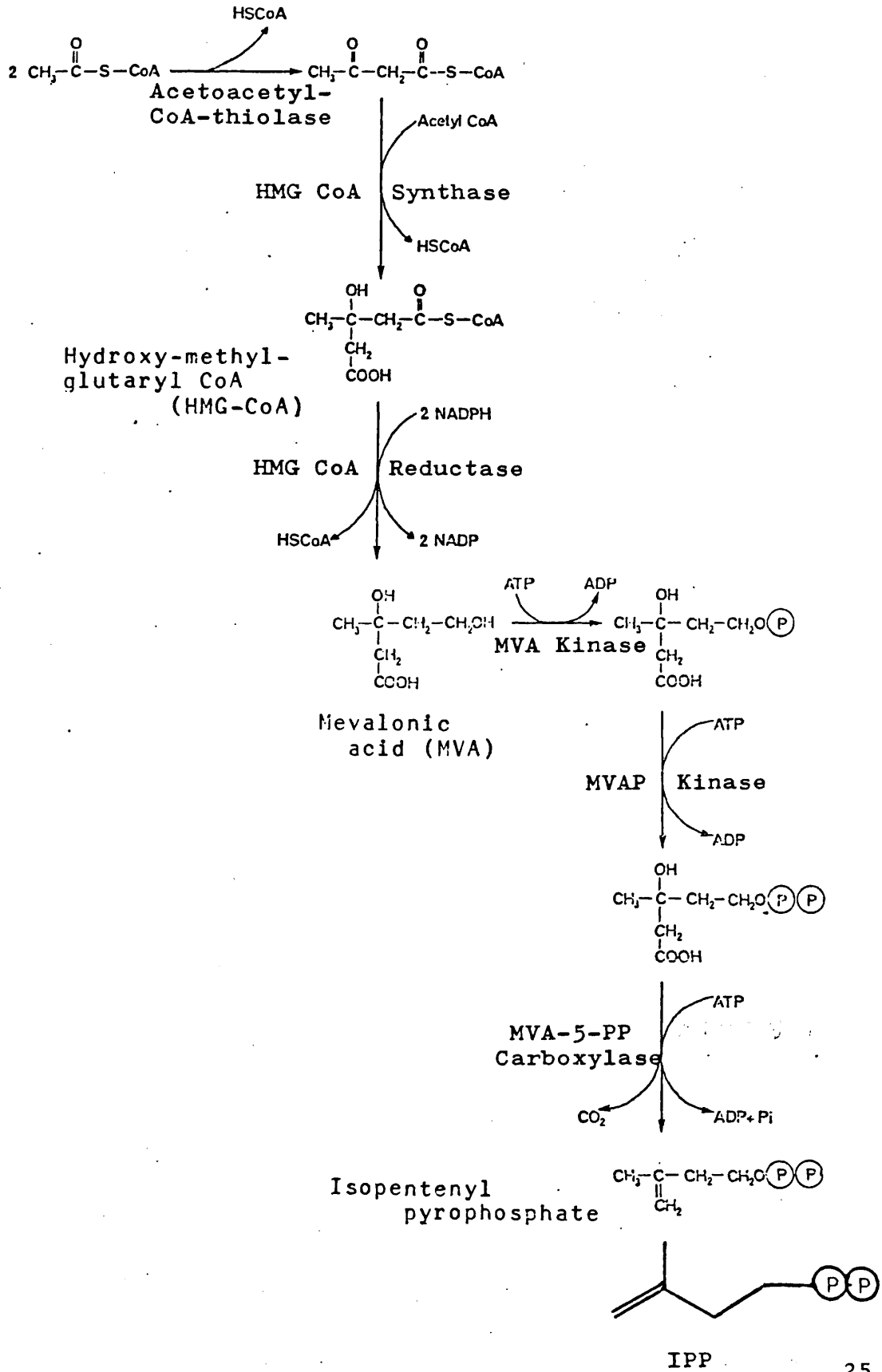
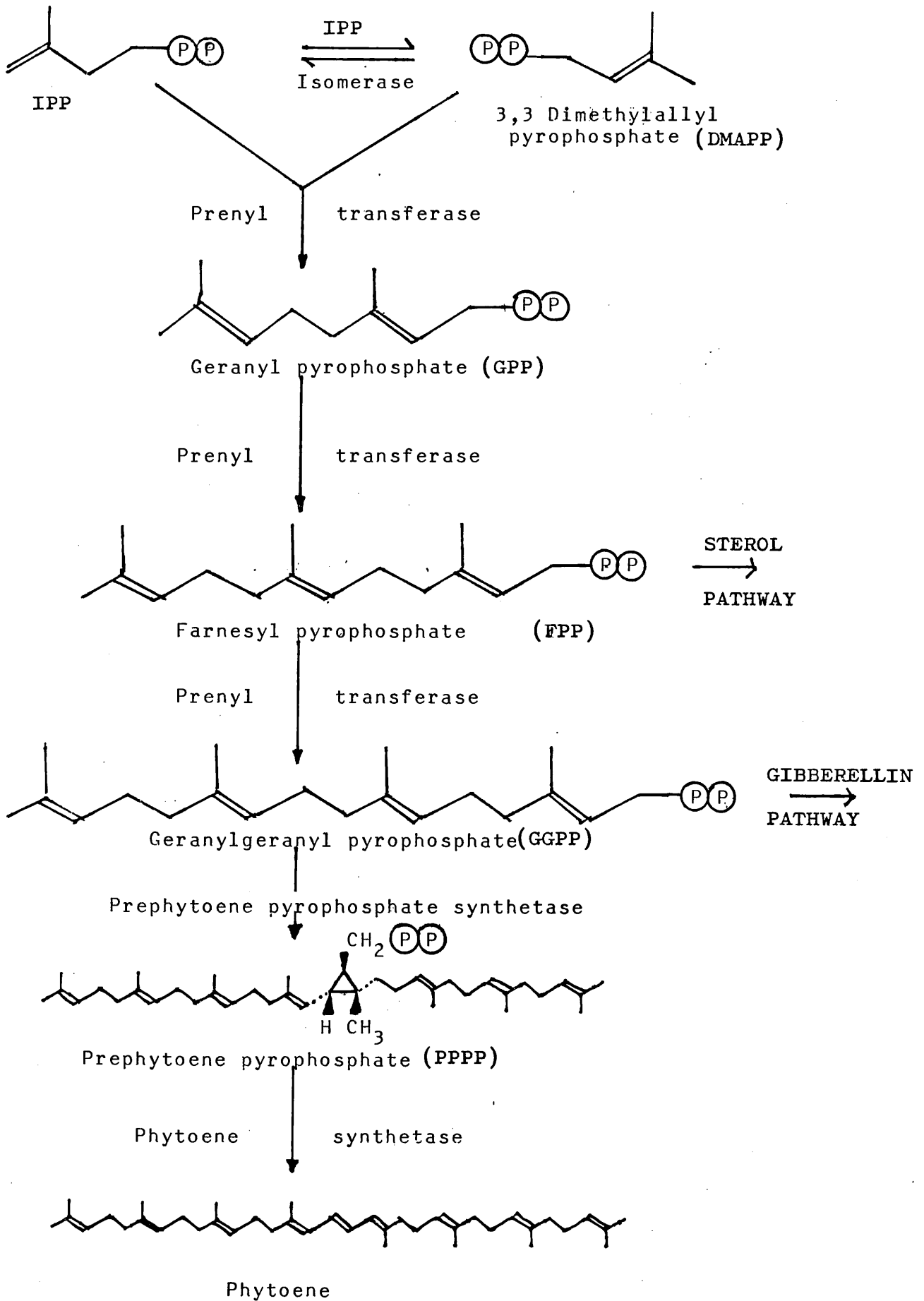
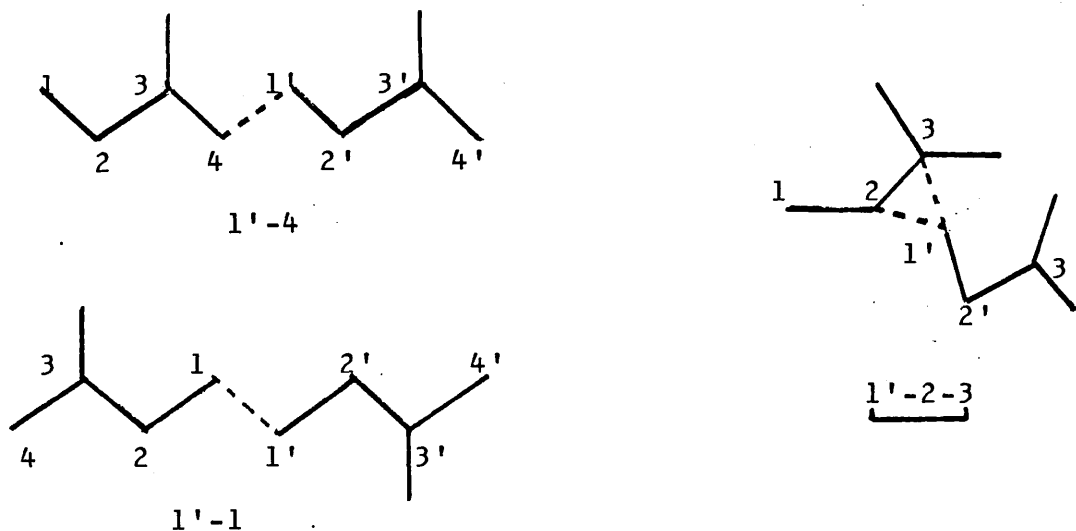


Fig 1.3 Conversion of IPP to Phytoene



A prenyl transfer reaction involves a condensation to connect 2-methylbutyl fragments to each other or other substrates, the regiochemistry of which is denoted by the numerical convention suggested by Poulter and coworkers (1977):



The premier prenyl transfer reaction is the 1'-4 condensation between IPP and an allylic pyrophosphate to generate the 5-carbon homologue of the allylic substrate; this is the basic chain elongation reaction for the biosynthesis of all the terpenoids.

The stereochemistry of the 1'-4 condensation was determined by Popjak and Cornforth (1966) studying FPP synthetase from rat liver and has been found to be a highly conserved property of the prenyl transferases (reviewed by Poulter and Rilling, 1983) having been isolated from yeast (Eberhardt and Rilling, 1975), Phycomyces blakesleeanus (Rilling, 1983) and avian liver (Reed and Rilling, 1975). These activities were all found to copurify with DMAPP transferase activity, suggesting both GPP and FPP are synthesized by the same enzyme.

However, Ogura and coworkers (1969) found the geranyl transferase activity of prenyl transferase from pumpkin

fruits was preferentially protected from heat denaturation in the presence of GPP, whilst DMAPP inhibited the use of *labelled* DMAPP but not of GPP. The same group later showed that an IPP analogue (4-methylpent-4-enyl pyrophosphate) was utilized as a co-substrate only with GPP (Ogura et al., 1974). Since it has been shown that the pumpkin FPP synthetase consists of 2 subunits (Reed and Rilling, 1975) it is possible that the two activities are located on different proteins in the same complex.

The enzymes that catalyze the 1'-4 condensation, farnesyl transferase reaction, forming GGPP have not been thoroughly investigated. The purified preparations of farnesyl synthetase on prolonged incubation could yield GGPP, but too slowly to be of any physiological significance and only with very high IPP and FPP concentrations (Eberhardt and Rilling, 1975). These experiments suggest other prenyl transferases may exist for GGPP formation.

GGPP Synthetase activity has been demonstrated in carrot roots (Nandi and Porter, 1964), the endosperm of immature Echinocystis macrocarpa, Greene seeds (Oster and West, 1968) and extracts of barley seed embryos (Davies et al., 1975). A number of properties have been determined for the partially purified enzyme from pumpkin seeds (Ogura et al., 1975) including the requirement for  $Mn^{2+}$  and the approximately equal ability to utilize DMAPP, GPP and FPP as the prenyl donor to IPP, suggesting a prenyl transferase of one activity and not different enzymes as the other experiments imply.

All farnesyl transferases isolated appear to synthesize the all-trans isomer of GGPP exclusively; as yet there is no evidence for formation of the cis isomer.



### 1.1.3 The Conversion of FPP to Squalene

Lynen and coworkers (1958) found yeast cell particulates converted FPP to squalene in the presence of NADPH. Squalene synthetase (EC 2.5.1.66) carries out this reaction and it is considered bound to the endoplasmic reticulum, unlike the enzymes that form FPP which are cytosolic (Gaylor, 1974).

Rilling (1966) isolated an intermediate between FPP and squalene from yeast cells which was established to be pre-squalene pyrophosphate (PSPP; Fig 1.4), (Epstein and Rilling, 1970). It was shown (Qureshi et al., 1973) that squalene synthetase from yeast exists in 2 forms, protomeric and polymeric, the former producing PSPP the latter converting it to squalene. It was also demonstrated that the two reactions can be regulated in different manners (Corey and Volante, 1976 and Agnew and Popjak, 1978), supporting the idea of individual catalytic sites.

Mechanistically there are 2 distinct reactions. The 1'-2-3 condensation, a prenyl transfer reaction in which FPP is both acceptor and prenylator i.e the alkylation of the C<sub>2</sub>-C<sub>3</sub> double bond of the acceptor to generate a protonated cyclopropane ring intermediate (Saunders et al., 1973) which yields PSPP by the loss of a proton from the C<sub>3</sub> cyclopropane carbon. This is followed by a 1'-2-3 to 1'-1 rearrangement. The mechanism of squalene synthetase (Fig 1.4) was determined by a number of groups (Edmond et al., 1971; Beytia et al., 1973 and McCorkindale, 1976).

### 1.1.4 The Conversion of GGPP to Phytoene

This step involves a head-to-head condensation of 2 GGPP molecules to form phytoene (Fig 1.5). Direct incorporation

Fig 1.4 Conversion of Farnesyl Pyrophosphate to Squalene

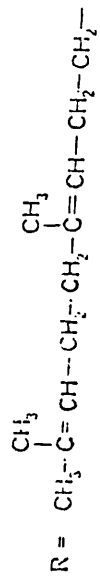
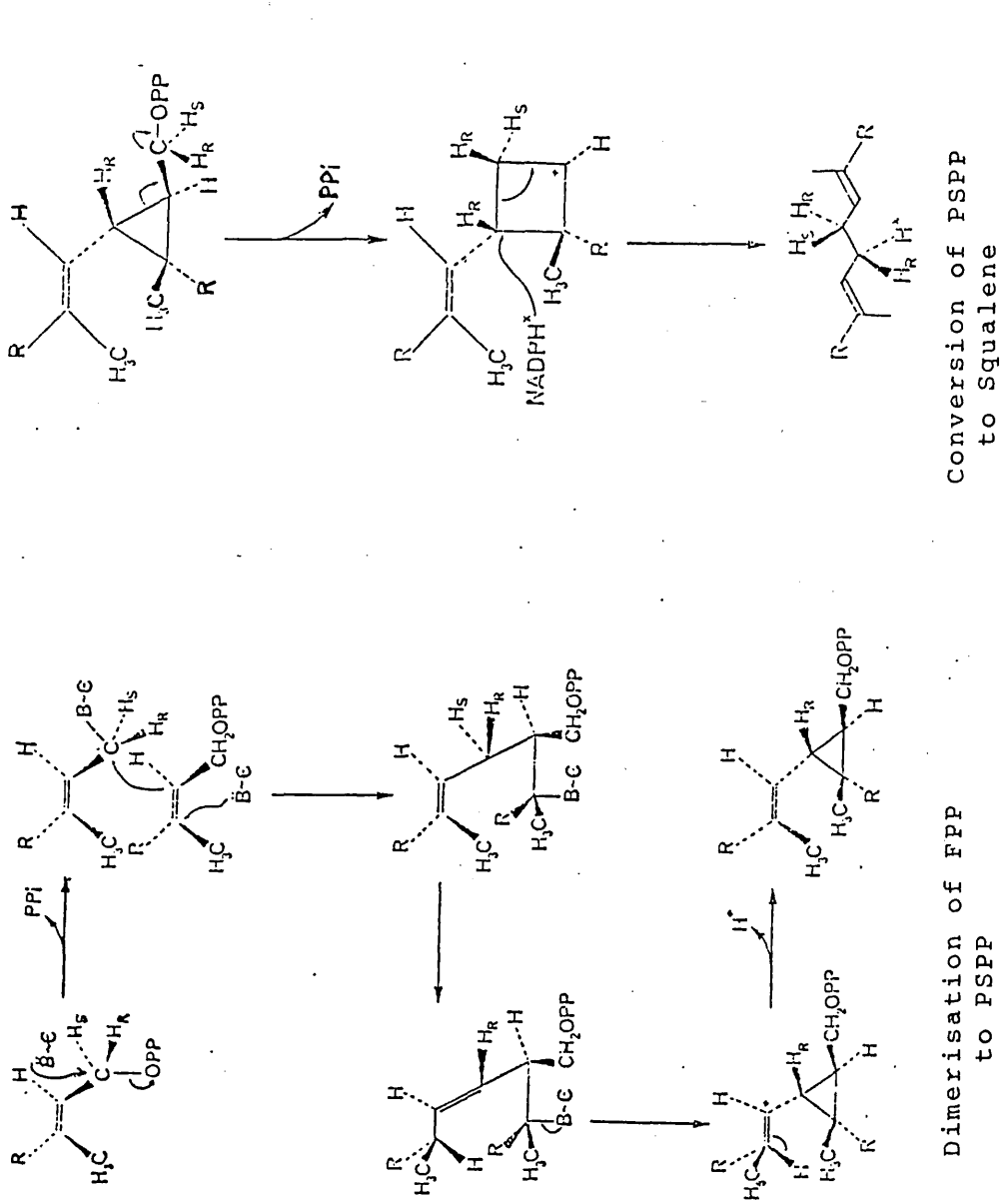
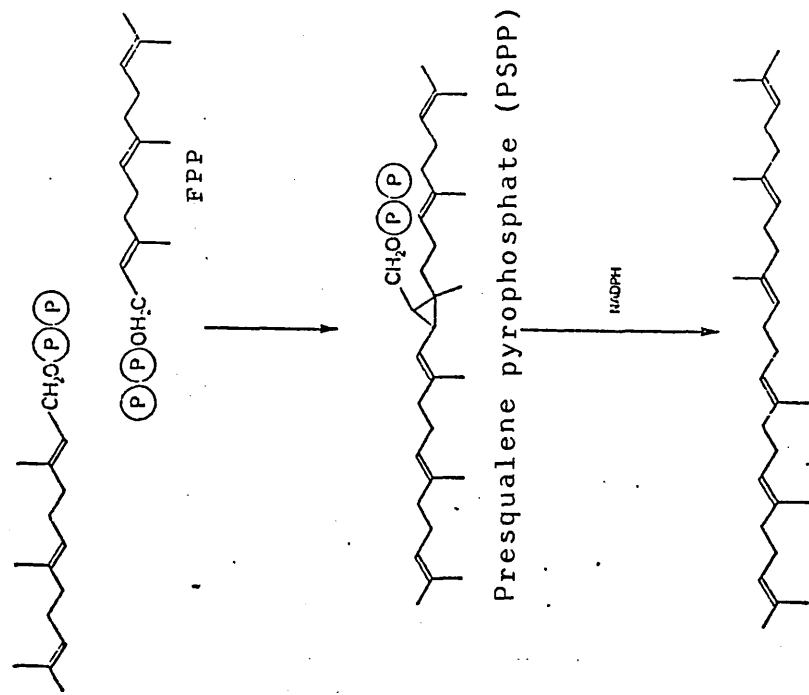
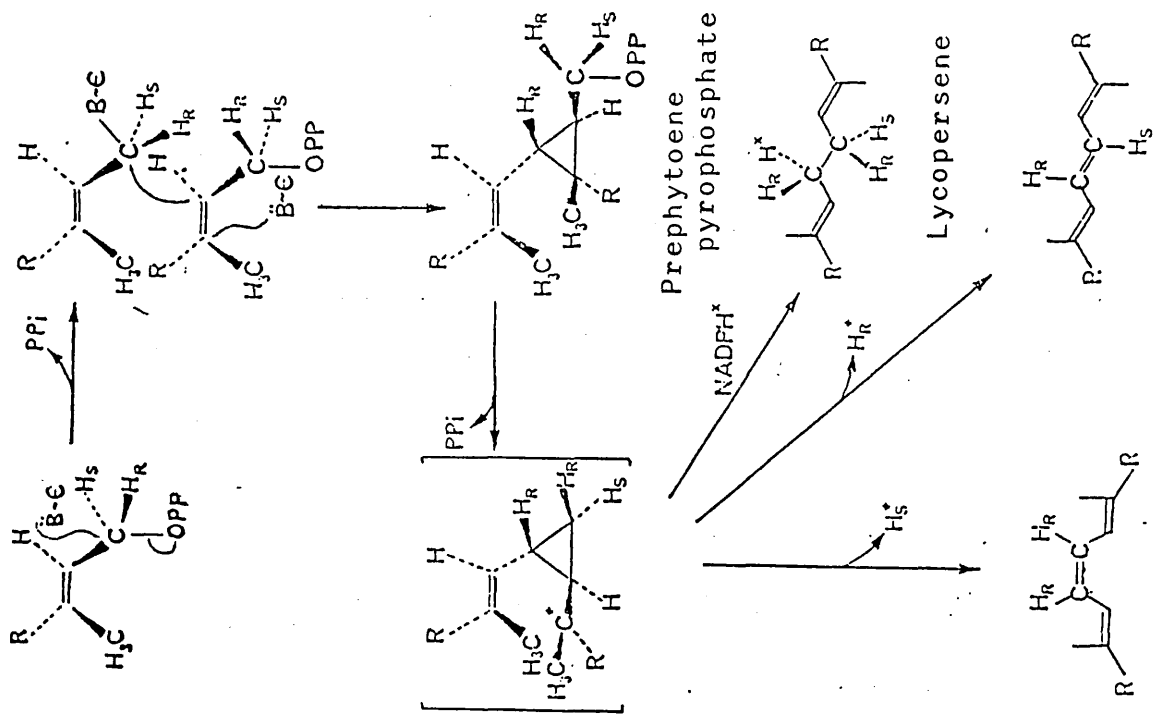
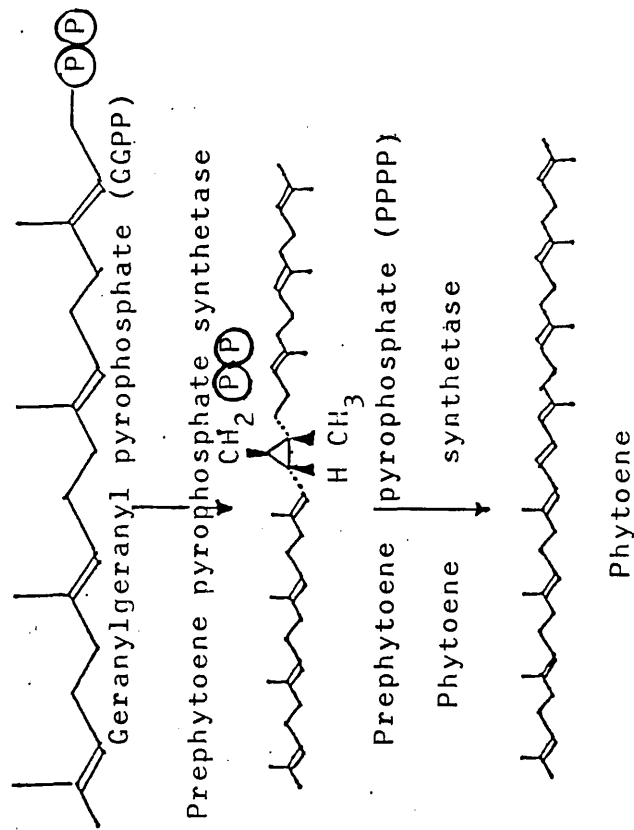


Fig 1.5 Conversion of Geranylgeranyl Pyrophosphate to Phytoene



of GGPP to phytoene has been demonstrated in a soluble tomato plastid enzyme system (Shah et al., 1965), cell free extracts of P.blakesleeanus (Lee et al., 1972) and a Mycobacterium sp. (Gregonis and Rilling, 1973) and in chloroplasts of Phaseolus vulgaris (Buggy et al., 1974); all require  $Mg^{2+}$  or  $Mn^{2+}$  as cofactors.

The condensation is analogous to the condensation of FPP to form squalene (1.1.3) via a cyclopropylcarbiny pyrophosphate intermediate prephytoene pyrophosphate (PPPP). This was first isolated as an intermediate in phytoene biosynthesis by Altman and coworkers (1972) from Mycobacterium sp.

A  $C_{40}$  intermediate between [ $^{14}C$ ]-GGPP and -phytoene from a tomato fruit plastid enzyme system was isolated (Qureshi et al., 1972) and called prelycopersene pyrophosphate (PLPP), since lycopersene formation was also seen. However, physical data showed it to be identical to the PPPP isolated by Altman.

If the condensation of GGPP molecules was completely analogous to that of FPP in squalene biosynthesis, then the first  $C_{40}$  hydrocarbon formed would be lycopersene. Whether this is an intermediate in phytoene biosynthesis has been a subject of considerable debate (reviewed by Spurgeon and Porter, 1983) but the bulk of evidence suggests it is not a normal intermediate and it is generally considered to be PPPP (Fig 1.5).

The condensation of GGPP molecules may lead to the formation of 15-cis phytoene or all-trans phytoene, depending on the stereochemistry of the hydrogen (H) removal (Fig 1.5), but in the majority of cases the isomer is predominantly the cis isomer (see Spurgeon and Porter, 1983). The mechanism, as for FPP condensation, is an initial 1'-2-3 condensation

followed by a 1'-1 rearrangement. The stereochemistry has been examined using MVA specifically labelled at C<sub>5</sub>. (3 RS, 5R)-[2-<sup>14</sup>C, 5-<sup>3</sup>H<sub>1</sub>]-MVA was incubated with tomato slices and the phytoene isolated showed a <sup>3</sup>H/<sup>14</sup>C atomic ratio of 8:8, indicating no loss of the 5R H (Williams et al., 1967). Using [2-<sup>14</sup>C, 5-<sup>3</sup>H<sub>2</sub>]-MVA the <sup>3</sup>H/<sup>14</sup>C ratio was approximately 14:8 in tomato slices (Williams et al., 1967) and the same in bean leaf chloroplasts (Buggy et al., 1969) suggesting the loss of 2 Hs, the pro-S Hs, leaving the pro-R Hs forming cis phytoene. In a Mycobacterium system where the all-trans isomer is predominant, it was found one pro-R and one pro-S H is retained (Gregonis and Rilling, 1974).

## 1.2 Carotenoids

The carotenoids are an important group of terpenoid compounds, which are generally composed of 8 isoprene units (C<sub>40</sub>). They are tremendously diverse in structure and well over 500 different carotenoids are known (see Davies, 1980).

They are mainly pigments with characteristic yellow, orange, red and purple colours and can be isolated from a wide range of organisms including animals, plants, fungi and algae.

### 1.2.1 Nomenclature

The rules for the nomenclature of carotenoids (Isler, 1971 and Commission on Biochemical Nomenclature, 1975) are designed to define precisely the structure of a given carotenoid. From these rules semi-systematic names are derived which assist communication and access to carotenoid literature. This is further aided by the use of trivial names which were generally assigned to a carotenoid when it was first

discovered and are of great value in natural product and biochemical work. The commonly used trivial names, semi-systematic names and structural formulae of all carotenoids encountered in this work are listed in Fig 1.6 (and Table 1.1).

### 1.2.2 Function

The functions of the carotenoids in photosynthetic tissue are well understood, acting as accessory or light-harvesting pigments and serving some photoprotective functions (Goodwin, 1980). In non-photosynthetic organisms such as the fungi however, their roles are less clear.

Photoprotection is a wide-ranging function attributed to the carotenoid pigments. First suggested by Griffiths (1955) and supported by work with a non-sulphur purple bacterium Rhodospseudomonas sphaeroides which is killed by light in the presence of  $O_2$  only in a carotenoid lacking mutant (Sistrom et al., 1956).

Goldstrohm and Lilly (1968) proposed the idea of carotenoids acting as filter pigments protecting a photosensitizer but most photosensitizers absorb light at different wavelengths from the carotenoids (Mathews, 1963 and Buchard et al., 1966) which precludes this possibility. It was proposed (Fujimori and Livingston, 1957) that carotenoids could interact with the triplet state of a sensitizer which being metastable can carry out abnormal photoreactions including photosensitized oxidations (Schneck, 1954). However it was shown (Foote and Deny, 1968) that the sensitizer must act directly with singlet  $O_2$  ( $^1O_2$ ) generated by;

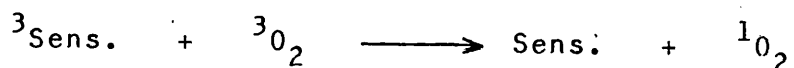
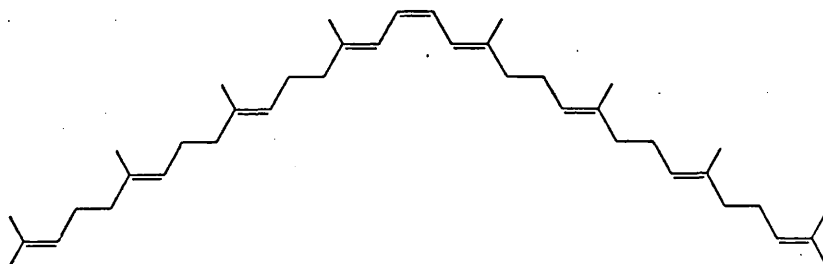


Fig 1.6 Commonly used Trivial Names, Semi-systematic Names and Structural Formulae of Carotenoids Encountered in this Work

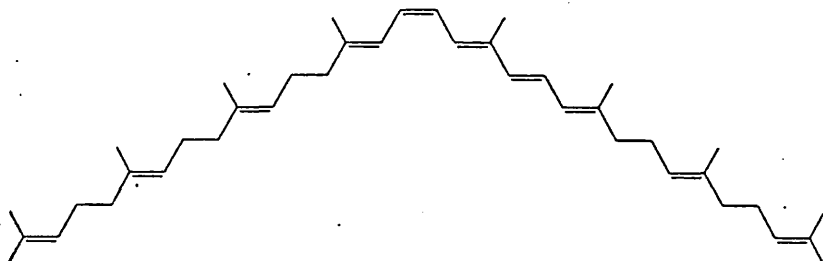
Phytoene, 7,8,11,12,7',8',11',12'-Octahydrolycopene, 15,15'-Dehydrolycopersene.  
 "Hexadecahydrolycopene" 15-cis-7,8,11,12,7',8',11',12'-Octahydro- $\psi,\psi$ -carotene

$C_{40}H_{64}$



Phytofluene, 7,8,11,12,7',8'-Hexahydrolycopene. "Dodecahydrolycopene"  
 15-cis-7,8,11,12,7',8'-Hexahydro- $\psi,\psi$ -carotene

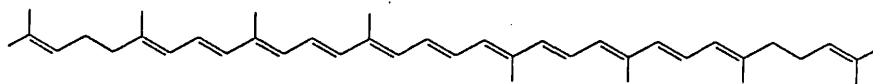
$C_{40}H_{62}$



Lycopene. Rhodopurpurin?

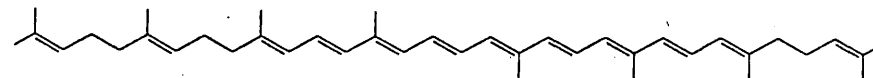
$\psi,\psi$ -Carotene

$C_{40}H_{56}$



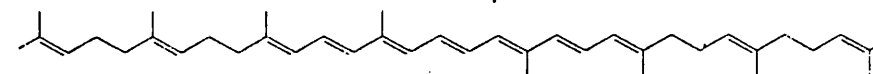
Neurosporene, 7,8-Dihydrolycopene, Flavorhodin? [513, 368], "Poly-cis- $\psi$ -carotene" [527, 605, 526].  
 "Tetrahydrolycopene" [719, 605, 604]; 7,8-Dihydro- $\psi,\psi$ -carotene

$C_{40}H_{58}$



$\zeta$ -Carotene, 7,8,7',8'-Tetrahydrolycopene. "5,6,7,8,5',6',7',8'-Octahydrolycopene"  
 "xi-Carotene"? 7,8,7',8'-Tetrahydro- $\psi,\psi$ -carotene

$C_{40}H_{60}$



3,4-Dehydrolycopene, Monodehydrolycopene; 3,4-Didehydro- $\psi,\psi$ -carotene

$C_{40}H_{54}$

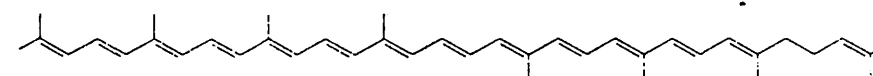
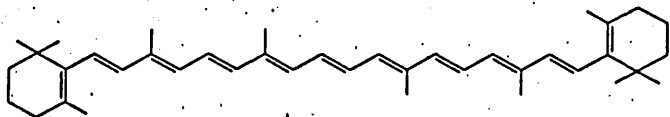


Fig 1.6 Contd.

$\beta$ -Carotene;  $\beta,\beta$ -Carotene

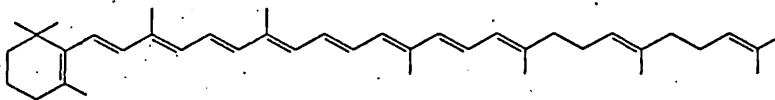


$C_{40}H_{56}$

$\beta$ -Zeaxanthin,  $\beta_1$ -Zeaxanthin  
"Pigment X"?

7,8'-Dihydro- $\gamma$ -carotene, "Carotene X"

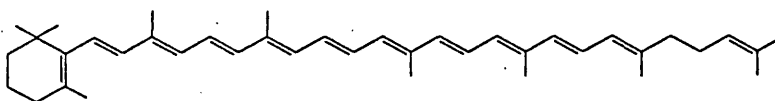
7,8'-Dihydro- $\beta,\psi$ -carotene



$C_{40}H_{58}$

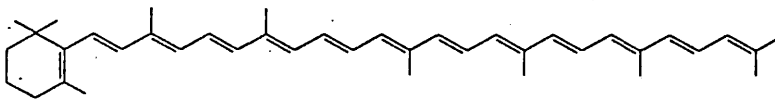
$\gamma$ -Carotene, "Sphaerobolin"

$\beta,\psi$ -Carotene



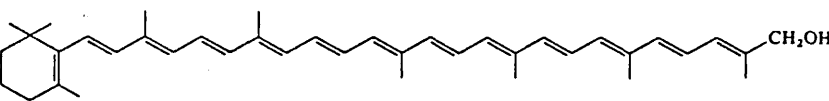
$C_{40}H_{56}$

Torulene. 3',4'-Dehydro- $\gamma$ -carotene; 3',4'-Didehydro- $\beta,\psi$ -carotene



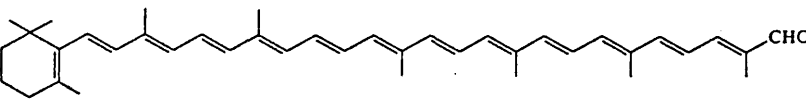
$C_{40}H_{54}$

"3',4'-Didehydro-18'-hydroxy- $\gamma$ -carotene"; 3',4'-Didehydro- $\beta,\psi$ -caroten-16'-ol



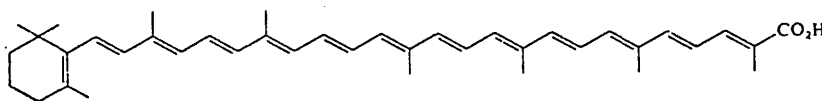
$C_{40}H_{54}O$

Torularhodinaldehyde, "3',4'-Dehydro-17'-oxo- $\gamma$ -carotene"; 3',4'-Didehydro- $\beta,\psi$ -caroten-16'-al



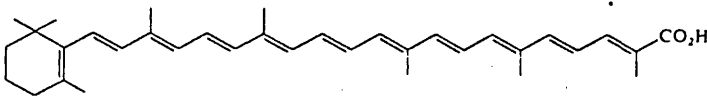
$C_{40}H_{54}O$

Torularhodin. "Torulencarboxylic(16') acid", "16'-Carboxyl-3',4'-dehydro- $\gamma$ -carotene",  
"Lusomycin"? 3',4'-Didehydro- $\beta,\psi$ -caroten-16'-oic acid



$C_{40}H_{52}O_2$

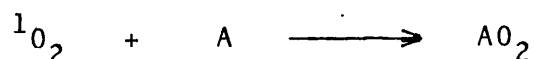
Neurosporaxanthin; 4'-Apo- $\beta$ -caroten-4'-oic acid



$C_{35}H_{46}O_2$

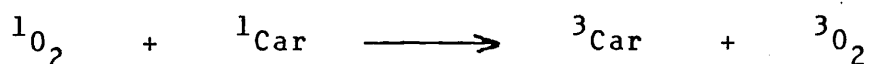


Consequently there may be lethal reaction:



Sens. = sensitizer;  ${}^1, {}^3$  denote singlet and triplet excited state;  
A = acceptor;  $\text{AO}_2$  = peroxide of A (lethal).

So it is probable that carotenoids photoprotect by quenching the singlet excited state of  $\text{O}_2$ :



This is further supported by the observation (Foote et al., 1970) of a cis to trans isomerization of carotenoids by  ${}^1\text{O}_2$  probably via  ${}^3\text{Car}$  (Fonken, 1967) which accompanies light absorption by chlorophyll pigments (Claes and Nakayama, 1959). So it is proposed the energy is dissipated from  ${}^3\text{Car}$  by collapse to all-trans  $\beta$ -carotene, for example.

For such reactions to occur the triplet energy level of the carotenoid must be below that of  ${}^1\text{O}_2$ . This is found to be the case for all carotenes with 9 or more conjugated bonds which is indeed found to be the minimum number required for maximum quenching efficiencies (Land, 1975).

Some reports suggest carotenoids may also protect against UV light damage, but detailed investigations may still be required. Coloured conidia from wild type Neurospora crassa are less sensitive to "black light" (300-425nm) in the absence of an exogenous photosensitizer than are conidia from albino strains; this is also observed at short wavelength UV radiation (Blanc et al., 1976).

Many biological phenomena described within plants and fungi are initiated or dependent upon illumination with "blue-

light" (reviewed by Senger, 1984). In fungi photoinduction of carotenogenesis and phototropism are examples of blue-light effects (Bergman et al., 1969). Action spectra of these responses were recorded by a number of workers and the carotenoids themselves were proposed to be involved (Curry and Green, 1959) though this was disputed (Delbruck and Shropshire, 1960) in favour of a flavin as the photoreceptor. Flavoproteins with action spectra analogous to the photo-response have been isolated from P.blakesleeanus (Bergman et al., 1969 and Ootaki and Wolken, 1973). Although the controversy between favins/flavoproteins and carotenoids/carotenoproteins is still pending, despite continuing research in the area, evidence in favour of the former is predominant (Presti and Delbruck, 1978). Furthermore, recently action spectra have been redetermined using more sophisticated techniques and there are suggestions that photoreception involves a number, possibly 2, of flavoproteins (Galland and Lipson, 1986).

Nevertheless, the involvement of carotenoids in photoresponses cannot be ruled out. However, the levels of carotenoids necessary for photoreception are far less than normally found in fungi say (Bergman et al., 1969) and so other functions for the carotenoids must be sought.

Formation of sporangiophores in P.blakesleeanus is inhibited under illuminated conditions in which  $O_2$  is limiting (Russo, 1977). Blue light reverses the inhibition but in carotene deficient mutants the threshold for the blue-light effect is raised 100 to 2000 times depending on the amount of  $\beta$ -carotene in the mycelium (Galland and Russo, 1979a). The raised threshold is overcome by the addition of retinol to the medium and the role of  $\beta$ -carotene in the system may

be to provide retinol as it does in animals (Galland and Russo, 1979b and 1.3.3).

Carotenoids may also be converted to sporopollenin and trisporic acids (1.3.3). Since carotenoid levels are found in both mycelia and sporangiophores (compared to the metabolites which are common to the latter) and in some fungi are produced in very large quantities, even in the absence of hyphae of opposite mating type, the production of these sex-metabolites is only a small part of carotenoid function in fungi.

### 1.2.3 Factors Controlling Carotenoid Biosynthesis

There are 3 main phases of carotenoid biosynthesis:

- i) an initial period of active synthesis leading to a maximum concentration.
- ii) an intermediate period with no qualitative or quantitative changes.
- iii) a final period during which the pigments disappear.

This, or a very similar pattern, is observed in a number of different strains (Champeau and Luteraan, 1946; Goodwin and Willmer, 1952; Vecher and Kulikova, 1968 and Hsu et al., 1974).

It is found in wild type P.blakesleeanus that the rate of carotenoid synthesis in the early phase of growth is slow with major synthesis coming when growth is complete (Goodwin and Willmer, 1952). In this case only sufficient carbon (C) is available for maximal growth and only excess C will stimulate carotenogenesis; generally a high carbon to nitrogen (C:N) ratio will be more effective in stimulating carotenogenesis.

In some cases there are marked qualitative changes during carotenogenesis, for example young red cultures of Rhizophlyctis rosea contain mainly lycopene while older cultures turn yellow due to a massive accumulation of  $\beta$ -carotene which masks lycopene (Davies, 1961). Similar changes are seen in other strains (Gribanovski-Sassu and Fopen, 1968 and Cerda-Olmeda and Torres-Matrinez, 1979).

#### 1.2.3.i Culture Conditions

Variations in the carbohydrate and nitrogen sources of basic culture media affect carotenogenesis in different fungal strains (reviewed by Goodwin, 1980). Rhodotorula sannei, for example, will not produce carotenoids when grown on glucose whereas glycerol is a most effective C source (Fromageot and Tschang, 1938). In P.blakesleeanus maltose and glucose are equally capable of supporting carotenogenesis whilst xylose and fructose, which can maintain growth, are much less capable (Garton et al., 1951). Valine or leucine as N sources greatly stimulate carotenogenesis in P.blakesleeanus (Goodwin and Lijinsky, 1952) and Rhodotorula gracilis (Vecher et al., 1967). This is found to be due to the formation of HMG-CoA via their enzymic conversion involving transamination.

Generally though, C and N sources best suited for carotenogenesis can only be determined empirically for specific strains and generalizations are not possible.

Different temperatures also affect carotenoid levels; in the main, high temperatures result in less pigment production.

Trace elements are found to have effects e.g N.sitophila, Co, Cu, Mn or Zn increased  $\beta$ -carotene production but only

Mn increased lycopene, neurosporene,  $\beta$ -carotene and phytofluene (Ishii, 1954). Also fluoride (F) added to growth medium increased carotenogenesis in P.blakesleeanus, possibly since it inhibits phosphatases which can dephosphorylate key intermediates in carotenoid biosynthesis (Desai et al., 1973).

On the whole though, culture conditions best suited for carotenoid production are dependent on the particular strain and inherent regulatory mechanisms.

#### 1.2.3.ii Non-Nutrient Chemicals

Sex-stimulated carotenogenesis was first observed in the heterothallic fungus Choanephora cucurbitarium (Barnett et al., 1956) and has since been observed in a number of organisms e.g P.blakesleeanus and Blakeslea trispora (reviewed by Goodwin, 1980). The biostimulator involved in this consists of carboxylic acids of which trisporic acid C is the major component derived from  $\beta$ -carotene (Caglioti et al., 1966; Bu'Lock et al., 1974 and Cerda-Olmeda and Torres-Martinez, 1974).

The primary function of trisporic acid, or a close metabolite thereof, is that of a sex hormone controlling zygothecium formation in Mucorales (Sutter, 1976) and in gameteogenesis in P.blakesleeanus (Barksdale, 1969). The effect is inhibited by cycloheximide (Thomas et al., 1967) and in the presence of diphenylamine (DPA) trisporic acid stimulates phytoene synthesis (Thomas and Goodwin, 1967) indicating that the metabolite probably functions by activating synthesis of an enzyme concerned with an enzymic step prior to phytoene synthesis. There is some evidence that trisporic acid is

formed from  $\beta$ -carotene, via retinol and retinal, so the system represents a positive feed back control (Bu'Lock et al., 1974).

Retinol and  $\beta$ -ionone were also found to stimulate carotenogenesis in P.blakesleeanus (Eslava et al., 1974) and B.trispora (Feofila and Bekhtereva, 1976) both effects were found to be inhibited by cycloheximide (Engel et al., 1953). Retinol stimulation was shown to be independent of sexual stimulation (Murillo and Cedra-Olmeda, 1976) whilst  $\beta$ -ionine and trisporic acid are competitive (Rao and Modi, 1977) suggesting that they have different points of action both stimulate de novo protein synthesis at a translational level (Feofila and Ar'Buzov, 1975).

There is also evidence for an element of negative feedback control since when  $\beta$ -carotene synthesis is hindered (either by mutation or chemical inhibition) formation of pre- $\beta$ -carotene carotenoids is stimulated (Murillo and Cerda-Olmeda, 1976). It was observed, in vitro, that  $\beta$ -carotene specifically inhibits cyclase activity using radioactivity from  $[2-^{14}C]$ -MVA to accumulate in lycopene and  $\gamma$ -carotene whilst  $\zeta$ -carotene, lycopene,  $\beta$ -zeacarotene and neurosporene (though not  $\beta$ -carotene) all inhibit phytoene desaturation (Bramley and Davies, 1976). The addition of phytoene or squalene to these systems further reduces terpenoid formation in general, perhaps acting between MVA and FPP (Bramley and Davies, 1976).

Citral inhibits carotene synthesis, but activates phytoene synthesis in Rhodotorula gracilis (Vecher and Kulikova, 1970) an effect akin to DPA action. In the same strain ammonium perchlorate is said to stimulate  $\beta$ -carotene synthesis over

that of torularhodin (Atamanyuk and Razumorskii, 1974).

High extracellular concentrations of cyclic adenosine 3',5'-monophosphate (cAMP;  $10^{-3}$  M or higher) inhibit carotenoid accumulation in N.crassa (Harding, 1973 and Turian and Khandjian, 1973) and since cAMP is known to play an important role in the regulation of genome expression it is suggested (Kritsky et al., 1982) to be involved in genetic regulation of N.crassa carotenogenesis.

### 1.2.3.iii Light

A number of studies indicate that photoinduction of carotenogenesis consists of a temperature independent photochemical reaction and a temperature dependent biochemical "dark" reaction (reviewed by Rau, 1980b and Harding and Shropshire, 1980). These phenomena found in a number of micro-organisms (reviewed by Rau, 1980a) including, Mycobacterium sp. (Rilling, 1964), N.crassa (Rau et al., 1967) Fusarium aquaeductuum (Rau, 1967a and b) and Rhodotorula minuta (Tada and Shiroishi, 1982). The only other fungi known to exhibit strict carotenoid photoregulation are listed below:

Aspergillus giganteus mut. alba

Cephalosporium diospyri

Dacryopinax spathularia

Fusarium aquaeductuum

Fusarium coeruleum

Fusarium oxysporum

Neurospora crassa

Neurospora sitophila

Pyronema confluens

fungi species in which carotenogenesis is reported to be strictly photoregulated continued;

Sphaerobolus stellatus

Syzygites megalocarpus

Verticillium agaricinum

(cited by Rau, 1980a)

Light frequently stimulates carotenogenesis in fungi which normally form reasonable amounts in the dark, e.g. P.blakesleeanus (Garton et al., 1951), N.crassa (Rau et al., 1967), R.rubra (Nakayama et al., 1954) and Penicillium oxysporum (Mase et al., 1957). In some strains qualitative carotenoid changes are induced by light in addition to quantitative changes.

The photoreceptor involved in photoinduction (1.2.2) has long been debated as to whether it is a carotenoid (Defabo et al., 1976) a flavin (Rau, 1967b) or even a porphyrin (Song et al., 1972) though current views (reviewed by Schrott, 1984) tend to favour the involvement of the flavin.

It is generally blue-light that photoactivates carotenogenesis and there are an increasing number of blue-light induced phenomena in plants and fungi such as phototropisms, photomorphogenesis and enzyme photoregulation such as the photoinhibition of alcohol dehydrogenase in P.blakesleeanus (reviewed by Senger, 1984). However, it has also been shown that red-light can induce carotenogenesis in the presence of redox-dyes such as methylene blue and toluidine blue, which presumably act as artificial photoreceptors (Harding, 1974).



After photoinduction there is a characteristic lag period of, for example, 4h in F.aquaeductuum (Rau, 1967a) and 40 to 90min in N.crassa (Rau et al., 1968). This period increased at lower temperatures in both cases. The occurrence of a lag phase suggests that synthesis of a new protein is associated with photoinduction and to support this, photostimulated protein synthesis has been demonstrated in V.agaricinum (Valadon et al., 1975), N.crassa (Subden and Turian, 1970) and in N.crassa and F.aquaeductuum (Mitzka-Schnabel et al., 1984).

This leads to the question as to whether the synthesis control is at a translational or transcriptional level. Actinomycin D (an inhibitor of transcription) partly inhibits photoinduced carotenogenesis in V.agaricinum (Valadon and Mummery, 1973 and Murillo, 1980) and distamycin A (a similar inhibitor of transcription) blocks photoinduction completely in F.aquaeductuum (Felbermier and Rau, 1976). These results suggest photoregulation is at the transcriptional level, which in turn implies a de novo synthesis of specific mRNAs on photoinduction. This has been demonstrated for N.crassa (Schrott and Rau, 1975), F.aquaeductuum (Felbermier and Rau, 1976) and in both strains (Schrott et al., 1975; reviewed by Rau, 1985).

Precisely which enzymes are photoinduced has long been an area of study and is complicated by the need for crude or purified enzyme preparations and functional in vitro assays. To date, very few carotenogenic cell free systems have been developed (reviewed by Bramley, 1985) including, from the fungi P.blakesleeanus (Yokoyama et al., 1962; Bramley and Davies, 1975 and de la Concha et al., 1983), N.crassa (Mitzka-Schnabel and Rau, 1981), B.trispora (Neujahr and

Bjork, 1970) and A.giganteus (El-Jack personal communication); in the higher plants Capsicum annum fruit (Camara et al., 1982), spinach (Lutke-Brinkhaus et al., 1983) and tomato fruit (Jungalwala and Porter, 1967); the prokaryotes Corynebacterium poinsettiae (Swift and Milborrow, 1981), Aphanocapsa ATCC6741 (Clarke et al., 1982), Flavobacterium dehydrogenans (Fahey and Milborrow, 1978) and Halobacterium cutirubrum (Kushwaha et al., 1976) and the only alga for which a cell free system has been developed capable of incorporating radioactivity from zeaxanthin to neoxanthin, peridinin and diadinoxanthin, Amphidinium carterae (Swift et al., 1982).

Using these in vitro systems a few enzymes have been determined conclusively to be photoinduced, or activity levels increased in light grown conditions (Table 1.2)

Table 1.2 Enzymes Involved in Terpenoid Biosynthesis Which have been Demonstrated to be Photo-induced or have Light-Activated Levels.

Enzyme	Light Effect	Organism	Reference
PPPP Synthetase	Total Induction	<u>Mycobacterium sp.</u>	Johnson et al., 1974
GGPP Synthetase	Increased Levels	"	"
Phytoene Synthetase	"	<u>F.aquaeductuum</u>	Rau, 1976
"	"	<u>N.crassa</u>	Spurgeon et al., 1979
Prenyl Transferase	"	"	"
HMG-CoA Reductase	"	<u>Rhodotorula minuta</u>	Tada and Shiroishi, 1982
Phytoene Synthetase	Total Induction	<u>A.giganteus mut. alba</u>	El-Jack, personal comm.

Table 1.2 Contd.

Enzyme	Light Effect	Organism	Reference
Desaturase	Total Induction	<u>A.giganteus</u> <u>mut. alba</u>	El-Jack, personal comm.
Cyclase	"	"	"

Addition of cycloheximide (a protein synthesis inhibitor) to F.aquaeductuum at various times after illumination, results in a differential inhibition of the synthesis of various carotenoids (Bindl et al., 1970) such that after photoinduction carotenogenic enzymes are synthesized sequentially; compared to the possibility that only the first enzyme of the pathway is photoinduced by their substrates. Further work with F.aquaeductuum (Lang and Rau, 1972) in which illuminated mycelia is kept anaerobically (thus inhibiting carotenogenesis but not protein synthesis) when subsequently exposed to O<sub>2</sub>, in the presence of cycloheximide, there is strict, concurrent formation of carotenoids without any lag period. This suggests that the enzymes are induced as a coupled group, though whether the genetic information is therefore expressed en bloc by means of a "carotenogenic operon" remains to be seen.

#### 1.2.3.iv Genetic Regulation

The ability of an organism to synthesize various carotenoids is dependent on specific genes. The pigment composition may be altered by differential expression of genes with light and certain other possible gene regulators (1.2.3.ii). These regulatory mechanisms may also be under genetic regulation. Mutants defective in carotenogenic genes allow elucidation

of the biosynthetic pathway and the few regulatory mutants in particular are extremely important in their potential value towards an understanding of control mechanisms. However, such mutations are relatively few and extensive studies are limited to certain organisms e.g N.crassa and P.blakesleeanus.

Mutations at 3 distinct genetic loci produce a number of albino phenotypes in N.crassa. al-1 mutants synthesize only phytoene indicating that the gene codes for phytoene desaturase (Goldie and Subden, 1973). al-3 mutation is defective in GGPP formation suggesting an inactive prenyl transferase, GGPP synthetase while al-2 mutants are defective in a particulate fraction involved in the GGPP conversion to phytoene i.e phytoene synthetase (Harding and Turner, 1981).

Coloured mutants of N.crassa have also been isolated but the extent of their blockage site has not been elucidated (Goldie and Subden, 1973). The white collar mutant of N.crassa lacks carotenoid biosynthesis in the mycelia but it is still present in the conidia suggesting that the genes for carotenogenesis are normal and the mutation is in the regulation of carotenoid biosynthesis (Harding and Turner, 1981). Since in the wild type mycelial carotenogenesis is subject to photocontrol (1.2.3.iii), further investigations of the white collar mutant may prove valuable in photoinduction studies.

It is with P.blakesleeanus that the most detailed carotenoid gene studies have been carried out. This work relies heavily on the use of mutants, for which a large number have been isolated and characterized, particularly in caroteno-

genesis (reviewed by Cerda-Olmeda, 1985; Table 1.3)

Table 1.3 Carotenogenic Mutants from P.blakesleeanus

Mutant Designation	Phenotype/Genotype
<u>carB</u>	Phytoene dehydrogenase deficient
<u>carR</u>	Cyclase deficient
<u>carA</u>	Regulatory, low carotenoid levels
<u>carS</u>	" superproducer of carotenoids
<u>carC</u>	Unknown, affects regulatory interplay between <u>carA</u> and <u>carS</u>
<u>carE</u>	Extranuclear, produces dark-grown protein- $\beta$ -carotene complex
<u>carD</u>	Carotenoid superproducer, further stimulation by chemicals
<u>carI</u>	No carotenoids under any growth conditions
<u>picA</u>	Partially defective photoinduction
<u>picB</u>	"
<u>madA,B,C,D,E and G</u>	Affected in phototropism, <u>madA,B</u> and <u>D</u> may also be in photocarotenogenesis

As in N.crassa, no mutants have been isolated which accumulate intermediates between phytoene and lycopene, suggesting that these steps are controlled by a single gene; heterokaryon studies support this concept (Aragon et al., 1976).

carB accumulates phytoene (Meissner and Delbruck, 1968) and carR mutants accumulate lycopene (Ootaki et al., 1973) so they are both structural mutants.

Regulation of carotene biosynthesis in P.blakesleeanus appears to involve feedback repression by  $\beta$ -carotene since reduction of the levels in the latter stimulates

carotenogenesis, which is seen in carR and carB mutants (Murillo and Cerda-Olmeda, 1976): this mimics the effects of  $\beta$ -carotene inhibitors (1.2.3.ii and .v). carS mutants accumulate excess  $\beta$ -carotene suggesting a loss of this repression (Murillo et al., 1978) while carA mutants synthesize very low levels of  $\beta$ -carotene (Ootaki, 1973) and so may be involved in a similar regulatory function in carotenoid biosynthesis as carS.

carC is defined as being whitish, accumulating low levels of  $\beta$ -carotene in the dark (Revuelata and Eslava, 1983), its precise function is unknown but it is suggested to influence regulatory interplay between carA and carS genes.

carE (da la Concha and Murillo, 1984) is unusual appearing to be extranuclear and involving a complex with  $\beta$ -carotene, but only in the dark.

Two recently isolated mutants suggest 2 other regulatory genes in carotenogenesis of P.blakesleeanus. carD (Bejerano et al., 1986) over-produces  $\beta$ -carotene (like carS) but is further stimulated by known activators such as retinol and dimethyl phthalate whereas in carI (Roncero and Cerda-Olmeda, 1982) there is no  $\beta$ -carotene production under any conditions.

picA and picB (Lopez-Diaz and Cerda-Olmeda, 1980 and Jayaram et al., 1979) are partially defective for photo-induced carotenogenesis, but otherwise normal while 7 independent genes have been affected in phototropism madA,B,c,d,e and G (Eslava et al., 1976) of which genes madA,B and D may also be involved in photoinduction (Jayaram et al., 1980).

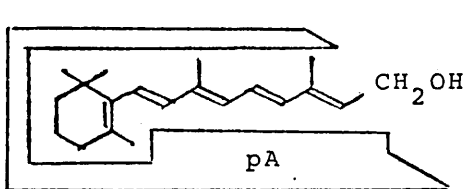
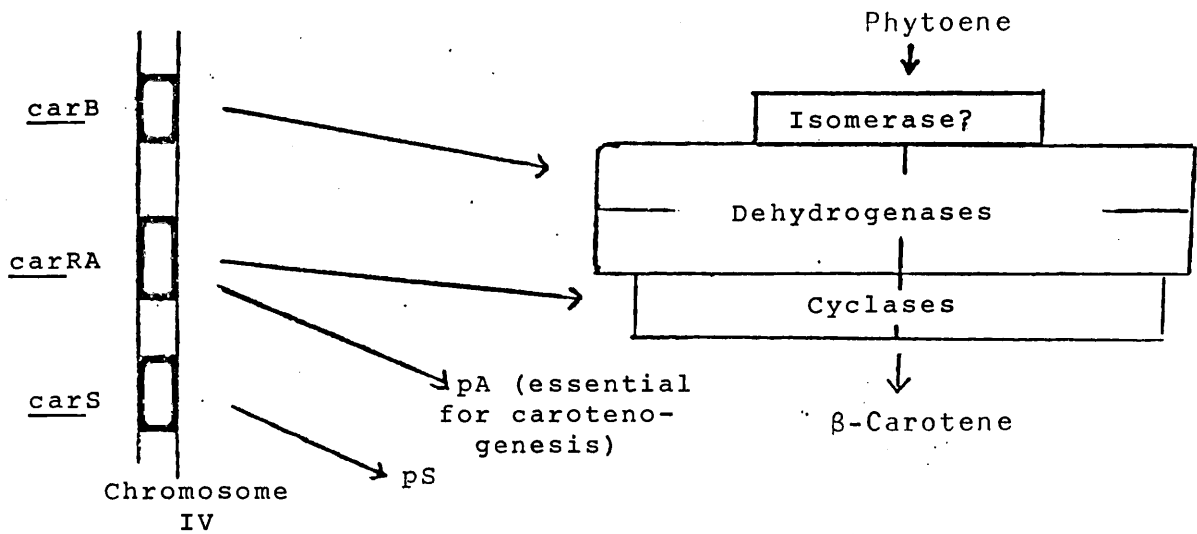
A scheme for the regulation of carotenogenesis in P.blakesleeanus was recently presented (Cerda-Olmeda, 1986) which

incorporates all the genes so far identified and the regulatory phenomena observed (Fig 1.7). It suggests carotenogenesis is blocked by a complex of  $\beta$ -carotene and 2 gene products from carS (pS) and the bifunctional gene carRA (pA) which is destroyed by light. The hypothesis encompasses most of the current observations in P.blakesleeanus and may be the first complete model for regulation of carotenogenesis. However, this is the theory for P.blakesleeanus and can only be presented due to the large amount of genetics that have been established for this strain. Obviously different fungal species may have different regulatory mechanisms and whether this model (if correct) is representative remains to be seen.

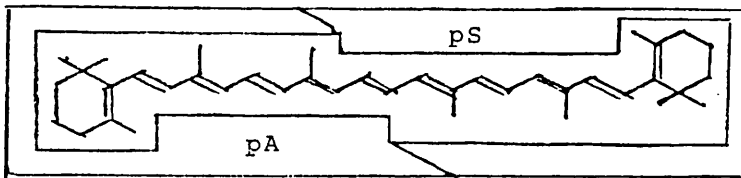
The anther smut Ustilago violacea accumulates lycopene and mutants have been isolated (Garber et al., 1975) accumulating  $\beta$ -carotene,  $\gamma$ -carotene and phytoene and other carotenoidless mutants. Three genes were postulated, w-gene for desaturation, o-gene for cyclization forming  $\gamma$ -carotene and y-gene for cyclization forming  $\beta$ -carotene: o- and y-gene products being inactive in the wild type.

Seven genes affecting carotenogenesis have been identified in photosynthetic bacteria Rhodospseudomonas capsulata (Marrs, 1982). Defects in crtB or crtE are carotenoidless, in mutants crtH and crtC phytoene and neurosporene accumulate respectively. The genes crtC, crtD, crtF and crtA are considered to code for the hydratase, 3,4-dehydrogenase, O-methylase and oxygenase respectively. All these genes, except crtH were mapped closely onto a 40 megadalton chromosomal segment involved with genes specific for photosynthetic membrane and photopigment synthesis.

Fig 1.7 Proposed Mechanism for Photoregulation of Carotenogenesis in *P.blakesleeanus* (Cerda-Olmeda 1986)



Pathway is Unblockable

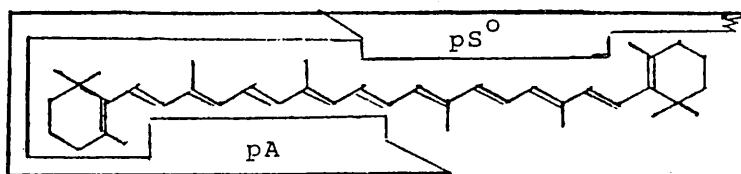
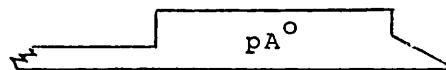


pA:  $\beta$ -Carotene:pS Complex  
Blocks Carotenogenesis and is Destroyed by Light

Mutants

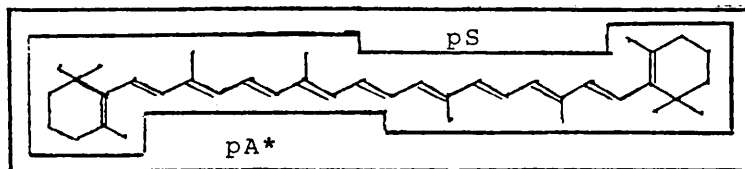
CarB or carR mutants do not produce  $\beta$ -carotene so pathway is unblockable

CarRA mutants pathway inoperative



CarS mutants pathway is operative and unblockable

CarA mutants pathway is overblockable





### 1.2.3.v Inhibitors of Carotenogenesis

Several chemical compounds inhibit carotenogenesis in various organisms and are used extensively in pathway elucidation and as herbicides (Yokoyama et al., 1981; Spurgeon and Porter, 1983; Clarke, 1984; Sandmann et al., 1984a; Bramley et al., 1984 and Sandmann and Boger, 1986a).

There are a number of different sites where inhibitors are found to act.

The bleaching herbicide dimethazone inhibits terpenoid accumulation and is considered to act within the prenyl pyrophosphate formation steps (Sandmann and Boger, 1986b).

Norflurazon acts on prephytoene pyrophosphate synthetase accumulating GGPP (Sandmann et al., 1980).

A number of inhibitors accumulate phytoene acting on the desaturase, such as DPA (reviewed by Sandmann et al., 1984a) while the recently developed cis-pyrones also accumulate  $\zeta$ -carotene (Sandmann et al., 1984b).

Several inhibitors block cyclization, accumulating lycopene, such as CPTA (2-(4-chlorophenyl)triethylamine) and nicotine (reviewed by Sandmann and Boger, 1986b ).

### 1.3 Carotenoid Biosynthesis

The biosynthesis of the carotenoids shares a common early pathway with the terpenoids (1.1.1) as far as GGPP at which point it is condensed to form the first carotenoid, committed to carotenogenesis, phytoene by the action of phytoene synthetase (1.1.4).

Beyond phytoene a large number of carotenoids can possibly be synthesised depending upon the organism and the enzyme systems present therein.

### 1.3.1 Phytoene Desaturation

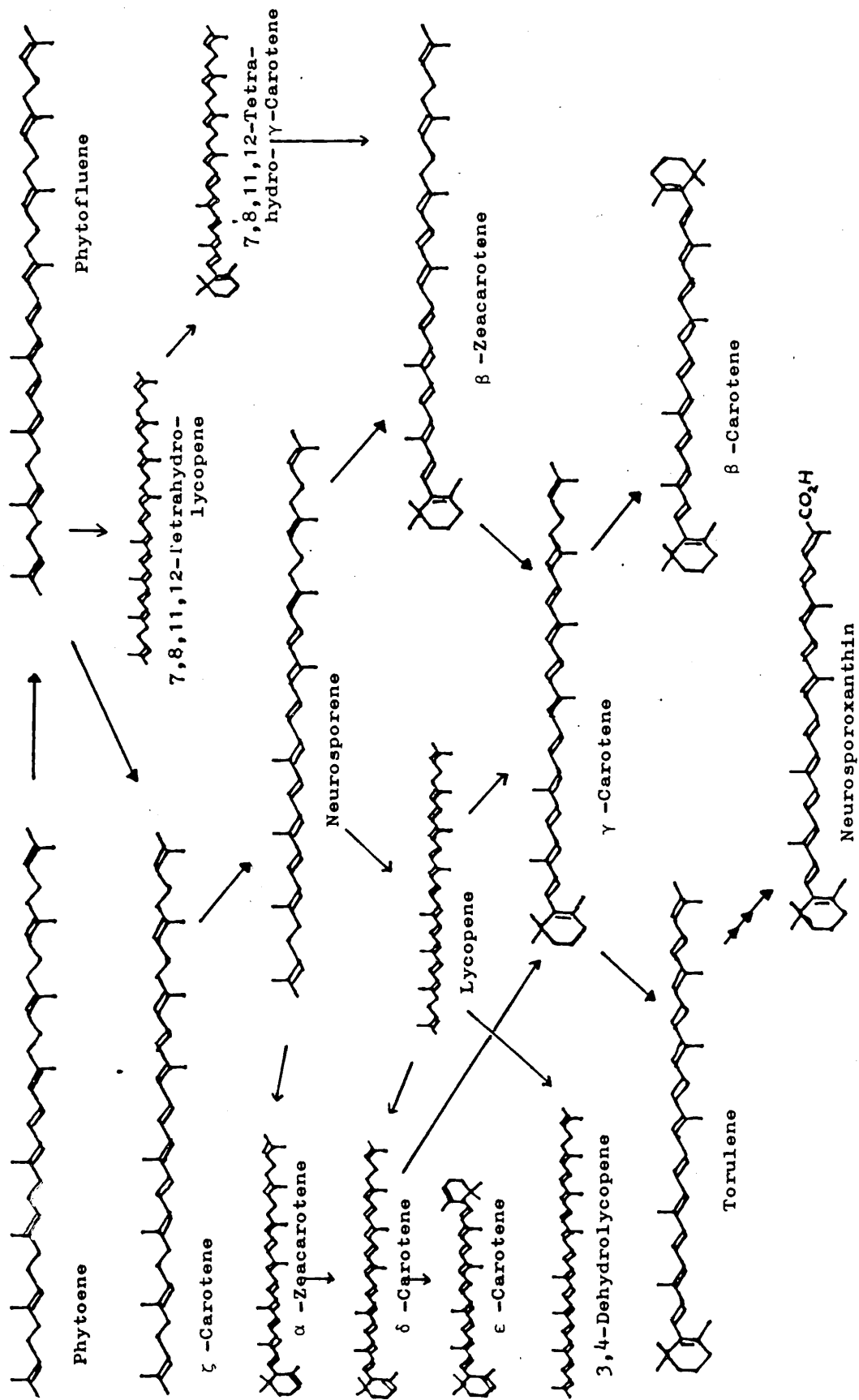
A stepwise desaturation of phytoene to lycopene by a series of didehydrogenations on alternative sides of an expanding polyene chromophore was proposed as the initial carotenogenic steps from phytoene in tomatoes (Porter and Lincoln, 1950) and the structures determined of the various intermediates (Davis et al., 1966). The sequence is now well authenticated (Fig 1.8) and involves, sequentially, phytofluene,  $\zeta$ -carotene, neurosporene and lycopene. It has been enlarged to include the unsymmetrical isomer of  $\zeta$ -carotene, 7,8,11,12-tetrahydrolycopene found in Rhodospirillum rubrum (Davies, 1970) and R.globiformis (Schmidt and Liaaen-Jensen, 1973) formed by 2 successive desaturations on the same side of the molecule. In Rhodopseudomonas viridis (Malhotra et al., 1970), Cellulomonas (Flavobacterium) dehydrogenans (Weeks, 1971), N.crassa (Davies et al., 1974) and P.blakesleeanus (Davies and Rees, 1973) both tetrahydrolycopene isomers exist together. It is not known which is involved in the pathway and both may possibly be involved (Goodwin, 1980).

In addition 3,4-dehydrolycopene is found in some fungi (Liaaen-Jensen, 1965) and 7,8,11,12-tetrahydro- $\gamma$ -carotene in Valencia oranges (Winterstein et al., 1960).

Evidence for the pathway comes mainly from:

- (i) mutant studies with many organisms containing the intermediates (1.2.3.iv)
- (ii) the use of inhibitors (1.2.3.v; Goodwin and Osman, 1954; Liaaen-Jensen et al., 1958 and Villoutreix, 1960)
- (iii) time course studies of synthesis after removal of inhibitors or incorporation of labelled precursors;

Fig 1.8 Desaturation of Phytoene and Possible Pathways of Reduction and Cyclisation



these studies both support (Beeler and Porter, 1963; Davies et al., 1963; Nusbaum-Cassuto et al., 1967 and Harding et al., 1969) and oppose (Purcell et al., 1959; Krzeminski and Quackenbush, 1960; Yokoyama et al., 1961; Purcell, 1964 and Karunakaran et al., 1966) the pathway.

Kinetic studies, however, are difficult to interpret unequivocally especially in cell-free systems of whole pathways and full proof will come from a clear enzymic demonstration of each step.

This evidence is accumulated from the development of various cell-free systems. Enzyme systems from certain genetic selections of tomato fruits incorporate radioactivity from [ $^{14}\text{C}$ ]-phytoene into phytofluene,  $\zeta$ -carotene, neurosporene and lycopene (Kushwaha et al., 1970). This was also achieved into phytofluene and lycopene by an enzyme system from spinach (Subbarayan et al., 1970). An enzyme system from an acetone powder of tomato fruit plastids was shown to incorporate radioactivity from [ $^{14}\text{C}$ ]-phytofluene into  $\zeta$ -carotene and from [ $^{14}\text{C}$ ]- $\zeta$ -carotene into neurosporene, lycopene and later cyclic carotenoids (Qureshi et al., 1974). Neurosporene to lycopene has been demonstrated with cell-free systems from H.cutirubrum (Kushwaha et al., 1976) and P.blakesleeanus (Bramley et al., 1977).

In higher plants, fungi and most bacteria, PPPP is converted predominantly into 15-cis phytoene (1.1.4); some non-photosynthetic bacteria such as Mycobacterium sp. (Gregonis and Rilling, 1973), F.dehydrogenans (Khan et al., 1975), H.cutirubrum (Kushwaha et al., 1972) and Sarcina flava (Al-Windawi, 1977) form the all-trans isomer.

As the more desaturated carotenoids are all-trans, isomerization must occur between cis phytoene and trans phytofluene. An experiment in which it was shown that all-trans phytoene is much better than 15-cis phytoene at diluting out incorporation from  $[2-^{14}\text{C}]$ -MVA into  $\beta$ -carotene by a cell-free system from P.blakesleeanus (Bramley, 1973), suggests that the all-trans isomer which is desaturated. Furthermore, the conversion of 15-cis  $[^{14}\text{C}]$ -phytoene into all-trans phytoene and phytofluene has been demonstrated in a cell-free system from Flavobacterium R1560 (Brown et al., 1975).

In higher plants however, the situation is a little different and it is suggested (Porter and Spurgeon, 1979) that all-trans carotenoids are synthesized via cis phytofluene which is isomerized to all-trans phytofluene in tomato fruit. A number of poly-cis carotenoids predominate in tangerine tomato fruit (Englert et al., 1979 and Clough and Pattenden 1979) and it is suggested that these are formed by an isomerization at 15-cis  $\zeta$ -carotene via 15-trans  $\zeta$ -carotene, whilst maintaining the other cis bonds in the structure (Goodwin, 1983).

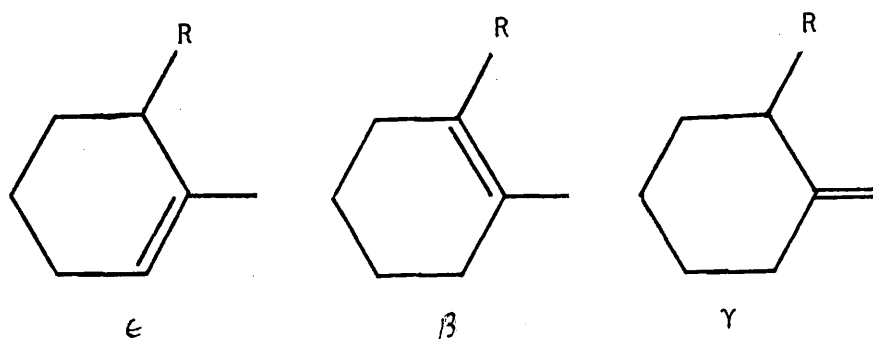
#### 1.3.1.1 Stereochemistry of Isomerization

Desaturation occurs around C atoms originating from C-2 and C-5 of MVA. Tomato slices, incubated with  $[2-^{14}\text{C}, (5\text{R})-5-^3\text{H}_1]$ -MVA and  $[2-^{14}\text{C}, 5-^3\text{H}_2]$ -MVA showed that desaturation eliminated the pro-4R H molecules (Williams et al., 1967). A similar experiment with  $[2-^{14}\text{C}, (2\text{R})-2-^3\text{H}_1]$ -MVA and  $[2-^{14}\text{C}, (2\text{S})-2-^3\text{H}_1]$ -MVA was not conclusive since the prenyl transferase(s) caused randomization of the label

between the 2S and 2R positions (Goad, 1970). This problem was overcome by synthesizing stereospecifically labelled GGPP using Echinocystis macrocarpa preparations and 2S and 2R tritiated [ $2-^{14}\text{C}$ ]-MVA substrates (West et al., 1980). The resultant [ $^{14}\text{C}, ^3\text{H}$ ]-GGPP, when incubated with tomato slices, showed that 2-pro-2S H molecules of MVA were eliminated during desaturation (Vose et al., 1980). The elimination of the pro-5R and pro-2S H of MVA has also been demonstrated with an enzyme system from a Flavobacterium sp. (McDermott et al., 1973).

### 1.3.2 Cyclization

Alicyclic end rings are a common feature of many natural carotenoids found in higher plants, some bacteria, fungi and algae. Three main ring types are known, the  $\beta$ -( $\beta$ -ionine) and  $\epsilon$ -( $\alpha$ -ionine) are relatively common but carotenoids containing the  $\gamma$ -ring have only been isolated from the fungus Culoscypha fulgens (Arpin et al., 1971) and the aphid Microsiphium lireodendri (Andrews et al., 1971). Furthermore, carotenoids containing the  $\epsilon$ -ring have not been found unequivocally in the fungi (Goodwin, 1976).



Alternative routes for cyclization are available depending on whether desaturation is completed or not. If desaturation is completed then lycopene would be cyclized, but cyclization can also occur at the level of neurosporene, as indicated by the occurrence of  $\alpha$ -zeacarotene and  $\beta$ -zeacarotene which may then be further desaturated. Cyclization of 7,8,11,12-tetrahydrolycopene is also known but is rare, 7',8',11',12'-tetrahydro- $\gamma$ -carotene has been isolated only from DPA inhibited cultures of P.blakesleeanus (Davies and Rees, 1973). The question as to whether neurosporene or lycopene is a primary substrate for cyclization has been debated for many years (see Britton, 1976). Genetic studies were carried out between  $\beta$ -carotene and lycopene accumulating strains of tomato and it was concluded that  $\beta$ -carotene was probably a cyclization product of lycopene (Porter and Lincoln, 1950).

It was observed that lycopene could be converted to  $\beta$ -carotene in carrot leaf chloroplasts (Decker and Kehleke, 1961) and into  $\beta$ -,  $\alpha$ - and  $\delta$ -carotene in isolated tomato plastids or spinach chloroplasts (Wells et al., 1964). However,  $\beta$ -zeacarotene was found in DPA inhibited cultures of P.blakesleeanus (Williams et al., 1965) and in N.crassa (Davies, 1973) suggesting cyclization may occur before complete desaturation.

Radioactivity from [ $^{14}\text{C}$ ]-lycopene could be incorporated into  $\beta$ -carotene in bean leaf chloroplasts and tomato plastids (Hill and Rogers, 1969) and from [ $^{15}, 15'\text{-}^3\text{H}_2$ ]-lycopene into  $\alpha$ -,  $\beta$ - and  $\gamma$ -carotene in the same systems (Kushwaha et al., 1969). Although these studies demonstrated that lycopene could be converted into  $\beta$ -carotene, they did

not necessarily eliminate the possibility of cyclization proceeding through neurosporene and  $\beta$ -zeacarotene.

The existence of 2 pathways to  $\gamma$ -carotene through either lycopene or  $\beta$ -zeacarotene (Fig 1.8) was demonstrated (Bramley and Davies, 1976) in that  $[2-^{14}\text{C}]$ -MVA, usually incorporated into  $\beta$ -carotene in *P.blakesleeanus*, was diluted out by the addition of  $\gamma$ -carotene and neurosporene, but not so effectively by either lycopene or  $\beta$ -zeacarotene. When the same system was incubated with  $[^{14}\text{C}]$ -neurosporene, equal amounts of incorporation were obtained in lycopene and  $\beta$ -zeacarotene suggesting both pathways are of equal physiological significance (Bramley et al., 1977).

Genetic studies on heterokaryons of *P.blakesleeanus* have indicated that a carotenogenic multi-enzyme complex exists consisting of 4 desaturases and 2 cyclases necessary for the conversion of phytoene into  $\beta$ -carotene (de la Guardia et al., 1971). Since  $\gamma$ -carotene can be synthesized from lycopene or  $\beta$ -zeacarotene it was suggested (Davies, 1973) that cyclase I is in the complex such that it can receive equally, the product of desaturase III (neurosporene) or desaturase IV (lycopene). As neurosporene and  $\gamma$ -carotene are substrates for the cyclases (Davies and Rees, 1973), it appears that these have specificity for desaturated, half carotene molecules, or perhaps only unsaturated 7,8-C bonds.

Although it was suggested (Porter and Lincoln, 1950) that the  $\beta$ -ring was isomerized from the  $\epsilon$ -ring, inheritance studies with tomatoes implied that both were formed independently from a common precursor (Tomes, 1967) and using stereospecifically labelled  $[^{14}\text{C}, ^3\text{H}]$ -MVA it was concluded cyclization involved formation of an active



cyclic intermediate stabilized by the loss of a H from C-4 ( $\epsilon$ -ring) or C-6 ( $\beta$ -ring) (Williams et al., 1967). It has also been demonstrated (Swift and Milborrow, 1981) that  $\epsilon$ -rings and  $\beta$ -rings cannot be precursors of  $\gamma$ -ring and (Milborrow, 1982) that the three ring types can not be interconverted.

### 1.3.3 Modifications and Further Metabolism of Carotenoids

There exist a large number and wide variety of modifications to carotenoids (reviewed by Spurgeon and Porter, 1983). In the fungi, modifications to carotenoids include the insertion of an oxygen function to xanthophylls (Goodwin, 1980). The most common of these are the introduction of a carbonyl group e.g. canthaxanthin ( $\beta, \beta$ -caroten-4,4'-dione) and oxidation of the terminal methyl group to a carboxylic acid e.g. torularhodin (Fig 1.6). Another modification involves the further desaturation at the 3,4 bond to give torulene (Fig 1.8).

In some fungi, such as N.crassa (Barber et al., 1966) and G.fujikuroi (Zalokar, 1957), a  $C_{35}$  carotenoid is found, the apocarotenoid neurosporoxanthin (NX). In this case one isoprene unit has been removed, presumably by oxidation of the corresponding  $C_{40}$  either torulene (Fig 1.8) or  $\gamma$ -carotene (Aasen and Liaaen-Jensen, 1965). Inhibitor studies suggest that  $\gamma$ -carotene is the most likely precursor (Valadon and Mummery, 1969), though this is by no means established: as in other parts of the pathway, 2 alternative possibilities need not be mutually exclusive.

$\beta$ -Carotene may be oxidatively cleaved to retinal which is then metabolised to trisporic acids, fungal hormones that regulate gametogenesis (Austin et al., 1969 and 1970). It

may also be oxidatively polymerised both in vitro and in vivo, to yield sporopollenin, a component of pollen grains and fungal spores (Shaw, 1971 and Gooday et al., 1973).

#### 1.3.4 Cofactor Requirements

A number of carotenogenic cell-free systems have been described for different organisms (reviewed by Bramley, 1985), but few attempts have been made to remove the endogenous cofactors to assess the true cofactor requirements.

A dialysed, cell-free extract from P.blakesleeanus incorporated radioactivity from [ $^{14}\text{C}$ ]-MVA into  $\beta$ -carotene was found to require NADP, NADPH, ATP and  $\text{Mg}^{2+}$  (Yokoyama et al., 1962). Later studies on a similar dialysed system from the carR mutant of P.blakesleeanus showed incorporation of [ $^{14}\text{C}$ ]-GGPP into lycopene was not dependent on NAD, NADP, NADPH or ATP, though their inclusion doubled incorporation into lycopene without an increase in total carotenoid incorporation (Lee and Chichester, 1969). A dialysed preparation from P.blakesleeanus carB10(-) (a phytoene accumulating mutant), showed no requirement for nicotinamide nucleotides or FAD for the synthesis of [ $^{14}\text{C}$ ]-phytoene from [ $2\text{-}^{14}\text{C}$ ]-MVA (Bramley, 1973).

#### 1.4 Gibberellins

The gibberellins (GAs) are generally recognized as regulators of the growth and development of higher plants. They are known constituents of many and presumably all higher plants (Bearder, 1983) and are major secondary metabolites of the fungus G.fujikuroi (Cross et al., 1964). G.fujikuroi is an important organism in GA studies since it produces far larger quantities of GAs than any higher plant

yet the presence of the GAs does not appear to be an essential requirement for any function of the fungus.

#### 1.4.1 Nomenclature

The GAs are cyclic diterpenoids which are subdivided into the C<sub>20</sub> GAs, in which the ring B of the ent-kaurene skeleton has undergone ring contraction to the ent-gibberellane skeleton, and the C<sub>19</sub> GAs are characterised by the (19-10)- $\gamma$ -lactone function and generally exhibit potent biological activity. This nomenclature is based on that of Rowe (1968) and a full list of GAs has been compiled (Bearder, 1980) which involves over 60 different GAs that have been structurally characterised, designated GA<sub>1</sub>, GA<sub>2</sub>, GA<sub>3</sub>, GA<sub>4</sub>....GA<sub>n</sub>.

The GAs that have been isolated from G.fujikuroi are illustrated in Fig 9, along with other diterpenoid metabolites implicated in GA biosynthesis studies.

#### 1.4.2 Function

It may be speculated that the GAs in G.fujikuroi act in some way to aid infection of a host plant since the fungus is parasitic, though there is no evidence for this possibility. It may also be suggested that a GA biosynthetic "operon" exists, which has a common genetic origin to that of plants, coming in some way from some early evolutionary "infection" of a transposable element. However GA biosynthetic pathway studies (1.5.3), showing major differences between the pathways of G.fujikuroi and higher plants, imply different origins of GA biosynthesis.

In plants, on the otherhand, GAs serve many wide and varied

Fig 1.9 Diterpenoid Metabolites  
of G.fujikuroi

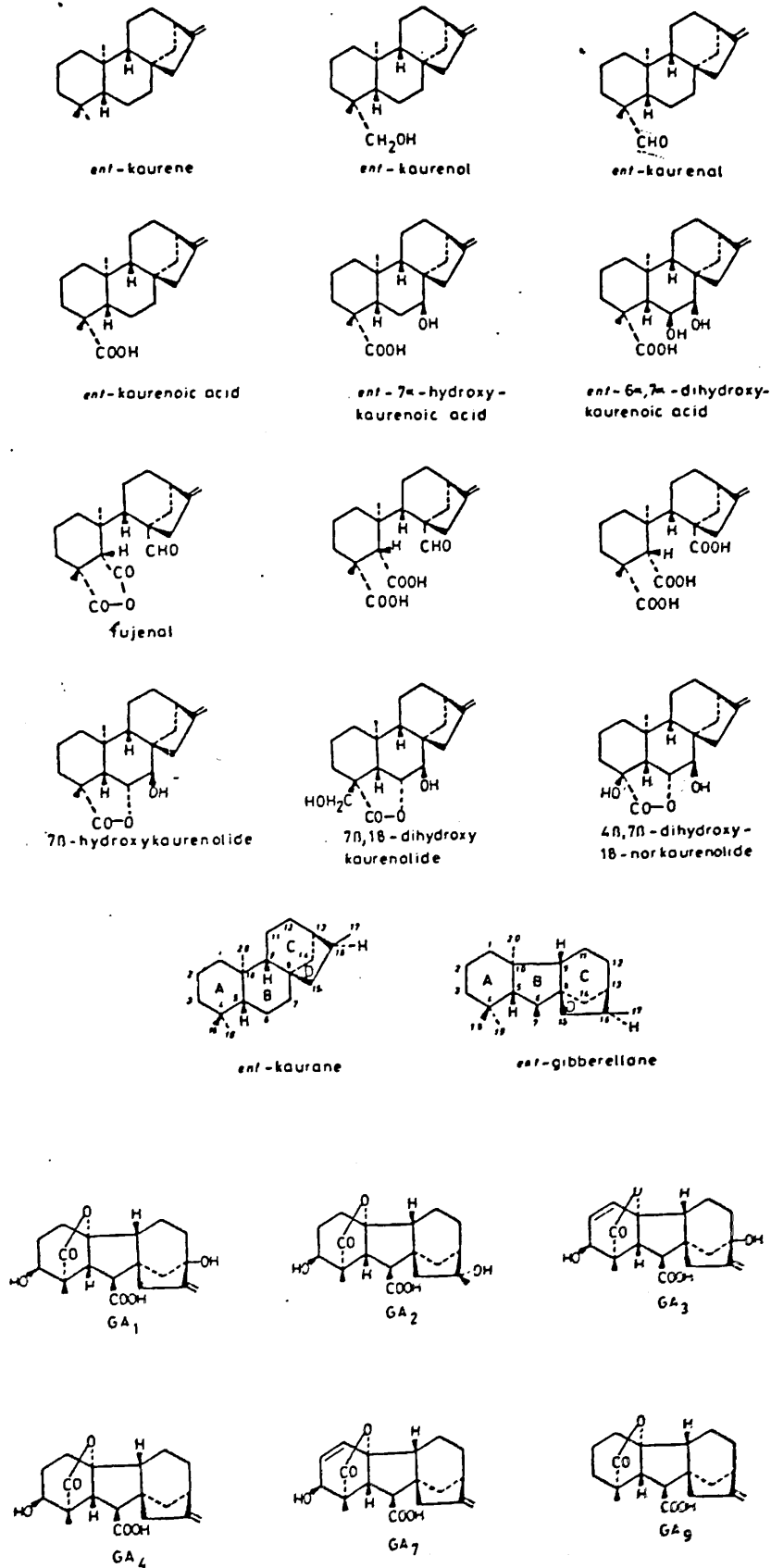
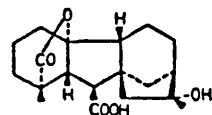
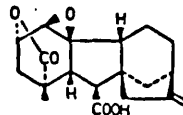


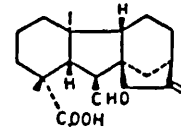
Fig 1.9 Contd.



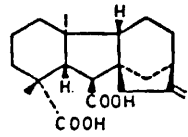
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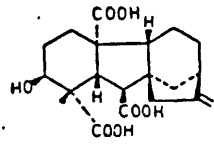
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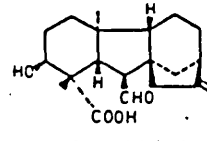
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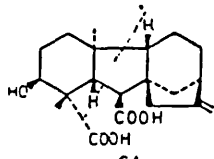
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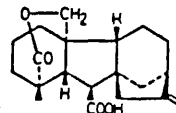
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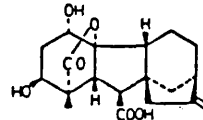
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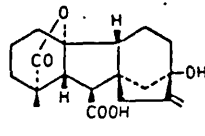
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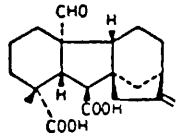
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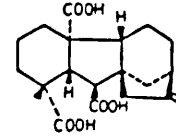
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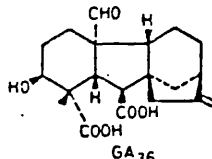
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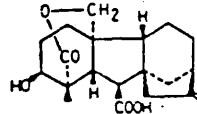
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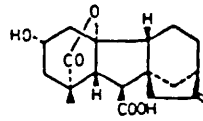
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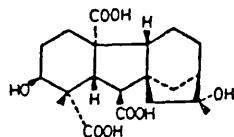
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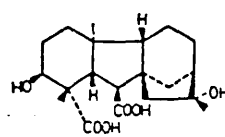
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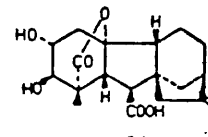
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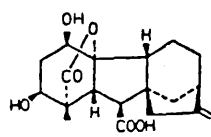
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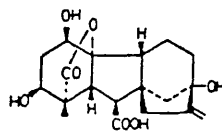
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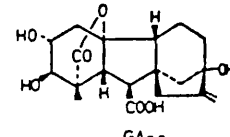
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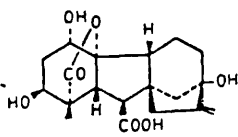
GA<sub>54</sub>



GA<sub>55</sub>



GA<sub>56</sub>



GA<sub>57</sub>

functions dependent upon the GA and the plant species involved (reviewed by Stoddart, 1983). Although relatively few of the identified GAs have been examined with respect to biological activity, they are used in industry to accelerate flowering and barley germination in the malting process and to increase yields in fruit production. These areas may be expanded upon when the effects of other GAs on higher plants are more fully understood.

#### 1.4.3 Factors Controlling GA Biosynthesis

GA production, as with carotenoids and other secondary metabolites, is generally late in growth, starting at the end of log. growth and increasing over the stationary phase. The regulation of GA production may share a common mechanism with early terpenoid pathway steps (1.1.1), with steps specific to GA production, having more individual regulation. However, the shortage of knowledge concerning those enzymes later in the pathway, compared to those involved in the common terpenoid pathway, means regulation of these steps is much less understood than in the earlier GA steps.

##### 1.4.3.i Culture Conditions

Using a constant level of N in the medium, after the N is exhausted, GA production is induced and increases with levels of C over a 5-30% glucose concentration range. At higher C concentrations the reverse is true and decreasing GA levels are seen with increasing C (Borrow et al., 1964a). The same workers showed with varying N levels, both the rate of GA production and overall productivity increase with increasing

N but that further increases cause a decrease in productivity and rates of production. This was one of the first studies of GA production regulation by culture conditions for G.fujikuroi and actually only GA<sub>3</sub> levels were analyzed.

Recent studies on G.fujikuroi (Aitken, personal communication) seem to show a relationship between C and N ratios (C:N) and GA levels identical to those already observed i.e. an increase in C:N levels causes an increase in GA<sub>3</sub> production. However, at higher levels of C, even at a high C:N level, a qualitative switch is seen and GA<sub>3</sub> production is almost completely replaced by increased GA<sub>4</sub> and GA<sub>7</sub> levels.

A number of studies on the effects of temperature on GA levels have been carried out in fermentations (reviewed by Jefferys, 1973) and tend to show optimum temperatures of 29-32°C (Borrow et al., 1964b). There is a suggestion (Sumiki et al., 1966) that a temperature of 32°C favours GA<sub>4</sub> and GA<sub>7</sub> production over GA<sub>3</sub>, though the pH of the medium may be more relevant to this change (Jefferys, 1973).

It is difficult to disentangle the effects of varied pH from those caused by various sources of N, though glycine use avoids the concurrent release of excess acid radicals during N assimilation. The pH has both quantitative and qualitative effects on GA production by G.fujikuroi, and it was shown that pH 3.0-5.5 range had no effect on GA<sub>3</sub> production (cited by Jefferys, 1973). A low range though, of pH 2-3 seems to favour mixtures of GA<sub>1</sub> and GA<sub>3</sub> (Fuska et al., 1962). High pH values such as pH 7, increase GA<sub>4</sub>, GA<sub>7</sub>, GA<sub>9</sub>, GA<sub>12</sub>, GA<sub>14</sub> and GA<sub>16</sub> (Cross, 1966).

#### 1.4.3.ii Metabolic Regulation

Metabolic regulation of the GAs shares some control common to the early terpenoid pathway steps. Inhibition of MVA kinase by GPP and FPP, but not by IPP and DMAPP, has been demonstrated in animal tissues (Dorsey and Porter, 1968 and Flint, 1970) and etiolated cotyledons of Phaseolus vulgaris (Gray and Kekwick, 1973). MVAPP Decarboxylase is subject to inhibition by its immediate products IPP and ADP (Skilleter and Kekwick, 1971). GA<sub>3</sub> was shown to stimulate MVA kinase activity in Corylus avellana embryonic axes (Shewry et al., 1974).

The cyclization of GGPP to ent-kaurene is a branch-point in the terpenoid biosynthetic pathway and a strong candidate for a regulatory point in GA biosynthesis. It has been suggested (Simcox et al., 1975) that it may be the A activity of kaurene synthetase (1.5.1) which is under regulation since there are a number of examples in which a lack of overall activity is associated with a lack of A activity only.

The most common GA derivatives are conjugates with glucose, the GA-glucosyl esters which are found in highest concentrations in mature seeds. Consequently, it is proposed that these esters have a storage role (Liebisch, 1974). On germination these esters appear to be hydrolyzed to free GAs by plant glycolytic enzymes. However, there is no compelling evidence that the ester formation and hydrolysis regulate GA levels during seed maturation and germination.

#### 1.4.3.iii Effects of Light

The effects of light on terpenoid levels are well documented with respect to the carotenoids (1.2.3.iii) showing that



certain steps common to the GA pathway are photoinduced and leading to the suggestion that GA levels may also be photo-induced.

Early reports on the stimulation of GA levels by light in G.fujikuroi (Zweig and Deny, 1959 and Mertz and Henson, 1967) and in P.sativum (Kohler, 1965). However, investigations of this type are limited and reports of light effects on GAs are intermittent with a bias towards higher plants.

It was shown that incubation of 10 day old Pisum shoot tips in solutions of chloramphenicol and cycloheximide resulted in a reduction of light-stimulated synthesis of ent-kaurene from MVA (Gomez-Nevarrette and Moore, 1978) suggesting photo-induction of one or more of the enzymes involved in ent-kaurene biosynthesis.

Light intensity has been shown to have significant effects on GAs, increasing levels in developing wheat grains, Triticum aestivum L. (Mengel et al., 1985). White light stimulated flower formation is accompanied by an increase in the flower GA content in Hyoscyanus niger L.

There are some indications that in higher plants light has an effect on GA metabolism. Light reduced the release of GAs from their glucosyl conjugates and increased the rate of conjugate formation in maize germinants (Rood et al., 1986). Since free GAs are more biologically active than their respective conjugates (Schneider, 1983) this has been implicated as the reason for increased GA activity causing germinating maize shoot elongation in the dark. A similar, though opposite effect is seen in Phaseolus coccineus seedlings in which GA<sub>4</sub> promotes stem growth in the light but not in the dark (Bowen et al., 1973). It has been shown

that GA<sub>4</sub> is metabolised more extensively in the dark (Bown et al., 1975) and proposed as the reason for the former phenomenon.

Whether the light-induced metabolism of GAs seen in the higher plants is significant in G.fujikuroi is unknown, but the possibility that the fungal GAs may be metabolised by the higher plants on infection, perhaps to more biologically active forms, cannot be ruled out.

Action spectra of ent-kaurene photoinduction in P.sativum (Choinski and Moore, 1980) indicated distinct peaks in the red and blue regions of the spectrum. The possibility of the involvement of phytochrome in the GA biosynthetic pathway has been predicted for some time and work so far suggests that the involvement may be quite complex, but further work is necessary to clarify this important aspect of GA biosynthesis.

#### 1.4.3.iv Inhibitors

The value of inhibitors of biochemical pathways in investigations of the pathway activities is well known and is discussed with respect to the carotenoid pathway (1.2.3.v).

Blockage of acetate incorporation into MVA was obtained with AMO-1618 (2'-isopropyl-4'-(triethylammonium chloride)-5'-methylphenyl piperidine-1-carboxylate; Douglas and Paleg, 1972).

AMO-1618 along with CCC (2-chloroethyltrimethylammonium chloride) also blocks the initial cyclization of GGPP (activity A) in G.fujikuroi extracts (Schechter and West, 1969) though their effects are less on certain higher plant systems (reviewed by Coolbaugh, 1983). There are a large

number of different potential inhibitors of ent-kaurene synthetase (Frost and West, 1977) towards both A and B activities.

Alaclor and Metolachlor (Wilkinson, 1981) also inhibit kaurene synthetase but also have other sites of action.

A number of different inhibitors exist for the cytochrome P-450 dependent oxidation of ent-kaurene to ent-kaurenoic acid such as triazoles, pyrimidines and diazetins and these have been used in computer modelling studies for kaurene oxidase from C.maxima (Muller et al., 1986).

### 1.5 GA Biosynthesis

Extensive studies on GA biosynthesis over the years using cell-free and whole systems, have established the general features of the pathway (reviewed by Bearder, 1983 and Coolbaugh, 1983). The relationship between GA and diterpene biosynthesis was established from early studies (Birch et al., 1958 and Cross et al., 1964). From the diversity of naturally occurring GAs (1.4.1) it is clear no single biosynthetic pathway exists in fungi and plants though it has been shown that the early step intermediates i.e MVA to GGPP (1.1.1), ent-kaurene and GA<sub>12</sub>-aldehyde are the same in G.fujikuroi and higher plants (reviewed by Bearder, 1983 and Coolbaugh, 1983). Thereafter the pathways diverge. Furthermore, with the exception of the soluble enzymes, kaurene synthetase from Marah macrocarpus (Duncan and West, 1981) and G.fujikuroi (Fall and West, 1971) and GA 2  $\beta$ -hydroxylase from P.vulgaris (Smith and MacMillan, 1984), the purification of enzymes from the pathway have not been

reported. Consequently knowledge of other pathway enzymes and GA biosynthesis is derived from studies with crude, cell-free preparations, mainly in the fungus G.fujikuroi and from immature seeds of M.macrocarpus, C.maxima and P.sativum.

Generally the pathway can be divided into 3 stages; the conversion of MVA to ent-kaurene by soluble enzymes; the oxidation of ent-kaurene by membrane associated enzymes to the first structural GAs; these are then further oxidized by soluble enzymes.

#### 1.5.1 MVA to ent-Kaurene

The conversion of MVA to ent-kaurene was first demonstrated (Graebe et al., 1965) in a cell-free preparation from the endosperm-nucellus of immature seeds of M.macrocarpus. It was later demonstrated in cell-free systems from seeds or young pods of P.sativum (Anderson and Moore, 1967), young seedlings of Ricinus communis (Robinson and West, 1970), P.sativum (Coolbaugh et al., 1973), Lycopersicum esculentum (Yafin and Schechter, 1975) and Zea mays (Hedden and Phinney, 1976), immature seeds from R.communis (Robinson and West, 1970), endosperm from C.maxima (Graebe, 1969) and mycelia from G.fujikuroi (Schechter and West, 1969). The highest rates of conversion were in the Marah and Cucurbita systems, in most other systems ent-kaurene was low and squalene and phytoene predominated (e.g Graebe, 1968 and Hedden and Phinney, 1976).

The enzymes responsible for ent-kaurene formation have already been discussed as far as GGPP (1.1.1) and are reviewed, with respect to GA biosynthesis, by Coolbaugh

(1983).

GGPP was first established as an intermediate in ent-kaurene production by Oster and West (1968) and its cyclization to form ent-kaurene has been shown to proceed in 2 steps (Fig 1.10; Schechter and West, 1969) by ent-kaurene synthetase activity A and activity B. The first step is probably a proton-initiated cyclization to a bicyclic intermediate, copalyl pyrophosphate (CPP; ent-trans-labda-8(16),13-dien-15-yl pyrophosphate) which can be incorporated into ent-kaurene in extracts of M.macrocarpus, R.communis and P.sativum (Simcox et al., 1982) and Helianthus annuus (Shen-Miller and West, 1982). ent-Kaurene synthetase was purified 170-fold in G.fujikuroi extracts (Fall and West, 1971) and had similar properties to a 5-fold purified fraction from M.macrocarpus (Frost and West, 1977), including different p<sub>H</sub> optima of A and B activities (7.5 and 6.9 respectively) and different susceptibilities to certain inhibitors. The 2 activities were resolved, showing CPP formed by system A was preferentially channelled to system B rather than equilibrating with free CPP in the system (Duncan and West, 1981).

The stereochemistry of ent-kaurene formation has been studied by a number of workers (reviewed by Hedden, 1983 and Coolbaugh, 1983).

#### 1.5.2 ent-Kaurene to GA<sub>12</sub>-Aldehyde

The second stage of GA biosynthesis is the conversion of ent-kaurene to GA<sub>12</sub>-aldehyde by a sequence of oxidative steps catalyzed by microsomal enzymes requiring a pyridine nucleotide (NADPH, preferentially) and O<sub>2</sub> for activity



(Graebe et al., 1980; Fig 1.10).

ent-[<sup>14</sup>C]-Kaurenol was isolated from incubations of [2-<sup>14</sup>C]-MVA with the Marah cell-free system (Graebe et al., 1965), subsequently ent-[<sup>14</sup>C]-kaurenal and ent-[<sup>14</sup>C]-kaurenoic acid were identified in similar incubations (Dennis and West, 1967). The biosynthesis of these ent-kaurenoids, ent-kaurene to ent-kaurenol to ent-kaurenal to ent-kaurenoic acid, was established by refeeding each intermediate and reisolation of the products (reviewed by Bearder, 1983). Later it was demonstrated there is a further hydroxylation of ent-kaurenoic acid in the Marah cell-free system (Fig 1.10).

The sequence ent-kaurene to ent-7β-hydroxy-kaurenoic acid has been demonstrated conclusively from the Cucurbita system (Graebe et al., 1974) and in crude homogenates of immature Pisum seeds (Graebe and Ropers, 1978). The conversion of ent-[<sup>14</sup>C]-kaurenoic acid has also been shown for a microsomal fraction from G.fujikuroi (West, 1973).

Using the Marah cell-free system it was shown that the enzymes for ent-kaurene to ent-kaurenoic acid are localised in the microsomal pellet of a high speed centrifugal preparation, requiring O<sub>2</sub> and a reduced pyridine nucleotide (preferentially, NADPH) suggesting these enzymes to be mixed-function oxygenases (Dennis and West, 1967). Furthermore, cytochrome P-450 was implicated at the active centre of the microsomal oxidases in M.macrocarpus (Murphy and West, 1969) and P.sativum (Coolbaugh and Moore, 1971) due to a 450nm-light-reversible CO inhibition. It was also found that ancyimidol inhibited kaurene oxidation, possibly by binding to cytochrome P-450, according to spectral studies

in M. macrocarpus (Coolbaugh et al., 1978). This inhibitor was less efficient at inhibiting kaurene oxidation in G. fujikuroi although other cytochrome P-450 binding inhibitors such as paclobutrazol do prevent kaurene oxidation in the fungus (Coolbaugh et al., 1982).

The hydroxylation of ent-kaurenoic acid to ent-7 $\beta$ -hydroxy-kaurenoic acid was at first thought to result from the same mixed function oxidase (Lew and West, 1971). However, the inhibitors of ent-kaurene oxidation, involving cytochrome P-450, had no effect on this step and so it is suggested that this does not involve the same enzyme, nor is it a cytochrome P-450 linked hydroxylase.

Although the inhibition of each step between kaurene and kaurenoic acid by, for example ancymidol, suggests that the enzyme binding sites may be very similar there is evidence that separate sites exist for each substrate. This includes the competitive inhibition of each step by the other substrates, such that the concentration for 50% inhibition was between 10 and 100 times higher than the  $K_m$  value for the substrate (Hirano, cited by West, 1980). Furthermore, the existence of the B1-41a mutant of G. fujikuroi, in which GA biosynthesis is blocked between ent-kaurenal and ent-kaurenoic acid lends additional support to the separate binding sites proposal (Bearder et al., 1974).

#### 1.5.2.i Formation of GA<sub>12</sub>-Aldehyde

GA<sub>12</sub>-Aldehyde formation was first observed in a cell-free system from C. maxima (Graebe et al., 1972) and later in cell-free systems from M. macrocarpus and G. fujikuroi (West, 1973) and P. sativum (Ropers et al., 1978). It was formed by a



single step from ent-7 $\beta$ -hydroxykaurenoic acid (Fig 1.10) in a reaction requiring NADPH and O<sub>2</sub> (Graebe and Hedden, 1974) so it is similar to the previous steps, although the involvement of cytochrome P-450 has not been demonstrated.

The mechanism of GA<sub>12</sub>-aldehyde formation involves a ring contraction of ring B from a 6 to 5 C-ring. Evidence for this mechanism and the suggested stereochemistry is reviewed by Hedden and coworkers (1978).

An interesting feature of the reaction is the accompanied formation of ent-6 $\beta$ ,7 $\beta$ -dihydroxykaurenoic acid in M.macrocarpus and G.fujikuroi (West, 1973) and C.maxima (Graebe et al., 1974). This activity has not been separated from GA<sub>12</sub>-aldehyde formation in C.maxima it forms about 50% of the conversion products of ent-7 $\beta$ -hydroxykaurenoic acid (Graebe and Hedden, 1974). This compound and its oxidative products are not precursors of the GAs and have no known function (Graebe et al., 1980). The very polar end-product is closely related to fujenal di-acid, a major metabolite in some cultures of G.fujikuroi, again with no known function (cited by Graebe et al., 1980). These oxidations, which have been shown to involve NADPH, are probably the result of a mixed-function oxidase similar to those already discussed (Hedden, 1983; Fig 1.10).

Another group of metabolites, the kaurenolides, are formed at this point in the pathway and, although early workers suggested their formation from ent-7 $\beta$ -hydroxykaurenoic acid (Evans et al., 1970), it has since been shown to probably be derived from ent-kaurenoic acid directly (Graebe et al., 1980; Fig 1.10). The complete pathway of kaurenolide biosynthesis has been determined in C.maxima endosperm micro-

some preparations (Hedden and Graebe, 1981) forming the endogenous product 7 $\beta$ ,12 $\beta$ -dihydroxykaurenolide and again involving NADP-requiring oxidases of the type that participate in the GA pathway. Indeed in some cases at least, the same oxidases may catalyze reactions in different pathways (Hedden, 1983). There is evidence that the kaurenolide pathway in G.fujikuroi is the same as that in C.maxima (Beale et al., 1982).

It may turn out that the formation of kaurenolides is an unavoidable consequence of GA biosynthesis since there are many indications that reactions involved in these apparently non-functional side-products are catalyzed by enzymes related to the main GA pathway (Hedden, 1983).

### 1.5.3 Pathways from GA<sub>12</sub>-Aldehyde

The later steps in GA biosynthesis are catalyzed by enzymes remaining in the 200000g supernatant fraction, the soluble nature of these enzymes might be expected since the substrates are more hydrophilic than the earlier, microsomal oxidation substrates. However, the available information on the soluble oxidases is much less than that towards the microsomal oxidases. Complete pathways to polar GAs have been demonstrated in vitro, in cell-free preparations from C.maxima endosperm (Graebe et al., 1974), immature seeds of P.sativum (Kamiya and Graebe, 1983) and suspensions of P.coccineus ( Ceccarelli et al., 1981 ). In addition, a single-terminal reaction has been studied from germinating seeds of P.vulgaris (Breidenbach, 1975 and Hoad et al., 1983).

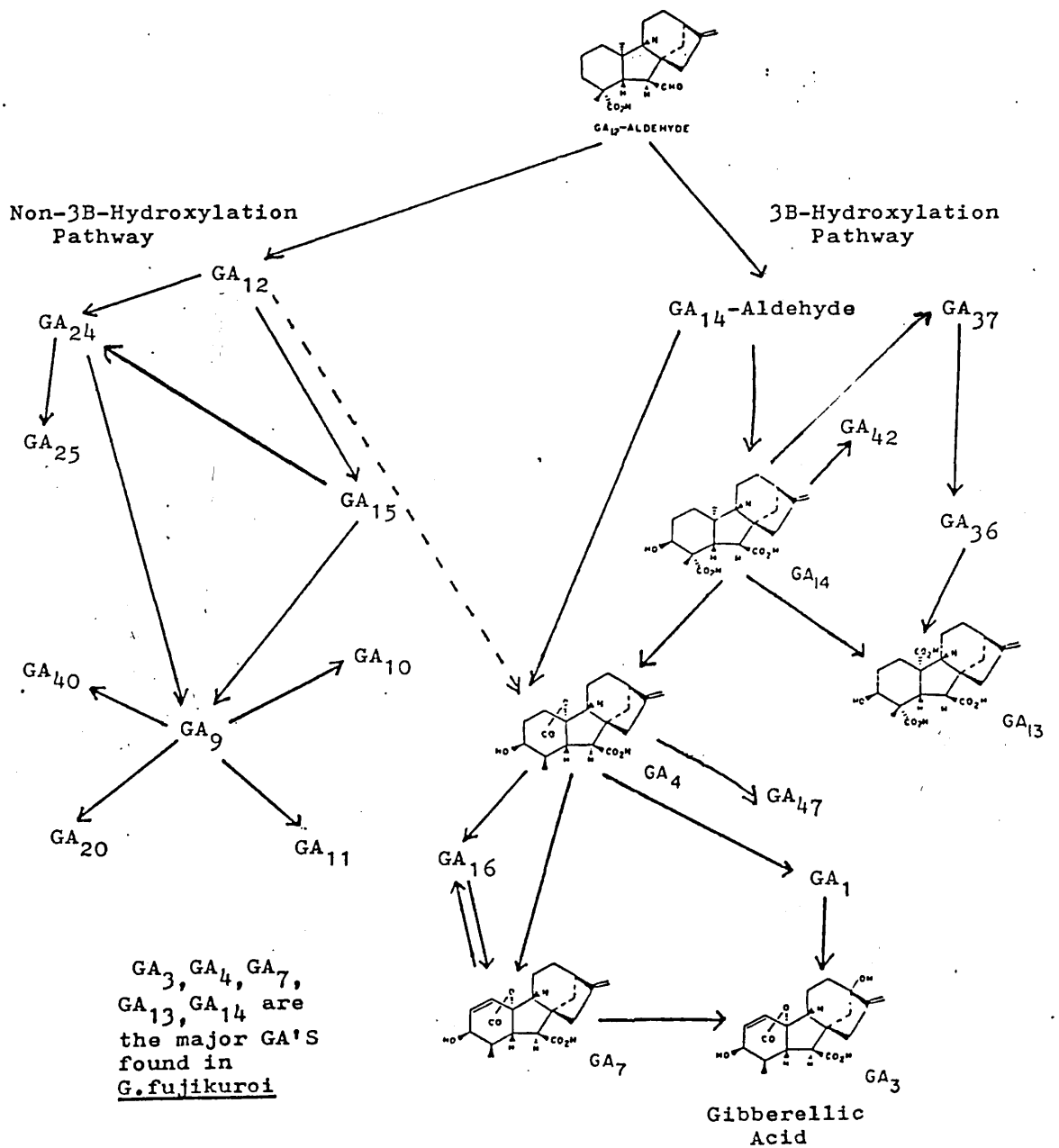
The biosynthetic pathways from GA<sub>12</sub>-aldehyde have been

established for G.fujikuroi (Hedden et al., 1978; Fig 1.11). These pathways are based on results from several studies using both GA-producing strains ACC 917, GF-1a and REC-193A and the mutants R-9 and Bl-41a. R-9 is blocked for 13-hydroxylation producing neither GA<sub>1</sub> nor GA<sub>3</sub> (Bearder et al., 1973; Spector and Phinney, 1968). Bl-41a is blocked between ent-kaurenal and ent-kaurenoic acid with a leakage of less than 3% for the conversion of [<sup>14</sup>C]-kaurenoic acid into [<sup>14</sup>C]-GA<sub>3</sub> (Bearder et al., 1974).

Bearder and coworkers (1975) studied the metabolism of substrates, either precursors of fungal GAs or analogues in resuspension cultures of G.fujikuroi. The results of these experiments, and others (reviewed by Hedden et al., 1978 and Bearder, 1983) lead to the proposed pathway (Fig 1.11).

There is a branch point at GA<sub>12</sub>-aldehyde in which microsomal oxidases convert GA<sub>12</sub>-aldehyde either to GA<sub>12</sub> or to 3β-hydroxy-GA<sub>12</sub>-aldehyde (GA<sub>14</sub>-aldehyde), the latter being quickly oxidized to GA<sub>14</sub> (Evans and Hanson, 1975). These are called the non-3-hydroxylation pathway and the 3-hydroxylation pathway, respectively. This is to be compared with the site of hydroxylation in higher plants which is found to be a C-13 in a number of species (Bearder, 1980). However, 13-hydroxylation is known to occur late in the pathway in G.fujikuroi in fact it is the final step in the biosynthesis of GA<sub>3</sub>. Thus, although the fungus hydroxylates at the same positions as many higher plant species, it does so in reverse order and probably uses a different type of enzyme for respective reactions. This suggests separate origins of GA biosynthesis in the fungus and higher plants (Hedden, 1983).

Figl.11 The Main Gibberellin Pathways in G.fujikuroi from GA<sub>12</sub>-Aldehyde



Unfortunately, since steps beyond GA<sub>12</sub> and GA<sub>14</sub> in G.fujikuroi have not been obtained in active form, it is not known whether or not the enzymes involved beyond these compounds are of the same type as those found in higher plants, for which cell-free systems have been developed and pathways established; particularly in C.maxima (reviewed by Hedden, 1983). Nevertheless, the pathways so far established in C.maxima and G.fujikuroi and the different respective GAs produced (GA<sub>3</sub> is the major GA in G.fujikuroi, Hedden et al., 1978; GA<sub>43</sub> is predominant in C.maxima, Graebe and Hedden, 1974) and the aforementioned hydroxylation-site differences, all suggest that while the MVA to GA<sub>12</sub>-aldehyde pathway is common to most GA-producing species, metabolism of GA<sub>12</sub>-aldehyde is a point at which major differences and divergences within the pathway are observed between species.

#### 1.6 Sterols (and Other Lipids)

There are 4 major lipid classes which go to make up the total lipids in fungi; fatty acids, phospholipids, acyl glycerols and sterols. The qualitative and quantitative levels of these classes vary between different species and different periods of growth and development within the same species (Weete, 1974 and Harwood and Russell, 1984).

Generally in fungi the major lipids which make up the classes are; myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid,  $\alpha$ - and  $\gamma$ -linolenic acid and arachidonic acid as the fatty acids; phosphatidyl -ethanol-amine, -choline, -glycerol and -inositol as the phospholipids; triacylglyceride as the major acylglycerol with ergosterol as the main sterol (Harwood and Russell, 1984)

### 1.6.1 Functions of the Sterols

Sterols are present in the membranes of plants, animals and fungi and serve an important biological function.

It was shown that the mean molecular area of a phospholipid monolayer was reduced in the presence of cholesterol and ergosterol (De Bernard, 1958). Later it was demonstrated (Demel et al., 1968 and Grunwald, 1974) that this condensation effect reduced the permeability of both artificial and natural membranes.

Addition of sterol to phospholipid dispersions caused a reduction in the crystalline and liquid-crystalline transition temperatures between the two phases (Ladbrooke et al., 1968). These alterations result from the sterols controlling the fluidity of the phospholipid hydrocarbon chains by disrupting the lattice in the crystalline phase and inhibiting chain flexing in the liquid-crystalline phase.

Arrhenius plots of ATPase activity of Mycoplasma cells, grown in the absence of cholesterol, showed discontinuities at temperatures corresponding to the transition phase of the membrane lipids; there were no discontinuities for cells grown in the presence of cholesterol (Rottem et al., 1973). These observations support the suggestion (Overpath et al., 1970) that hydrocarbon chains of membrane lipids must be in a fluid state if transport and catalytic functions are to proceed normally and that the major function of sterols is to maintain this membrane fluidity over a wide temperature range.

### 1.6.2 Photoregulation

Despite the multitude of literature on carotenoid photo-

regulation, which includes common terpenoid pathway steps involved in sterol synthesis (1.2.3.iii), there is very little evidence of a similar effect within sterol biosynthesis (nor in any other lipids).

In the fungus A.giganteus mut. alba it has been shown that total lipid extract levels are increased, by up to 10 fold, in light-grown tissues. More detailed analysis revealed that this increase is seen in all the major lipid classes i.e the sterols, phospholipids, fatty acids and acylglycerols (El-Jack, personal communication). In developing wheat seedlings it was seen that the concentrations of acyl glycerols increase in the chloroplasts with increasing light levels (Lechowicz et al., 1986). However, further reports implying lipid photoinduction are rare and deeper investigations, especially in A.giganteus mut. alba where the phenomenon appears to be a blue-light effect (El-Jack, personal communication) should prove interesting.

### 1.7 Sterol Biosynthesis

Sterols are a group of triterpenes within the terpenoid family. As with the carotenoids and the GAs the initial steps in the biosynthesis are common to the terpenoid pathway and in the case of the sterols this is as far as FPP (1.1.1). FPP is then condensed with another FPP molecule to form the PSPP intermediate which then forms squalene (1.1.3), the first triterpene formed and committed to sterol biosynthesis.

### 1.7.1 Biosynthesis of Lanosterol

Squalene could be converted to lanosterol (lanosta- $\Delta^{8,24}$ -dienol) and cholesterol by rat liver homogenate, requiring NADPH and  $O_2$  (Tchen and Bloch, 1957). It was confirmed that squalene 2,3-oxide was an intermediate (Corey et al., 1966) and later it was shown that the second reaction occurs anaerobically (Dean et al., 1967). The cyclization of squalene 2,3-oxide to lanosterol has been demonstrated in cell-free systems from *P. blakesleeanus* (Mercer and Johnson, 1969) and *Cephalosporium caerulens* (Kawaguchi et al., 1973) indicating that the sequence may be similar in all fungi (Fig 1.12).

### 1.7.2 Biosynthesis of Ergosterol

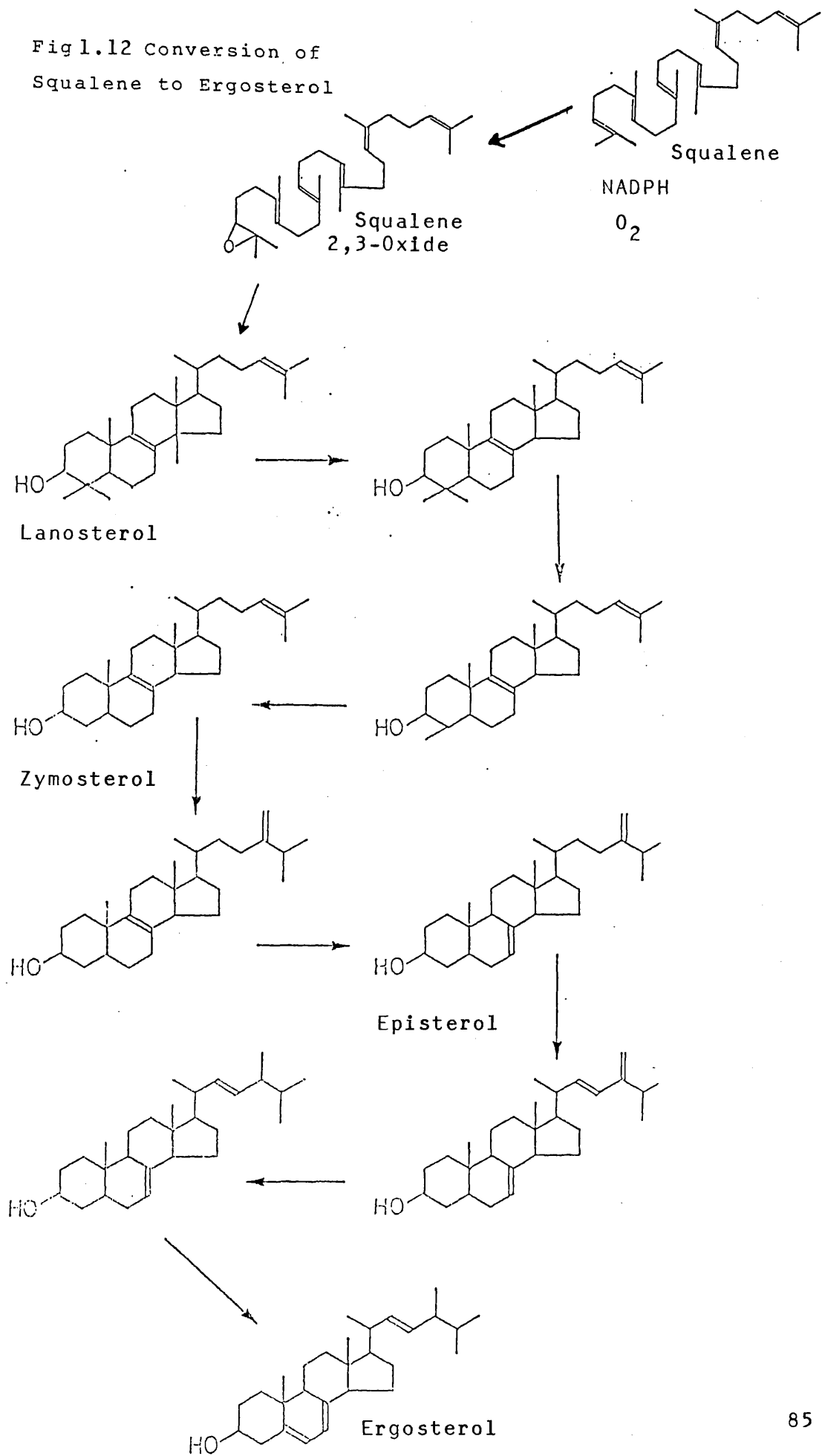
The major sterol in most fungi is ergosterol (ergosta- $\Delta^{5,7,22}$ -trienol; Weete, 1973) which was first shown to be produced from lanosterol in yeast (Schwenk and Alexander, 1958). The conversion involves 5 reactions:

- a) loss of the methyl groups at C-4 and C-14
- b) reduction of the C=24 bond
- c) formation of a 5,6-double bond and isomerization of the 8,9- to a 7,8-double bond
- d) methylation at the C-24
- e) formation of a C=22 bond.

In the formation of the principle 4-desmethyl sterols, the same steps are present in most organisms but quantitatively important intermediates and the reaction sequence differ, not only between major taxonomic groups, but also between tissues and developmental stages of the same organism (Weete, 1973). This leads to difficulties in interpretations of experiments in which proposed intermediates are incorpor-



Fig1.12 Conversion of Squalene to Ergosterol



ated into products due to the lack of specificity of the enzymes involved. Despite this, a major route for ergosterol production has been proposed for yeast (Fryberg et al., 1973; Fig 1.12).

Several nystatin-resistant mutants (a polyene antibiotic which complexes with membrane sterols; Kinsky, 1967) were isolated from yeast (Trocha et al., 1974). Each of these involved only single mutations suggesting that the later stages of ergosterol biosynthesis are a combination of 5 basic processes, which may occur in any order, although some routes are quantitatively more important.

The ~~formati~~ formation of zymosterol (cholesta- $\Delta^{8,24}$ -dienol) by a yeast enzyme preparation is inhibited by CO (Alexander et al., 1974) suggesting that demethylation reactions involve a microsomal, mixed-function oxidase and cytochrome P-450 (Gaylor, 1974).

The C-24 methyl group of ergosterol is derived from methionine (Alexander et al., 1957) via S-adenosyl methionine (SAM; Parks, 1968). When a mutant of N.crassa was grown on deuterated SAM only 2,  $^2\text{H}$  atoms were found on the C-24 methyl group of ergosterol (Jaureguiberry et al., 1965). Since the conversion of lanosterol to ergosterol also involves the migration of a H from C-24 to C-25 (Akhtar et al., 1967) it is probable that the methyl group is transferred to the  $\Delta^{24(25)}$ -double bond forming a carbonium ion which migrates from C-25 to C-24 and loses a H to form the 24-methylene sterols e.g episterol (Russell et al., 1967) this may then be reduced to 24-methyl sterols (Goulston and Mercer, 1969).

The  $\Delta^{7(8)}$ -bond is formed by the reversible isomerization of the  $\Delta^{8(9)}$ -bond (Fryberg et al., 1973), while the intro-

duction of the  $\Delta^5$ -double bond probably involves a mixed-function oxidase since it requires  $O_2$  (Frantz et al., 1959) and is inhibited by cyanide (Dempsey, 1969).

The introduction of the 22,23-trans double bond involves elimination of the 22-pro-S and 23-pro-S H atoms in Aspergillus fumigatus (Bimpson et al., 1969); this probably occurs after the reduction of the  $\Delta^{24(25)}$ -bond since sterols with the conjugated  $\Delta^{22,24(25)}$ -double bond system have not been identified in plants or fungi (Weete, 1973).

### 1.7.3 Esterification of Sterols

Steryl esters are found to represent about half the total sterol content in P.blakesleeanus mycelia (Bartlett and Mercer, 1974). At most stages of growth the same range of fatty acids is found within the steryl esters (triacyl glycerols and phospholipids) but there are quantitative differences in the type of fatty acid esterified to the sterols (and to glycerol; Mercer and Bartlett, 1974). Furthermore, the proportion of each sterol in the free and esterified form, varies widely with the stage of growth, indicating that esterification is not a random process (Bartlett and Mercer, 1974).

P.blakesleeanus grown on starvation medium showed no breakdown of the steryl esters, indicating that they do not serve as energy reserves (Bartlett and Mercer, 1974). Competition between esterification and demethylation of 4 $\beta$ -methyl sterols has been observed in cell extracts of rat liver (Brady and Gaylor, 1971), implying that esterification may serve a regulatory function towards lanosterol metabolism (Grunwald, 1975).

#### 1.7.4 Sterol Carrier Proteins

All early precursors of the sterols, up to FPP, are water soluble, but subsequent intermediates and sterols themselves are lipophilic. Washed rat liver microsomes exhibited low enzymic activity to reduce cholesta- $\Delta^{5,7}$ -dienol to cholesterol (Ritter and Dempsey, 1970), but the same workers showed that it could be enhanced by addition of a heat-stable, non-dialyzable, trypsin-sensitive factor from a 100000g supernatant fraction. It was partially purified and termed sterol carrier protein (SCP).

SCP from rat liver cells was found to enhance squalene biosynthesis in microsomal preparations from yeast (Rilling, 1972) and an analogous protein was isolated from the yeast.

3 Distinct carrier proteins, SCP<sub>1</sub>, SCP<sub>2</sub> and SCP<sub>3</sub>, involved in the cyclization of squalene; the C-4 demethylation and the  $\Delta^{7(8)}$ -bond reduction respectively, were isolated (Scallen et al., 1975). Evidence for SCPs involved in other steps between squalene and cholesterol was also obtained. These workers also suggested that since many other reactions in lipid metabolism are stimulated by the presence of a 100000g supernatant fraction, carrier proteins may be involved in other reactions involving water-insoluble proteins.

#### 1.8 Intracellular Sites of Terpenoid Biosynthesis

It has been observed that throughout growth the  $\beta$ -carotene concentration in the cell vacuoles of P.blakesleeanus remained constant, whereas the concentration in the lipid globules increased (Riley and Bramley, 1982). The lipid globules were suggested to be either the site of biosynthesis, or the primary accumulation site prior to metabolism into retinol and trisporic acid; the lipid globules have been

implied as the carotenogenic site in Mucor hiemalis (Herber, 1974) and Peredinium foliaceum (Withers and Haxo, 1978).

A number of cell-free systems have been obtained capable of synthesizing phytoene (reviewed by Bramley, 1985) including fungi, such as P.blakesleeanus (Bramley and Davies, 1975) and N.crassa (Spurgeon et al., 1979); plants, such as tomatoes (Anderson and Porter, 1962) and spinach (Subbarayan et al., 1970) and bacteria such as Flavobacterium R1560. In the majority of cases a particulate fraction is necessary, thereby suggesting that phytoene synthetase is membrane bound, while the previous enzymes are soluble (Gregonis and Rilling, 1974); the enzyme appears to be soluble in Halobacterium cutirubrum (Kushwaha et al., 1976). However, phytoene synthetase in P.blakesleeanus is only partially pelleted at 105000g and can be almost entirely released from the membrane with high molarity salts, suggesting that it is in fact a loosely bound, peripheral membrane protein (Bramley and Taylor, 1985). The desaturase and cyclase activities in carotenogenesis were shown to be tightly bound to the membrane by the same workers.

In GA biosynthesis, cell-free systems for ent-kaurene production from MVA, have been developed in G.fujikuroi (Evans and Hanson, 1972) and a number of higher plant systems (1.5.1) which show these early pathway enzymes are soluble and located in the cytosol. It has further been shown that the enzymes between ent-kaurene and GA<sub>12</sub>-aldehyde hydroxylation products, in the same systems, (reviewed by Coolbaugh, 1983), are microsomal, while the later steps of GA biosynthesis are catalyzed by enzymes located in the cytosol.

It is not known if the GAs, which are readily released from

the fungus into the surrounding medium (Jefferys, 1973), are secreted actively or by general diffusion. It is possible that the later membrane based steps culminate in the formation of secretion vesicles to aid transport from the cells (Stoddart, 1983).

In sterol biosynthesis, as for the other terpenoids, FPP synthesis is cytosolic, but then squalene synthetase is found to be microsomal in most sources so far examined (Popjak and Agnew, 1979) as are the biosynthetic steps between squalene and ergosterol: squalene synthetase appears to be soluble in P.blakesleeanus (Bramley, 1973)

The early, common terpenoid biosynthetic steps, are cytosolic involving soluble enzymes. The points at which the pathways diverge, to produce the sterols, GAs and carotenoids say, the enzymes involved become membrane bound. Furthermore, it is suggested that the first, branch-point enzymes are peripheral, loosely bound membrane proteins i.e squalene synthetase, phytoene synthetase and kaurene synthetase (1.1.3, 1.1.4 and 1.5.1). This compartmentization within the overall terpenoid biosynthesis, may prove an important regulatory feature (Fig 1.13).

### 1.9 Quinones

Quinones are found chiefly in higher plants, fungi and bacteria and in the animal kingdom in arthropods and echinoderms. Their appearance in other phyla is rare apart from the widely distributed "bioquinones" involved in cellular respiration and photosynthesis. Brief mention should be made of this latter group, especially the ubiquinones which, as their name implies, are extremely ubiquitous in nature. These



metabolites include in their structure an isoprenoid side chain, of varying unit-length (6-10) depending on source. These will be found in fungi involved in respiration and indeed coenzyme Q<sub>10</sub> has been isolated from G.fujikuroi ATCC 12616 (Lavate and Bentley, 1964). Consequently they are included in the terpenoid biosynthetic pathway, and although not investigated in these studies, should be borne in mind when considering the whole pathway (Fig 1.1).

Anthraquinones (not derived from the terpenoid pathway) comprise the largest group of quinones found in fungi, lichens and higher plants and also in bacteria and animals (reviewed by Thomson, 1971). The naphthaquinones (not from terpenogenesis) are more sporadically distributed and, in contrast to the anthraquinones, there are none in Penicillia and only one representative in the Aspergilli; Fusarium spp. elaborate a small group of naphthaquinones. The third major group of quinones, the benzoquinones are common to higher plants and fungi, with some representatives in the animal kingdom e.g coenzyme Q. Other groups of quinones occur with limited members and wide-ranging occurrence, however, this study is only concerned with a few fungal quinones which are not found in these classes.

#### 1.9.1 Functions

As indicated, the naturally occurring quinones are extremely ubiquitous and can range from pale yellow to almost black in colour. However, though they are widely distributed and exist in large numbers with a great structural variety, they make relatively little contribution to natural colourings, compared to say, the carotenoids or anthocyanins.



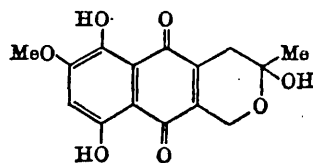
Instead they display a range of different functions, depending on their source; in Fusarium spp. a number of quinones and their derivatives are produced which have been shown to possess toxic properties. One such quinone is 0-demethylanhydrofusarubin (Fig 1.14), a naphthaquinone that has been isolated from F.javanicum (Arnstein et al., 1945), F.solani (Ruelius and Gauhe, 1950), F.martii (Pfiffner, 1963), F.oxysporum (Carlile, 1965) and F.monoliforme (Cross, et al., 1973). This compound has been shown to have entomopathogenic properties (Cole et al., 1981). Whether this is the primary function of this particular quinone is uncertain, but G.fujikuroi does produce a number of mycotoxins which have been shown to affect plants, insects and bacteria (Thomson, 1971), echinoderms (Singh, 1967), man and other animals (though here they are not prominent pathogens and generally occur as either colonisers of tissues damaged by other organisms or trauma, or as systematic invaders in hosts who are compromised in their resistance by, for example, immunosuppressive drug treatment).

#### 1.9.2 Biosynthesis

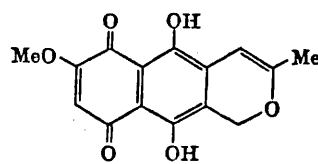
Like other key, secondary metabolites, the quinones are derived from a few key intermediates, principally acetate, shikimate and mevalonate. The biogenesis of the naphthaquinones has received relatively little attention but the structures of a number of compounds (including fusarubin and derivatives) were correctly predicted on the basis of acetate biogenesis (Birch, 1954). 0-Demethylanhydrofusarubin is considered to be derived from the acetate-malonate pathway via a polyketide intermediate (Fig 1.13;

Fig 1.14 Some Quinones found in G.fujikuroi

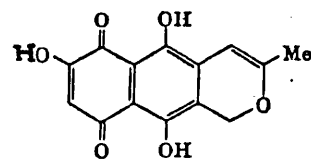
Polyketone chain  
built up from  
acetate, and  
possible synthetic  
route of quinones



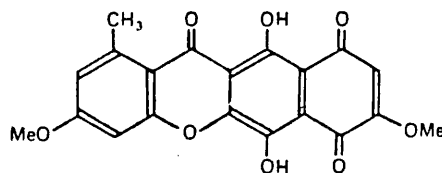
Fusarubin



Anhydrofusarubin



O-Demethyl  
anhydrofusarubin



Bikaverin

Thomson, 1971). These reactions are entirely analogous to those of the fatty acid biosynthetic pathway except there is no reduction of the ketones, as is seen in the latter.

In fungi, fatty acid biosynthesis is mediated by a single, multi-enzyme complex (Lynen, 1967). It is likely that polyketide biosynthesis involves a similar multi-enzyme complex and, indeed, a 6-methyl salicylic acid synthetase has been isolated and purified from Penicillium patulum (Dimroth et al., 1970).

Assuming that polyketide synthetases are distinct from the fatty acid synthetase and not simply degenerate forms of it which have lost their reducing capacity, it is possible that they may have evolved through gene duplication of those genes whose products form the fatty acid synthetase, with subsequent mutation of the second copy. It would also be interesting to know whether there is any link between fatty acid formation and polyketide biosynthesis. Certainly a number of species of Fusarium are known to accumulate quite large quantities of lipids (Table 1.4).

Table 1.4 Production of Lipids by Fusarium.

Species	Lipid (% w/w)
<u>F.bulbigenum</u>	50
<u>F.graminearum</u>	31
<u>F.lini</u>	35
<u>F.lycopersici</u>	35-40
<u>F.oxysporum</u>	29-34

(Ratledge, 1982)

In a study of the lipids from 15 species of Fusarium it was

shown (Gorbik et al., 1980) that some species produced as much as 35-50% of their dry weight as lipid, and that of this lipid 70-80% was in the form of triglycerides. These high fatty acid levels do suggest a possible link with the polyketide pathway, which is also highly active in Fusarium, and further provoke considerations over functions: are high levels a result of a high activity in the common pathways to produce necessary polyketides, or are the latter unimportant bi-products of a mutagenically derived pathway resulting from large fatty acid requirements?

### 1.9.3 Regulation

O-Demethylanhydrofusarubin is a product of secondary metabolism. Generally, secondary metabolism in the filamentous fungi is complex and many strains are able to produce numerous metabolites, often involving quite different pathways under apparently the same growth conditions (reviewed by Campbell, 1984). They may in fact be produced sequentially, either because they represent different stages in a single biosynthetic pathway, or because the production of each is controlled by the physiological status of the fungus, which itself is time dependent in a batch culture. Consequently, determining regulation of a quinone, such as O-demethylanhydrofusarubin in G.fujikuroi is difficult, coupled to the production of other metabolites, and uncertainty of function.

However, the production of bikaverin, an anthraquinone and a polyketide metabolite requiring both malonyl and acetyl CoA and possessing a marked and reasonably specific anti-protozoal activity (Balan et al., 1970) along with a vacuolation effect on many fungi, being termed a vacuolation factor

(Robinson et al., 1969), was studied in G.fujikuroi (Bu'Lock et al., 1974). From these studies the essential conclusion was that bikaverin production is initiated by a limitation in the availability of the N source (as in the GAs 1.4.3.i and carotenoids 1.2.3.i). But a greater understanding of such quinone regulation will only come with a fuller insight into the polyketide pathway.

#### 1.10 Gibberella fujikuroi (Fusarium monoliforme)

G.fujikuroi is a member of the Ascomycetes class, the higher, or true fungi. G.fujikuroi is the perfect state or anamorph of this fungi, while F.monoliforme is the imperfect state or telomorph. The fact that there are 2 forms of the organism has complicated the taxonomy since each form has been classified separately, with the imperfect form being classified within the class Deuteromycotina, the Fungi Imperfecti which have lost the sexual cycle.

One form of classification which leads to the least confusion involves identical grouping until reaching the genera where the perfect form is in the Gibberella genus, while the imperfect form is placed in the Fusarium genus. (Table 1.5)

#### Table 1.5 Taxonomy of G.fujikuroi (F.monoliforme)

Kingdom: Plant

Division: Mycota

Sub-division: Eumycotina

Class: Ascomycetes

Higher or true fungi; mycelium usually septate; vegetative reproduction often by conidia (or by budding); sexual reproduction involves ascospores; ascospores produced within asci.

Sub-class: Euscomycetidae

Asci produced in ascocarps mostly from ascogenous hyphae asci typically unitunicate, if bitunicate then borne in apothecium; ascocarps of various types.

Series: Prenomycetes

Ascocarp closed, mostly ostiolate.

Order: Hypocreales

Ascocarps and stromata, if present, brightly coloured, soft, fleshy or waxy.

Family: Nectriaceae

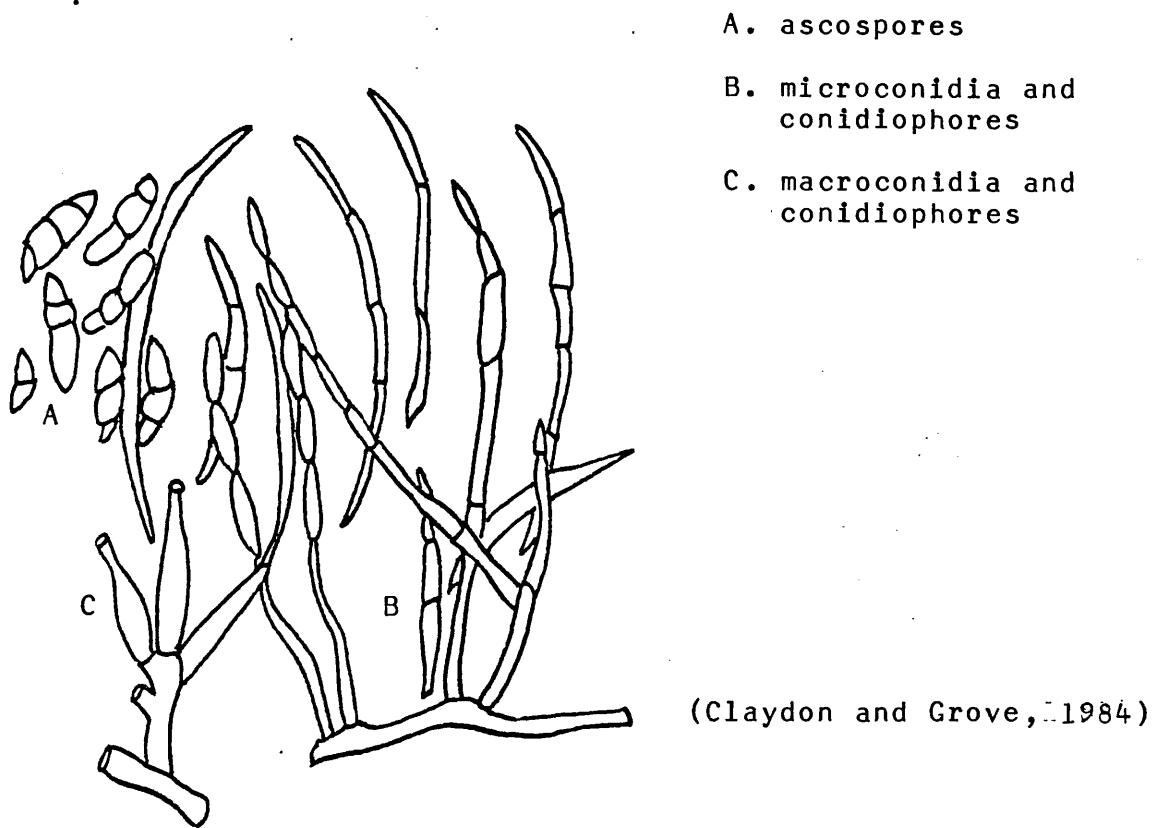
Perithecia discrete or formed superficially in groups on flat subicular stroma usually red or orange. Perithecium has an ostiole with periphyses and contains pseudoparaphyses. The ascospores are hyaline and two-celled.

Genus: Fusarium = Genus: Gibberella  
Section: Liseola Gibberella fujikuroi  
Fusarium moniliforme

F.moniliforme produces both macro- and micro-conidia, which is unusual among fungi, however, the former are more rare with the microconidia being produced as chains, 5-12 in length, 1.5 by 2.5µm in size (Fig 1.15).

It is a major parasite of many hosts such as rice, sugarcane, maize and sorghum causing seedling blight, scorch, foot rot, stunting and hypertrophy. This is especially a problem in rice, causing "bakanae" disease or "foolish seedling" disease, the most serious disease of rice in Asia. Such diseases are a result of growth stimulating hormones, the gibberellins (GAs), produced in large amounts by the fungus, though harmless to the organism itself.

Fig 1.15 G.fujikuroi (F.monoliforme)



#### 1.10.1 Mutants of G.fujikuroi

A number of mutants of *G.fujikuroi* were obtained using UV radiation and N-methyl-N'-nitro-N-nitroso-guanidine as mutagens on the uninucleate microconidia (Avalos et al., 1985). The drug was more efficient at producing mutants which were selected by the mutagenic effects on the fungal colony size, surface texture, colour and overall morphology (Plate 1.1a and b). Those mutants which were available for characterization are classified, along with initial characterizations in Table 1.6.

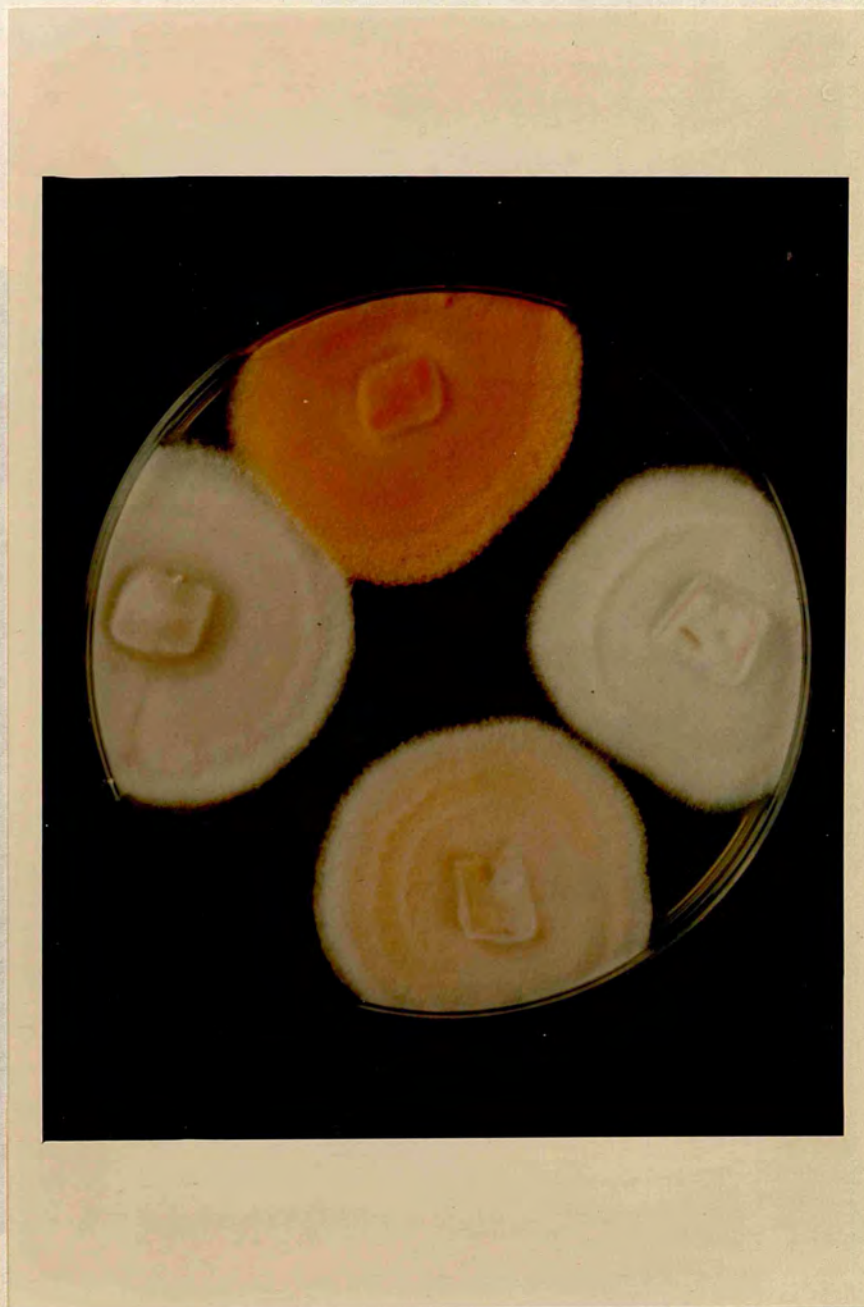
#### 1.11 Aims

The main aims of this work centred around the character-

Plate 1.1a Light-Grown G.fujikuroi Mutants

SG43

SG22



Wild Type

SG78



Plate 1.1a Light-Grown G.fujikuroi Mutants

SG68

SG4



SG75

SG76



Plate 1.1b Dark-Grown G.fujikuroi Mutants

SG43

SG22



Wild Type

SG78



Plate 1.1b Dark-Grown G.fujikuroi Mutants

SG68

SG4



SG75

SG76

Table 1.6 Phenotype of G.fujikuroi Mutants

Strain Designation	Phenotype of Strain
S1	Wild type strain of <u>G.fujikuroi</u> IMI58289; orange when grown in light, white in dark.
SG4	White mutant lacking carotenoids and, reportedly, GAs, probably blocked between FPP and GGPP.
SG 22	Deep orange mutant, producing the same carotenoids as S1 but increased levels in light and dark grown cultures.
SG43	White mutant accumulating phytoene only in light, probably blocked in phytoene dehydrogenase.
SG48	Orange mutant; orange at 30°C, at 23°C pale orange, both in dark, a temperature dependent mutant affected in photo-regulation.
SG68	Red mutant accumulating torulene, blocked between torulene and the end-product carotenoid, NX.
SG75	Mutant synthesizing GAs but not carotenoids so probably blocked between GGPP and phytoene i.e prephytoene synthetase or phytoene synthetase.
SG76	Mutant analogous to SG75.
SG78	White mutant accumulating high amounts of phytoene, so like SG43 blocked in phytoene dehydrogenase, but further affected in regulation levels, as SG22.

SG4, SG22, SG43 and SG48 were derived from S1

SG68, SG75, SG76 and SG78 were derived from SG22

ization of a number of mutants of G.fujikuroi (Table 1.6). These mutants were to be examined more closely within the terpenoid pathway i.e the carotenoids, GAs and sterols, including the earlier pathway, common intermediates: the aims included developing appropriate systems for such

analyses.

From this it was intended to establish a terpenoid pathway for G.fujikuroi, especially in the carotenogenic pathway since this is where most structural mutations are located.

In conjunction with this work the mutants were to be analyzed with respect to regulation of the terpenoid biosynthetic pathway. Photoregulation is known to occur in carotenogenesis in a number of fungi (1.2.3.iii). It was intended to study this phenomenon in G.fujikuroi and to further determine if these effects could be seen in the rest of the terpenoid pathway e.g GA and sterol biosynthesis for which photoregulation studies are extremely limited (1.4.3.iii and 1.6.2).

Aims also included examining other regulatory mechanisms towards the terpenoid pathway as a whole in G.fujikuroi, such as media effects and age of cultures.

CHAPTER II  
MATERIALS AND METHODS

"Though this be madness, yet  
there is method in it"

W.Shakespeare

## 2.1 Materials

### 2.1.1 Chemicals

Laboratory chemicals, including growth media components, were purchased from Sigma, Poole, Dorset; Oxoid Ltd. Basingstoke, Hants; BDH Chemicals Ltd. Poole Dorset; Difco, Detroit, Michigan USA. They were of analytical grade wherever possible.

Analytical grade solvents were used throughout, purchased from various suppliers, except for use in high performance liquid chromatography (HPLC) where HPLC-grade methanol and water were used, obtained from May and Baker Ltd. Dagenham, Kent.

Neutral alumina for column chromatography was purchased from Woelm GmbH, Eschwege, West Germany and Supplied by Koch-Light Ltd. Haverhill, Suffolk.

Kieselgel G for thin layer chromatography (TLC) plates and ready prepared Kieselgel G (Merck 5735) and neutral alumina (Merck 5550) plates, were supplied by BDH Ltd. Poole, Dorset.

The GAs, GA<sub>4</sub> and GA<sub>7</sub> were kindly donated by Dr P.Hedden, Long Ashton Research Station, Bristol. GA<sub>13</sub> and GA<sub>14</sub> were gifts of Professor M.Murofushi, Dept. of Agricultural Chemistry, Japan. GA<sub>9</sub> was a gift of Dr G.Sandmann, Fakultat Biologie der Universitat Konstanz, West Germany.

The HPLC column was a reverse phase, Spherisorb S50DS column from Hichrom C.B.Scientific Ltd. Berks.

Geranylgeraniol was kindly donated by Professor G.Pattenden, Dept. of Chemistry, University of Nottingham.

### 2.1.2 Radiochemicals

[<sup>3</sup>H]-Kaurene and non-tritiated kaurene, and [<sup>3</sup>H]-kaurenoic

acid were gifts of Dr D.Lawrence, ICI Plant Protection Division, Jealott's Hill, Bracknell, Berks.

R-[2-<sup>14</sup>C]-MVA lactone (50mCi/mmol) was supplied by Amersham, Bucks.

### 2.1.3 Organisms

Gibberella fujikuroi (Fusarium moniliforme) CMI 58289, was obtained from the Commonwealth Mycological Institute, Kew.

G.fujikuroi mutants of CMI 58289, designated SG4, SG22, SG43, SG48, SG68, SG75, SG76 and SG78 were all kindly donated by J.Avalos, Departamento de Genetica, University of Seville, Spain.

G.fujikuroi ACC 917 was obtained from ICI, Plant Protection Division, Jealott's Hill, Bracknell, Berks.

Neurospora crassa ACC 10816 and strains 4014 and 911, were obtained from the Fungal Genetics Stock Centre, College of Health Sciences, University of Kansas, USA.

Phycomyces blakesleeanus C5 carB10(-) and C115 carS42 mad-107(-), were donated by E.Cerda-Olmeda, Departamento de Genetica, University of Seville, Spain.

Aspergillus giganteus mutant alba strain 101.64, was obtained from the Centrealbureau voor Schimmelcultures, Baarn, The Netherlands.



## 2.2 Methods

### 2.2.1 Growth and Maintenance of Organisms

i) G.fujikuroi and mutants were maintained on a nutrient broth agar (Avalos et al., 1985) termed 'Spanish Agar', which contained per litre of distilled water:

Glucose	30g
NaNO <sub>3</sub>	3g
KH <sub>2</sub> PO <sub>4</sub>	1g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.5g
KCl	0.5g
H <sub>3</sub> BO <sub>4</sub>	10µg
CuSO <sub>4</sub>	100µg
FeCl <sub>3</sub>	200µg
MnCl <sub>2</sub>	20µg
MoO <sub>4</sub> .Na	20µg
ZnSO <sub>4</sub>	2mg
Yeast Extract (Difco)	4g
Peptone (Bacto-tryptone Difco)	8g
Agar	16g

After 7 days growth, the agar slopes were stored at 4°C and subcultured every 3 months.

Liquid media used for liquid shake cultures were either termed 'Spanish Medium' (Avalos et al., 1985) and designated 'DG', or 'American Medium' (Schechter and West, 1969) designated 'AM'. DG was as Spanish Agar but without the yeast extract, peptone or agar. AM contained per litre of distilled water:

Glucose	80g
KH <sub>2</sub> PO <sub>4</sub>	1g
MgSO <sub>4</sub>	0.5g
NH <sub>4</sub> NO <sub>3</sub>	1g
FeSO <sub>4</sub> .7H <sub>2</sub> O	2.5mg
CuSO <sub>4</sub>	375µg
MnSO <sub>4</sub>	250µg
ZnSO <sub>4</sub>	3.8mg
Ammonium molybdate	250µg

Before inoculation into liquid medium, the cultures were grown on a special carbon-limiting medium designed to produce large numbers of conidia and termed 'Conidia Agar' (Avalos et al., 1985). This contained per litre of distilled water:

Yeast Extract	1g
$\text{NH}_4\text{NO}_3$	1g
$\text{KH}_2\text{PO}_4$	1g
$\text{MgSO}_4$	0.5g
Agar	16g

This agar (2ml) was dispensed into small bottles (5ml) and inoculated from stocks. Conidial cultures were grown for 5-7 days in the dark at 30°C. To inoculate shake cultures, these slopes were suspended in sterile water (3ml) and the conidia were gently suspended with a sterile loop. This suspension (1ml) was used as a routine inoculum of the liquid media (500ml): one loop of inoculum was used to re-inoculate a fresh agar slope.

ii) N.crassa and mutant strains were maintained on a special 'Conidiating Agar Medium' (Frost, 1962) which contained per litre of distilled water:

Ammonium Tartrate	5g	} i.e Frie's Minimal Medium
$\text{NH}_4\text{NO}_3$	1g	
$\text{KH}_2\text{PO}_4$	1g	
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5g	
NaCl	0.1g	
$\text{CaCl}_2$	0.1g	
Glycerol	15g	
Glucose	2.5g	
Malt Extract	2.5g	
Bacto-Peptone (Difco)	0.5g	
Yeast Extract	2.5g	
Agar	20g	

Cultures were grown for 2 days at 30°C and then stored at

room temperature.

The liquid culture medium (Bradford and Gibgot, 1963) contained, in addition to Frie's minimal medium, per litre of distilled water:

Yeast Extract (Difco)	5g
Sucrose	10g

The liquid medium (500ml) was inoculated from the conidial suspension (ca  $2 \times 10^{10}$  conidia) after filtering through glass wool, to remove hyphae and agar.

iii) P.blakesleeanus was maintained on agar slopes of Sutter's medium IV, a sporangiophore medium which contained per litre of distilled water:

Asparagine	2g
Agar	20g
Glucose	20g
Salt Solution Concentrate	20ml

The salt soln. concentrate contained, per litre of distilled water:

$\text{KH}_2\text{PO}_4$	250g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	25g
Thiamine-HCl	100mg
$\text{CaCl}_2$ Soln. 14% (w/v)	10ml
$\text{CHCl}_3$	3ml
Trace Elements Soln.	5ml

The trace elements soln. contained, per litre of distilled water:

Citric Acid	2g
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$	1.5g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	30mg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	300mg
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	50mg

Suspensions of sporangia from 7 day old agar slopes were

used to inoculate liquid shake medium (Than et al., 1972) which contained per litre of distilled water:

Glucose	25g
Yeast Extract (Difco)	500mg
MgSO <sub>4</sub>	500mg
Thiamine-HCl	0.25mg
L-Asparagine	1.25mg
L-Leucine	1.25mg
KH <sub>2</sub> PO <sub>4</sub>	1.5g

iv) A.giganteus was maintained on Wickerham's medium (Wickerham, 1951) which contained per litre of distilled water:

Malt Extract (Difco)	3g
Yeast Extract (Difco)	3g
Mycological Peptone	5g
Glucose	10g

The same medium was used for agar slopes with the addition of agar (2%). The slopes contained agar (ca 10ml) in bottles (ca 20ml) and were inoculated from stock cultures. They were grown in the light at 25°C for 7 days. A conidial suspension was obtained by addition of sterile water (10ml) to a slope: an aliquot of this suspension (3ml) was sufficient for a standard inoculum into shake medium (ca 300ml).

#### 2.2.2 General Growth Conditions

Static, agar plate and slope cultures were generally grown at 25°C and, if light was necessary, 10cm from a bank of fluorescent lights, Sylvania, 60 Watt, F30T12/WW/RS, Canada.

Shake cultures were mainly grown in flasks (21) containing liquid medium (500ml) in a Gallenkamp, shaking incubator at 25°C, shaking at 160 rpm, under a bank of 40 Watt lights, Philips MCFE 15/35-1", producing 48 Wm<sup>-2</sup>, (1.8X10<sup>4</sup> Lux).

Dark-grown cultures were grown under the same conditions with the flasks enveloped in light-tight, black plastic sheets.

The length of time of growth for liquid shake cultures was dependent on the strain being used and the experiment to be carried out, as detailed in the Results sections.

### 2.2.3 Harvesting

Mycelia was harvested from liquid shake cultures by filtering through muslin cloth and squeezing to hand dryness. They were stored at  $-70^{\circ}\text{C}$  or used immediately.

### 2.2.4 Extraction of Total Lipids.

Freshly harvested mycelium was homogenized using a Silverson Laboratory Mixer Emulsifier, Silverson Machines Ltd. Waterside, Chesham, Bucks, twice with methanol (2 vols) and then four times with acetone (2 vols). These extracts were bulked in a light-tight container.

### 2.2.5 Saponification

The 'hot' ( $60^{\circ}\text{C}$ ) method of saponification was found to be more effective than the 'cold' (room temp.) method (Goodwin, 1975) and involved boiling the total lipid extract for exactly 10min in a MeOH:60% KOH soln. (9:1, v/v) in minimal light over a water bath.

### 2.2.6 Isolation of the Carotenoids

Neutral carotenoids were isolated from the alkaline methanol soln. after saponification, by partitioning with  $\text{Et}_2\text{O}$  (3-4 times) until no more colour was removed. The ethereal extracts were washed with water until neutral, bulked and

left to dry over anhydrous  $\text{Na}_2\text{SO}_4$  for no less than 1h at  $4^\circ\text{C}$  in a sealed, dark vessel, under an atmosphere of  $\text{N}_2$ .

The acidic carotenoids were removed from the aqueous phase after removal of the neutral carotenoids by acidifying with  $\text{HOAc}$  (8.6M), to pH 4.0 and partitioning against  $\text{Et}_2\text{O}$  until the epiphase was colourless (5-6 times). The ethereal extracts were washed with distilled water until neutral, bulked and dried over anhydrous  $\text{Na}_2\text{SO}_4$  in the same manner as for the neutral carotenoids. In some cases the aqueous washings had to be re-extracted since they were initially acidic enough to contain acidic compounds.

### 2.2.7 Separation of Carotenoids

#### i) Column Chromatography:

Neutral or acidic carotenoid extracts were applied to a grade III  $\text{Al}_2\text{O}_3$  column in petrol ( $40^\circ\text{-}60^\circ\text{C}$ ); the column was normally 10g of  $\text{Al}_2\text{O}_3$  in a 1.3cm diameter column. Preliminary separation was achieved on this column, the number of fractions and the eluting solvent depending on the sample in question and are detailed in Plate 3.1 and Plate 3.2).

#### ii) Thin Layer Chromatography

Further separation and purification of the carotenoids, after column chromatography, was achieved by TLC. 2 TLC plate systems were developed for the neutral carotenoids:

Kieselgel G was used to separate phytoene and phytofluene using 3% toluene/petrol ( $60^\circ\text{-}80^\circ\text{C}$ ) as a developing solvent. These carotenoids were visualized using UV light by means of the fluorescent dye,  $\text{F}_{254}$ , contained within the gel (the Rfs in Table 2).

Table 2.1 Rf Values of Carotenoids on Different TLC Systems

Carotenoid	A	Rf Value in System		
		B	C	D
$\beta$ -Carotene	-	-	0.88	-
$\beta$ -Zeacarotene	-	-	0.80	-
$\zeta$ -Carotene	-	-	0.65	-
$\gamma$ -Carotene	-	0.78	0.35	-
Neurosporene	-	0.68	0.24	-
Torulene	0.49	-	-	0.44
Lycopene	0.44	0.43	-	0.36
3,4 Dehydro-lycopene	-	-	-	0.10

System:

A; MgO/Kieselgel G (1/1, w/w); Toluene

B; " ; 90% Toluene/Petrol (60°-80°C)

C; " ; 50% "

D; Al<sub>2</sub>O<sub>3</sub>; 10% Toluene/Petrol (60°-80°C)

Table 2.2 Rf Values of Terpenoids on Different TLC Systems

Terpenoid	Rf Value in System			
	E	F	G	H
Kaurene	-		0.73	0.54
Squalene	-	0.43	0.72	0.40
Phytoene	-		0.54	0.30
Phytofluene	-		0.34	
$\beta$ -Carotene		0.42	0.27	0.29
$\beta$ -Zeacarotene	0.85	0.40	0.26	0.27
$\zeta$ -Carotene		0.34	0.25	
$\gamma$ -Carotene	0.57	0.32	0.10	
Neurosporene	0.43	0.27		0.10
Torulene		0.25	-	
Lycopene	0.40	0.24	-	

System:

E; Kieselgel G; 20% Toluene/Petrol (60<sup>o</sup>-80<sup>o</sup>C)

F; " 15% "

G; Al<sub>2</sub>O<sub>3</sub>; 3% Toluene/Petrol (60<sup>o</sup>-80<sup>o</sup>C)

H; Kieselgel G; Petrol (60<sup>o</sup>-80<sup>o</sup>C)





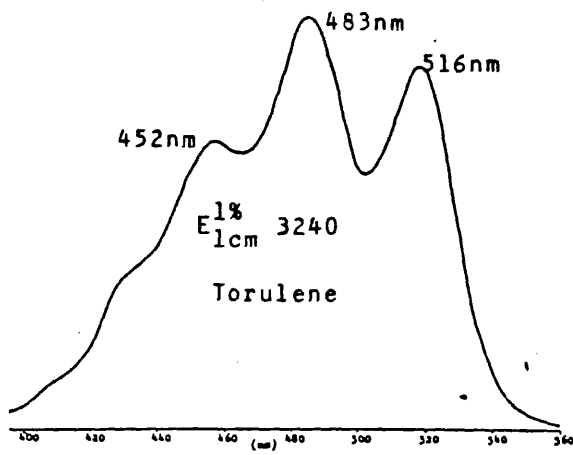
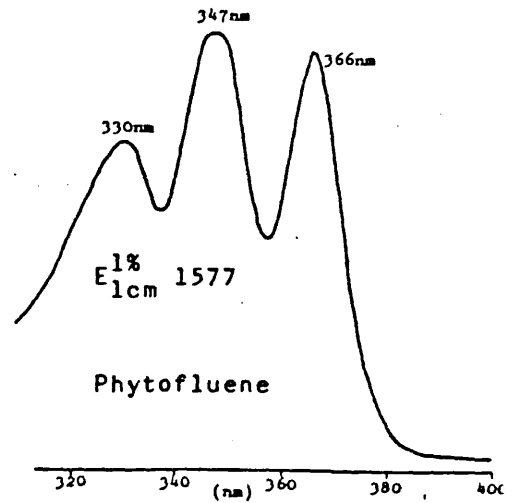
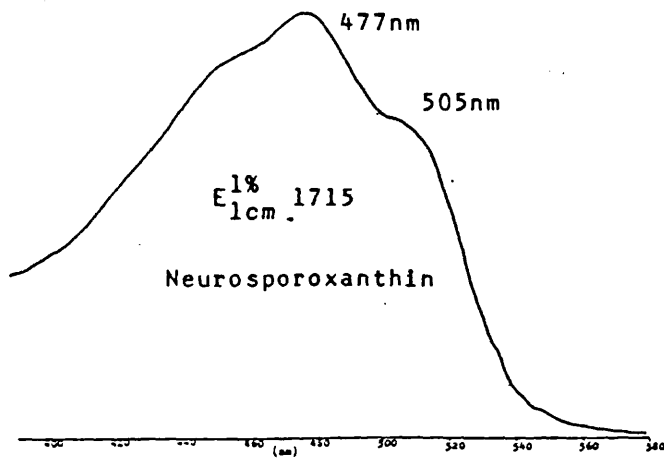
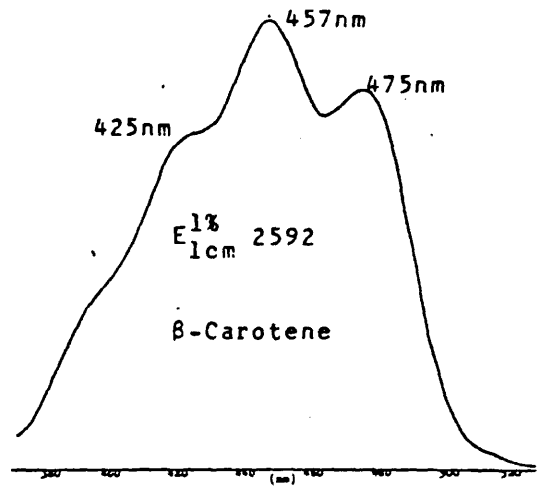
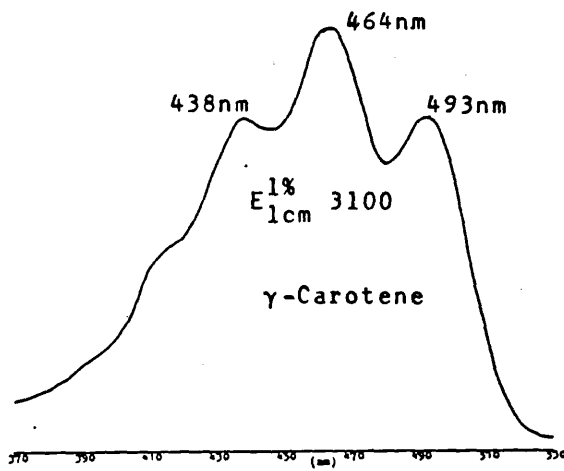


Fig 2.1 Absorption Spectra of Carotenoids in Petrol (40°-60°C)



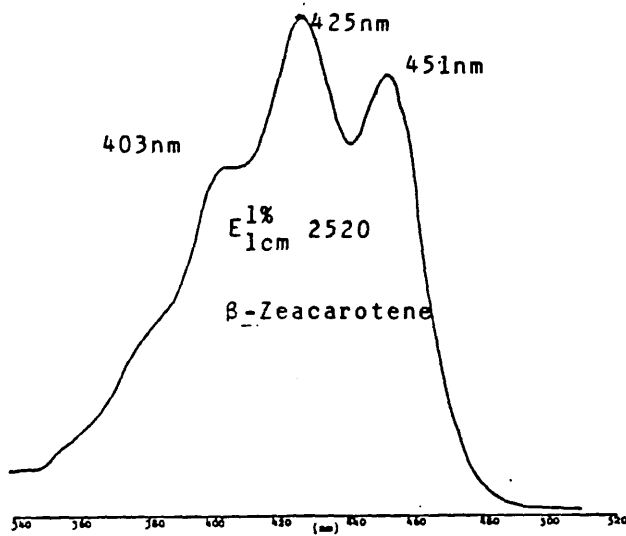
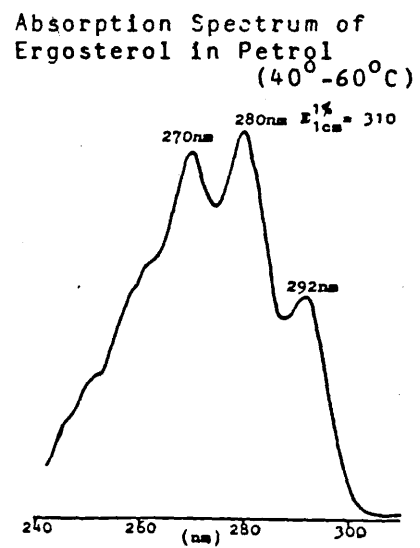
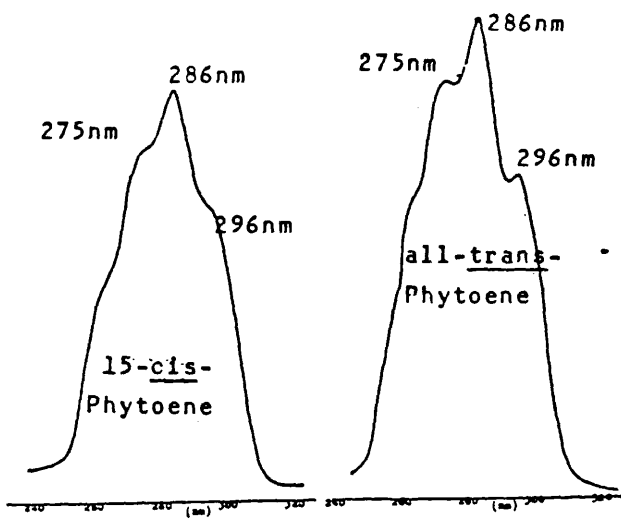
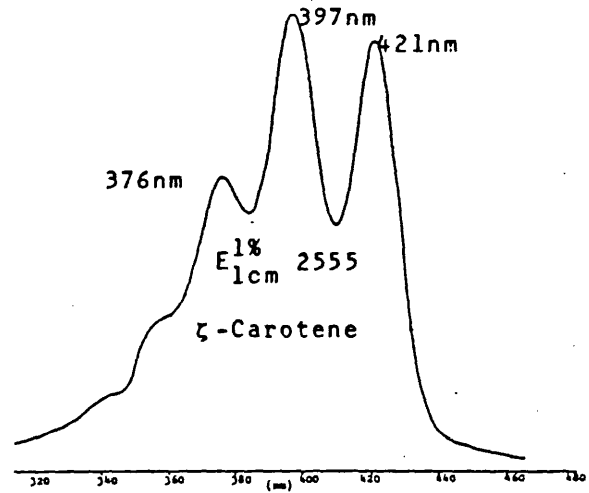
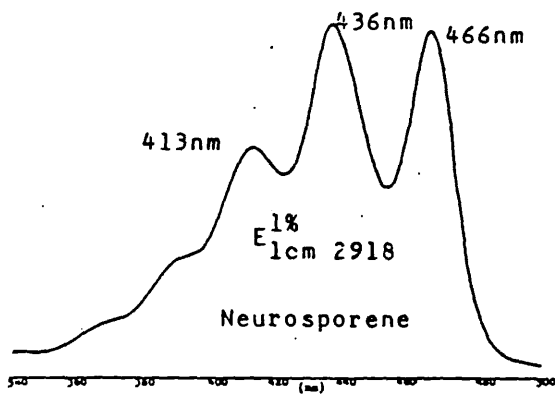


Fig 2.1 Absorption Spectra of Carotengids in Petrol ( $40^{\circ}$ - $60^{\circ}\text{C}$ )



eluted with 2% Et<sub>2</sub>O/petrol (40°-60°C) while the trans-isomer was eluted with 4% Et<sub>2</sub>O/petrol (40°-60°C) (Than et al., 1972).

#### 2.2.10 Precautions for Working with Carotenoids

Carotenoids, being inherently unstable, were handled under careful conditions. All light exposure was kept to a minimum, TLC was carried out in a dark-room as was removal of carotenoids from TLC plates, columns were wrapped in light-proof, black plastic sheets and all container vessels were wrapped in foil. Whenever possible, all carotenoid solutions were kept on ice. Carotenoid solutions stored for long periods of time were kept at -70°C under an atmosphere of N<sub>2</sub>.

#### 2.2.11 Other Lipid Determinations

The total lipid was determined by gravimetric analysis, as was the unsaponified lipid, using a fraction of the saponified extract.

Ergosterol was assayed spectrophotometrically using an  $E_{1\text{cm}}^{1\%}$  of 310 at 281.5nm and a correction for irrelevant UV-absorption using the formula:

$$E_{281.5} = 0.654 (5 \cdot E_{281.5} - 3 \cdot E_{276.5} - 2 \cdot E_{289.0})$$

(Glover, 1964)

#### 2.2.12 GA Extraction

The GAs were extracted using an adapted method (Reeve and Crozier, 1978).

The medium was extracted by adjusting to pH 8.0 using 60% KOH and partitioning (X3) against petrol (40°-60°C).

The aqueous phase was then adjusted to pH 2.5 with 8.6M HOAc and partitioned (at least 5 times) with EtOAc. This

removed the bulk of the free GAs (Durley and Pharis, 1972). The fraction was then dried over anhydrous  $\text{Na}_2\text{SO}_4$  for at least 1h at  $4^\circ\text{C}$ . The fraction was then taken up in a suitable volume of HPLC grade MeOH and passed through a  $0.2\mu\text{m}$  bacterial filter before HPLC.

GAs were extracted from the mycelia by homogenisation with a Silverson homogeniser (2.2.4) using cold MeOH (2 vols, X3). The methanolic extracts were then adjusted to pH 8.0 with 60% KOH and partitioned in the same way as the medium extraction.

Before extraction the medium or mycelial methanolic extracts had an internal standard of sorbic acid introduced (100 $\mu\text{g}$ /500ml of media or 20g of fresh weight tissue).

#### 2.2.13 GA Separation

Separation of the GAs was carried out using HPLC and an adapted method (Barendse et al., 1980; ICI, 1982 and Koshioka et al., 1983).

The instrument consisted of a Pye Unicam LC-XPD pump, an LC-XP Gradient Programmer, an LC-UV detector and a Phillips PM8252 dual-pen recorder. A 'Ramona Raytest', supplied by Lablogic, Sheffield, radioactivity monitor could be used for radiolabelled GA analysis.

The conditions found to be best suited for separation of GAs were:

Solvent flow rate	0.75 ml/min
UV detector wavelength	206 nm
Sample Volume	20 $\mu\text{l}$
Recorder chart speed	1 cm/min
Column	Spherisorb S50DS

The separation procedure consisted of a programmed, gradient

elution, composed of 4 programmed segments:

Segment	Time (min)	Eluent Gradient or Isocratic	Pressure (psi)
0	15	40% MeOH in Phosphoric Acid : Isocratic	187
1	10	40% MeOH in Phosphoric Acid to 60% MeOH in Phosphoric Acid : Gradient (1.0)	187 to 147
2	15	60% MeOH in Phosphoric Acid : Isocratic	147
3	0	60% MeOH in Phosphoric Acid to 100% MeOH : Gradient (1.0)	147 to 67
4	15	100% MeOH : Isocratic	67

The identification of the individual GAs separated was achieved by co-chromatography with authentic standards and RRTs from the literature (see Fig 4.1).

#### 2.2.14 Preparation of a Cell-Free System from G.fujikuroi

The method used to prepare cell-free extracts was an adapted method (Bramley, 1973).

The harvested mycelium was frozen at  $-70^{\circ}\text{C}$  and then lyophilised using a freeze-drier, under about 0.02 mbar vacuum. The freeze-dried material could be stored for up to 1 month at  $-70^{\circ}\text{C}$  in an air tight container containing silica drying bags to maintain anhydrous conditions; after this time cell-free activity became considerably reduced.

The freeze-dried mycelium was rubbed through a laboratory test sieve, 355 $\mu\text{m}$  aperture (Endecotts Ltd. London), onto foil and weighed. It was immediately resuspended, in a 50ml centrifuge tube, with ice-cold 400mM Tris-HCl buffer, pH 8.0 containing 5mM DTT, in a 1:8 (w/v) ratio. The mixture was stirred into a uniform, smooth paste over ice. The prepara-

tion was then centrifuged for 30min, at 4°C at 10000g. The supernatant, termed the S<sub>10</sub>, was used for in vitro incubations to study the terpenoid pathway.

To isolate the soluble enzymes from the mycelium the S<sub>10</sub> was centrifuged for 90min, at 4°C at 105000g. The supernatent, termed the S<sub>105</sub> fraction contained 3 distinct layers (Fig 2.2). Just above the pellet (P<sub>105</sub>) was a grey, turbid layer above which was a larger, clear, straw-coloured layer, whilst floating on the top was a clearer, lipid-like layer which became more opaque on standing. The middle, straw coloured could be separated carefully with a Pasteur pipette, or by puncturing the centrifuge tube at the bottom of the layer and allowing it to flow out, without contamination from other layers.

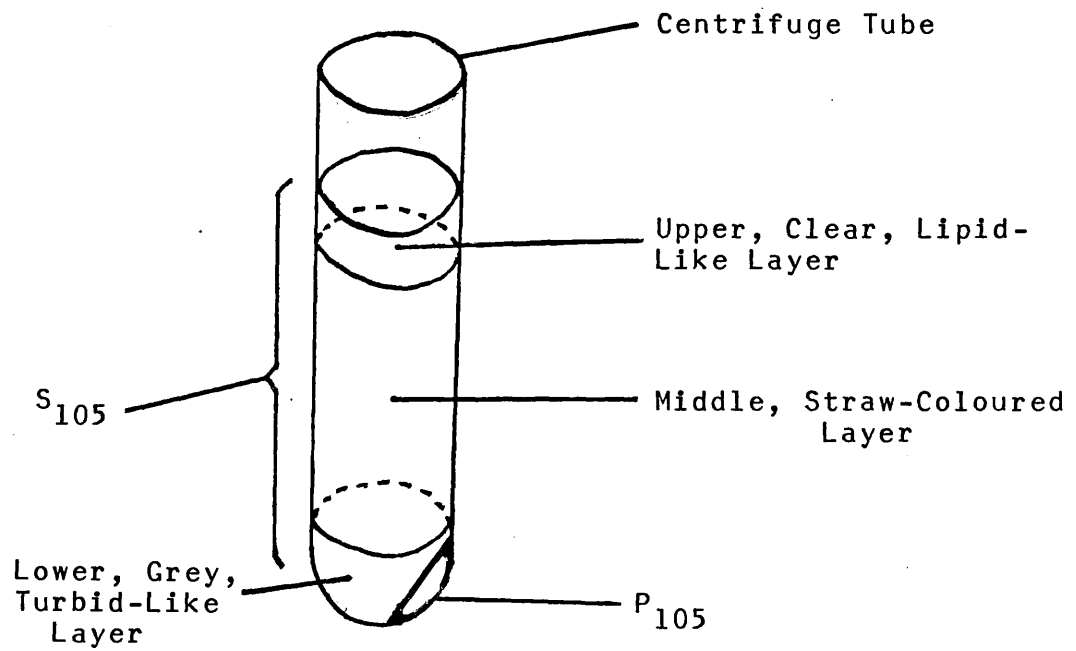
#### 2.2.15 Cell-Free Incubations

Cell-free incubations were performed in tapered, glass centrifuge tubes (10ml) which were wrapped in foil. The standard incubation contained:

R-[2- <sup>14</sup> C]-Sodium mevalonate	0.25μCi
ATP	10μmol
NAD	1μmol
NADP	1μmol
FAD	1μmol
MnCl <sub>2</sub> ·4H <sub>2</sub> O	6μmol
MgCl <sub>2</sub> ·6H <sub>2</sub> O	4μmol
S <sub>10</sub> and S <sub>105</sub> fraction	200μl
KF	10mmol
400mM Tris-HCl buffer pH 8.0 containing 5mM DTT	up to 500μl total vol.

The order of addition of the incubation components was standardised. An appropriate amount of R-[2-<sup>14</sup>C]-MVA lactone

Fig 2.2 Illustration of Centrifuge Tube  
Containing 105000g Preparation of  
G.fujikuroi Extracts





was converted to its sodium salt after evaporation of the containing solvent under  $N_2$ . NaOH (375ng) was added such that the amount of  $Na^+$  was in excess to the  $[2-^{14}C]$ -MVA concentration (5 $\mu$ l). ATP, NAD, NADP and FAD dissolved in 400mM Tris-HCl buffer pH 8.0 containing 5mM DTT (275 $\mu$ l) were added to this followed by the KF (10 $\mu$ l of a 0.325M soln.). Finally a stock soln. (10 $\mu$ l) of 0.2M  $MgCl_2 \cdot 6H_2O$  and 0.3M  $MnCl_2 \cdot 4H_2O$  was added, followed by the cell extract (200 $\mu$ l of  $S_{10}$  or  $S_{105}$ ). The mixture was incubated at 37 $^{\circ}$ C in a covered, shaking water bath for an appropriate period of time (2h unless indicated otherwise).

It was found that the cofactor/precursor soln. could be made up for any number of incubations and dispensed accordingly, ready to receive the cell-free extracts.

Incubations were stopped by placing over ice and adding cold MeOH (1ml).

#### 2.2.16 Phosphatase Assay

The alkaline phosphatase assay used was an adapted method (Bramley, 1973).

It involved the use of pNP as a substrate which produced pNP by phosphatase action. This was assayed using a Beckman 3600 spectrophotometer at 405nm by formation of the yellow coloured pNP. A standard calibration curve of pNP was constructed (Fig 5.1).

The assay was carried out on  $S_{10}$  preparations of mutant SG78 G.fujikuroi CMI 58289, grown on different media and prepared in different buffers (5.3.1).

400mM potassium phosphate buffer pH 7.5 containing 5mM DTT,  
with and without KF

400mM Tris-HCl buffer pH 8.0 containing 5mM DTT, with and  
without KF

These  $S_{10}$  preparations (250 $\mu$ l) were made up to a suitable volume (1ml) with the appropriate buffer and the pNPP (250 $\mu$ l of a 0.1M soln.) was added. The mixtures were incubated at 30 $^{\circ}$ C in a shaking water bath and samples (200 $\mu$ l) taken at appropriate times for assay. These were added to buffer (3ml) to stop the reaction and the absorbance recorded at 405nm. Formation of pNP was plotted against time and the phosphatase activity in each preparation determined (5.3.1)

Standard alkaline phosphatase activity was assayed in a similar manner using a standard alkaline phosphatase solution (1mg/ml). An aliquot of this (250 $\mu$ l) was diluted (made up to 1ml) with the appropriate buffer followed by addition of 0.1M pNPP (250 $\mu$ l).

#### 2.2.17 [ $^{14}$ C]-Labelled Terpenoid Extraction

The extraction procedures for the [ $^{14}$ C]-labelled terpenoids were adapted from the method of (Bramley and Davies, 1975). After the incubation had been stopped, an aliquot of carrier terpenoid (1ml) was added which contained, per 1ml of petrol (40 $^{\circ}$ -60 $^{\circ}$ C):

Terpenoid	Amount ( $\mu$ g/ml)	Source
Phytoene	150	<u>P.blakesleeanus C5</u> C5 <u>carB</u> 10(-)
Phytofluene	trace	"
$\beta$ -Carotene	100	<u>P.blakesleeanus C115</u> <u>carS</u> 42 <u>mad107</u> (-)
Lycopene	100	Ripe tomatoes
Squalene	100	Sigma, Poole, Dorset

The contents of each incubation tube were then washed into a separating funnel (100ml) containing saturated NaCl solution (ca 50ml) with Et<sub>2</sub>O (ca 20ml). The separating funnel was wrapped in black, light-proof cloth and shaken gently. The

hypophase was then discarded (except when studying the intermediate terpenoid pyrophosphates; 6.2.4 ) and the epiphase delivered into a test-tube containing anhydrous  $\text{Na}_2\text{SO}_4$  (ca 1g). The tube was wrapped in foil and kept stoppered, under  $\text{N}_2$  at  $4^\circ\text{C}$  for 1h. The dried solution was washed through a sintered-glass funnel (porosity 3) into a foil-covered, round-bottomed flask (100ml) with  $\text{Et}_2\text{O}$  (10-20ml). This solvent was then evaporated, under vacuum, to a minimal volume (ca 1ml). The solution was then transferred, with washings (X3, 2ml) of  $\text{Et}_2\text{O}$ , into a graduated, conical, glass centrifuge tube (10ml) wrapped in foil and on ice. At this point, a fraction of the sample (1%) could be assayed by liquid scintillation counting, to approximate the total amount of  $^{14}\text{C}$ -labelled incorporation into the terpenoids.

The solution was then evaporated to near dryness (ca 50-100 $\mu\text{l}$ ) under a stream of  $\text{N}_2$ , or by use of a Univamp centrifugal evaporator, supplied by Uniscience Ltd. London. The sample was now ready for separation by TLC.

#### 2.2.18 Separation of Neutral Terpenoids

The terpenoid extract from 2.2.17, was applied onto a TLC plate as a thin band together with  $\text{Et}_2\text{O}$  washes (X2, 50-100 $\mu\text{l}$ ). The TLC plate was 0.2mm thick and 5X20cm and normally Kieselgel G. The developing system depended upon the terpenoids to be separated (Table 2.2).

In most cases the first TLC system was not enough for complete terpenoid separation and purification. Consequently, bands were scraped off into tissue-plugged funnels and eluted with  $\text{Et}_2\text{O}$  (6-10ml) into conical, glass, centrifuge tubes, wrapped in foil over ice. This was carried out with great

care to ensure minimal terpenoid breakdown (2.2.7). The solvent was then taken to a small volume (50-100 $\mu$ l) under N<sub>2</sub> or with a centrifugal evaporator and applied to a second TLC system (Table 2.2).

#### 2.2.19 Visualization of [<sup>14</sup>C]-Labelled Terpenoids

The carrier terpenoids in the incubation extracts were present in sufficient quantity to enable location of the [<sup>14</sup>C]-labelled terpenoids by sight. This was easy with the coloured carotenoids, but squalene, phytoene and phytofluene had to be visualized under UV light or by staining with I<sub>2</sub> vapour. The latter technique involved heating iodine crystals until they began to vaporize and then placing them in a chamber containing the developed plates to be visualized. This method of visualization was only used if no further separation of terpenoids was necessary. Once the markers had been located, care against terpenoid breakdown could be ignored since this would not affect the levels of radioactivity.

An alternative method for locating [<sup>14</sup>C]-labelled incubation products involved the use of a 'spark chamber' (Birchover Instruments, Letchworth, Surrey). This could detect [<sup>14</sup>C] on TLC plates in excess of 1000 dpm/cm<sup>2</sup> which could be recorded by means of a Polaroid camera. This method was used alone or in conjunction with marker location and was particularly useful in identifying incorporation into compounds other than those for which marker was available.

#### 2.2.20 Radioassay

This was achieved by liquid scintillation counting with a Beckman LS7500 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 (3ml) consisting of 2,5 diphenyloxazole (PPO) in toluene (5g/l) and assayed to levels of 2  $\sigma$  % error.

When assaying total terpenoid fractions the Et<sub>2</sub>O sample (max. vol. 200 $\mu$ l) was treated in the same way.

#### 2.2.21 Separation of Other Terpenoids

The method for terpenoid extraction, along with carotenoids, squalene and kaurene, also isolated other terpenoids such as the oxidative products of kaurene i.e kaurenoic acid, kaurenol and kaurenal and the alcohol derivatives of the intermediate prenyl pyrophosphates: the former being intermediates in the GA biosynthetic pathway (1.5.2) the latter, possible artifacts due to phosphatase and pyrophosphatase activities in the cell-free extracts (5.3.1).

All of these [<sup>14</sup>C]-labelled compounds remained at the origin of the first TLC system used in terpenoid separation (system H, Table 2.2 ) and were resolved by a series of additional TLC systems (Table 2.3a and b).

The origin of the first TLC system was quickly scraped off and eluted with Et<sub>2</sub>O (6-10ml) through a plugged, glass funnel over ice and reduced to a minimal volume (50-100 $\mu$ l) in a centrifugal evaporator. This fraction was applied to a second TLC system i.e Kieselgel G developed with toluene: EtOAc (9:1, v/v; Kushwaha et al., 1977). [<sup>14</sup>C]-labelled radioactive bands were visualized using a spark chamber, then

Table 2.3a Rf Values of Radiolabelled Bands from the Origin of System H (Table 2.2), Chromatographed on TLC System I

Band	Rf
Origin	-
1	0.15
2	0.25
3	0.39
4	0.52

System:

I; Kieselgel G; Toluene/EtOAc (9/1, v/v)

Table 2.3b Rf Values\* of Radiolabelled Bands from TLC System I (2.3a), Chromatographed on TLC System J

Band/s from Origin	Rf	Band/s from 1	Rf	Band/s from 2	Rf	Band/s from 3	Rf	Band/s from 4	Rf
A	0.6	B	0.36	D	0.30	F	-*	J	*Origin
		C	0.53	E	0.50	G	0.17		
						H	0.27		
						I	0.40		

System:

J; Kieselgel G, activated\* with 5% paraffin/petrol(60<sup>o</sup>-80<sup>o</sup>C);  
MeOH/Paraffin Saturated H<sub>2</sub>O (4/1, v/v)

Identification of Bands:

A; Geraniol  
 B; Geranylgeraniol  
 C; Kaurenoic Acid  
 D; Kaurenol?  
 E; Farnesol  
 F; Kaurenal?  
 G; Prephytoene Alcohol?  
 H; Unknown  
 I; "  
 J; "

\* The activation of the TLC plate leaves a strip of unactivated Kieselgel G which acts as the origin for sample application (2.2.21). The Rf value is taken from the paraffin-activated origin, not the sample origin.

scraped and eluted in the usual manner. The compounds from this TLC system were then developed on a third, reverse TLC system (Kushwaha et al., 1977). This adapted method comprised of Kieselgel G pre-developed (5% paraffin/petrol (60°-80°C)) such that the developing solvent left a band of normal phase Kieselgel G at the top of the TLC plate (ca 3cm). This strip was then used as the origin for the bands eluted from the previous plate. The reverse phase TLC plate was developed in MeOH:paraffin saturated H<sub>2</sub>O (4:1 v/v; ca 3h). Bands from the third TLC system were visualized with the spark chamber and radioassayed. If possible, the [<sup>14</sup>C]-labelled compounds were identified by co-chromatography of appropriate standards e.g kaurenoic acid, geraniol, geranylgeraniol and farnesol.

#### 2.2.22 Extraction and Separation of [<sup>14</sup>C]-Labelled Prenyl Pyrophosphates

The cell-free incubations produced prenyl pyrophosphates which were water soluble and were not extracted with the other terpenoids. These compounds were extracted and separated by two methods, one via a direct extraction of the prenyl pyrophosphates, the other by an indirect extraction and separation of the alcohol derivatives formed by acid hydrolysis of the prenyl pyrophosphates (Kushwaha et al., 1977).

i) The direct method involved initial partition of the incubation mixture against Et<sub>2</sub>O (2.2.17). The aqueous phase was then extracted using n-BuOH (X3, ½ vol.) in a separating funnel (100ml) containing the incubation and H<sub>2</sub>O. NaCl saturated H<sub>2</sub>O was not used since the NaCl interferes with the n-BuOH in the extraction procedure (2.2.17).

The n-BuOH extracts were bulked and left to stand (1h) to

allow  $H_2O$  to separate, which was then removed by pipette. It was then reduced in volume, under vacuum, transferred to a conical glass tube and further reduced under  $N_2$  over a hot-plate. The n-BuOH was applied to TLC with a single n-BuOH wash (50 $\mu$ l). A number of TLC systems were employed depending on the prenyl pyrophosphates under scrutiny (Table 2.4).

ii) The indirect method involved initial partitioning against  $Et_2O$  (2.2.17; NaCl saturated  $H_2O$  could be used), followed by addition of conc. HCl to the aqueous fraction (200 $\mu$ l per 1.5ml). The mixture was incubated in a shaking water bath (37 $^{\circ}C$ ; 30min). The hydrolysis was stopped by addition of EDTA (0.25M; equal volume to conc. HCl) and conc.  $NH_4OH$  (X2 vol. of conc. HCl) then adjusted to pH 8.0. The basic, aqueous fraction was then extracted with  $Et_2O$  (X3;  $\frac{1}{2}$  vol.) which was left over anhydrous  $Na_2SO_4$  (1h). The fraction was then treated for subsequent application to TLC (2.2.17). The main TLC system used for separation of alcohol derivatives was system I (Table 2.3a,b and 2.5); though other systems were used (Table 2.5).

The [ $^{14}C$ ]-labelled alcohol derivatives of the prenyl pyrophosphates were localised by use of the spark chamber; identification by co-chromatography with standards (Table 2.5).

#### 2.2.23 Protein Determinations

These were carried out routinely using Bradford's protein reagent (Bradford. 1976) which comprised:

Coomasie blue G (10mg) dissolved in 95% EtOH (5ml) and 85%



Table 2.4 Rf Values of Prenyl Pyrophosphates on Different TLC Systems

Prenyl Pyrophosphate	Rf in TLC System		
	K	L	M
PPPP	0.52	0.58	-
GGPP	0.40	0.44	0.44
FPP	0.26	0.30	-
GPP	0.13	0.20	-

System:

K; Kieselgel G; EtOAc/iso-PrOH/NH<sub>4</sub>OH, 3/4/3

L; Kieselgel H; CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O, 60/40/9 (Kushwaha et al., 1977)

M; Kieselgel G; Pr-1-OH/NH<sub>4</sub>OH/H<sub>2</sub>O, 6/3/1 (Sagami et al., 1977)

Table 2.5 Rf Values of the Alcohol Derivatives of Prenyl Pyrophosphates on Different TLC Systems

Alcohol	I	Rf in TLC System		O
		J	N	
Geraniol	-	0.60	} 0.67	0.30
Farnesol	0.25	0.50		0.38
Geranyl-geraniol	0.15	0.36	0.58	0.50
Prephytoene Alcohol	0.39	0.17	0.30	0.77

System:

I; Kieselgel G; Toluene/EtOAc (9/1, v/v) (Sagami et al., 1977)

J; Kieselgel G activated with 5% Paraffin/Petrol (60°-80°C);  
MeOH/Paraffin saturated H<sub>2</sub>O (4/1, v/v)

(Kushwaha et al., 1977)

N: as J, except MeOH/Paraffin Saturated H<sub>2</sub>O (22/3, v/v)

(Kushwaha et al., 1977)

O: Kieselgel G; Toluene/EtOAc (4/1, v/v)

"

orthophosphoric acid (10ml) made up to a total volume (100ml) by slow addition of H<sub>2</sub>O, followed by filtering the solution.

The reagent (0.95ml) was added to a protein sample (50μl) and the absorbance recorded at 595nm spectrophotometrically.

The properties of the reagent deemed it necessary to carry out a protein standardization each time an assay was performed. The normal standard range for the reagent was 0-200μg/ml (for a 50μl aliquot) of bovine serum albumin solution.

#### 2.2.24 Purification of Phytoene Synthetase

Attempts were made to purify phytoene synthetase from G.fujikuroi. For the purification an assay had to be developed, complicated by the fact that phytoene is inherently unstable (2.2.10) and radiolabelled GGPP and PPPP (enzyme substrates, 1.1.4) were not readily available for large numbers of assays.

##### i) Coupled Assay

The principle of this technique was to use a cell-free system to generate [<sup>14</sup>C]-GGPP and -PPPP which when coupled to phytoene synthetase would produce [<sup>14</sup>C]-phytoene which could be assayed. This method was an adaption of the principle involved when a phytoene-generating system from P.blakesleeanus was coupled to Aphanocapsa homogenates to incorporate [<sup>14</sup>C]-MVA into carotenoids (Sandmann and Bramley, 1985).

The method involved a preincubation step which produced [<sup>14</sup>C]-GGPP and -PPPP (along with -IPP, -GPP and -FPP). This was followed by a coupling step in which the substrates were used by another cell-free system. A number of non-phytoene producing strains were analyzed and G.fujikuroi mutant SG4 was determined to be the best for the pre-

incubation step (6.2.5).

Cell-free preparations of light-grown G.fujikuroi mutant SG78 were used for the coupling step as a source of phytoene synthetase.

The pre-incubation step included in the incubation:

$\underline{R}$ -[2- $^{14}\text{C}$ ]-Sodium mevalonate	0.25 $\mu\text{Ci}$
ATP	10 $\mu\text{mol}$
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	6 $\mu\text{mol}$
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	4 $\mu\text{mol}$
KF	10mmol
$\text{S}_{10}$ from SG4	200 $\mu\text{l}$
400mM Tris-HCl buffer pH 8.0 containing 5mM DTT	325 $\mu\text{l}$ total vol.

This was prepared in the same way as the standard cell-free incubation (2.2.15) except that the ATP was taken up in a smaller vol. of buffer (100 $\mu\text{l}$ ). After the incubation period (90min), a high concentration of un-labelled sodium mevalonate (700 $\mu\text{g}$ ) was added in a small volume (5 $\mu\text{l}$ ) in order to stop any further incorporation of  $\underline{R}$ -[2- $^{14}\text{C}$ ]-MVA. Immediately after this, the coupling system was added to the incubation comprising:

ATP	10 $\mu\text{mol}$
NAD	1 $\mu\text{mol}$
NADP	1 $\mu\text{mol}$
KF	10mmol
SG78 purified, cell-free extract fraction	200 $\mu\text{l}$
400mM Tris-HCl buffer pH 8.0 containing 5mM DTT	310 $\mu\text{l}$ total vol.

The total coupled system (635 $\mu\text{l}$ ) was then incubated for a further period of time (10min) before the reaction was stopped with cold MeOH (1ml).

#### ii) Coupled Assay Extraction and Separation for Phytoene

The extraction of the coupled system was identical to the

normal terpenoid extraction method (2.2.17) except that lycopene was not included in the carrier terpenoids. The separation of phytoene comprised 2 successive TLC systems, system H (Table 2.2 ) from which the carotenoid band containing  $\beta$ -carotene and phytoene (Rf 0.30) was eluted and chromatographed on TLC system G (Table 2.2 ) from which phytoene (Rf 0.54) could be isolated.

Care was taken to ensure only the specific bands of interest were removed from the TLC plates.

### iii) Polyethylene Glycol Precipitation

Total S<sub>105</sub> from SG78 was dispensed (200 $\mu$ l) into a series of ultracentrifuge tubes and to each was added an equal vol. of buffer (400mM Tris-HCl pH 8.0 with 5mM DTT) containing varying concentrations of PEG (0-20%). The samples were mixed on a vortex mixer and after incubation on ice (1h), centrifuged in an ultracentrifuge (5min). The precipitate was resuspended in buffer by occasional agitation (1-2h) over ice and assayed, along with the supernatant, by coupled assay.

## CHAPTER III

### RESULTS

Identification of Carotenoids,  
Ergosterol and O-Demethylanhydro-  
fusarubin in the wild type and  
Mutants of Gibberella fujikuroi

"All colours will agree in the dark"

Francis Bacon

### 3.1 Introduction and Aims

The carotenoid producing mutants of G.fujikuroi, SG22, SG43, SG48, SG68, and SG78 (Table 1.6; Avalos et al., 1985) plus the wild type strain CMI 58289 were analyzed for their carotenoid and ergosterol contents under light- and dark-grown conditions. Additional determinations were made of the total, saponifiable and unsaponifiable lipids under the same growth conditions.

Carotenogenesis is known to be photoinduced in a number of organisms (1.2.3.iii), and initial investigations suggested this to be the case in G.fujikuroi (Avalos et al., 1985). Furthermore, some of the mutants are considered (Avalos et al., 1985) to be affected in the photoinduction mechanism and analysis of these mutants may reveal the steps of the terpenoid pathway that are photoregulated.

Other mutants were affected in structural genes of the carotenogenic pathway itself, accumulating pathway intermediates such as SG43, SG68 and SG78 (Table 1.6). Since carotenoid distribution varies tremendously between organisms (1.3.2), identification of carotenoid intermediates may lead to the elucidation of the specific carotenogenic pathway in G.fujikuroi; the structural mutants may aid such investigations.

Recent studies with A.giganteus (El-Jack, personal communication) have shown that not only carotenoids, but other lipids, including ergosterol, are photoinduced. Since the sterols form part of the terpenoid pathway, which is photoregulated with respect to carotenogenesis, the total lipids, including ergosterol, were measured in light- and dark-grown cultures of the mutants to examine photo-effects on the lipids of

### G.fujikuroi.

Under certain conditions, G.fujikuroi produces a series of quinones (1.9). Attempts were made to identify these pigments.

### 3.2 Additional Methods

The carotenoids of acidic and neutral mycelial extracts (2.2.6), were separated on neutral alumina grade III columns. Separation of the neutral carotenoids (Plate 3.1) produced 5 fractions which were eluted with increasing polarity solvents (Petrol (40<sup>o</sup>-60<sup>o</sup>C)-5% Et<sub>2</sub>O/Petrol (40<sup>o</sup>-60<sup>o</sup>C). These fractions were then further purified by TLC (2.2.7).

An elution profile of the neutral carotenoids (Fig 3.1) indicated the need for further purification .

Although the neutral fraction contained mainly neutral carotenoids, a certain amount of neurospoxanthin (NX) was also present in the fraction (Plate 3.1). This suggests that the acidic/neutral separation technique (2.2.6) is not completely effective. This was also found with the acidic extracts on an alumina column. The separation of bands from the acidic fraction showed that in addition to the expected quinone(s) and NX, there were contaminant levels of the neutral carotenoids (Plate 3.2).

The dark, naphthaquinone band at the top of the alumina column (Plate 3.2) did not interfere with NX purification, since the polarity of the former was far greater than that of NX.

The combination of these column systems and appropriate TLC systems (Table 2.1 and 2.2) allowed separation of up to 9 individual carotenoids, and although the initial acidic/neutral partition was not completely efficient, it

Fig. 3.1 Elution Profile of Neutral Carotenoids on Alumina Grade III Column

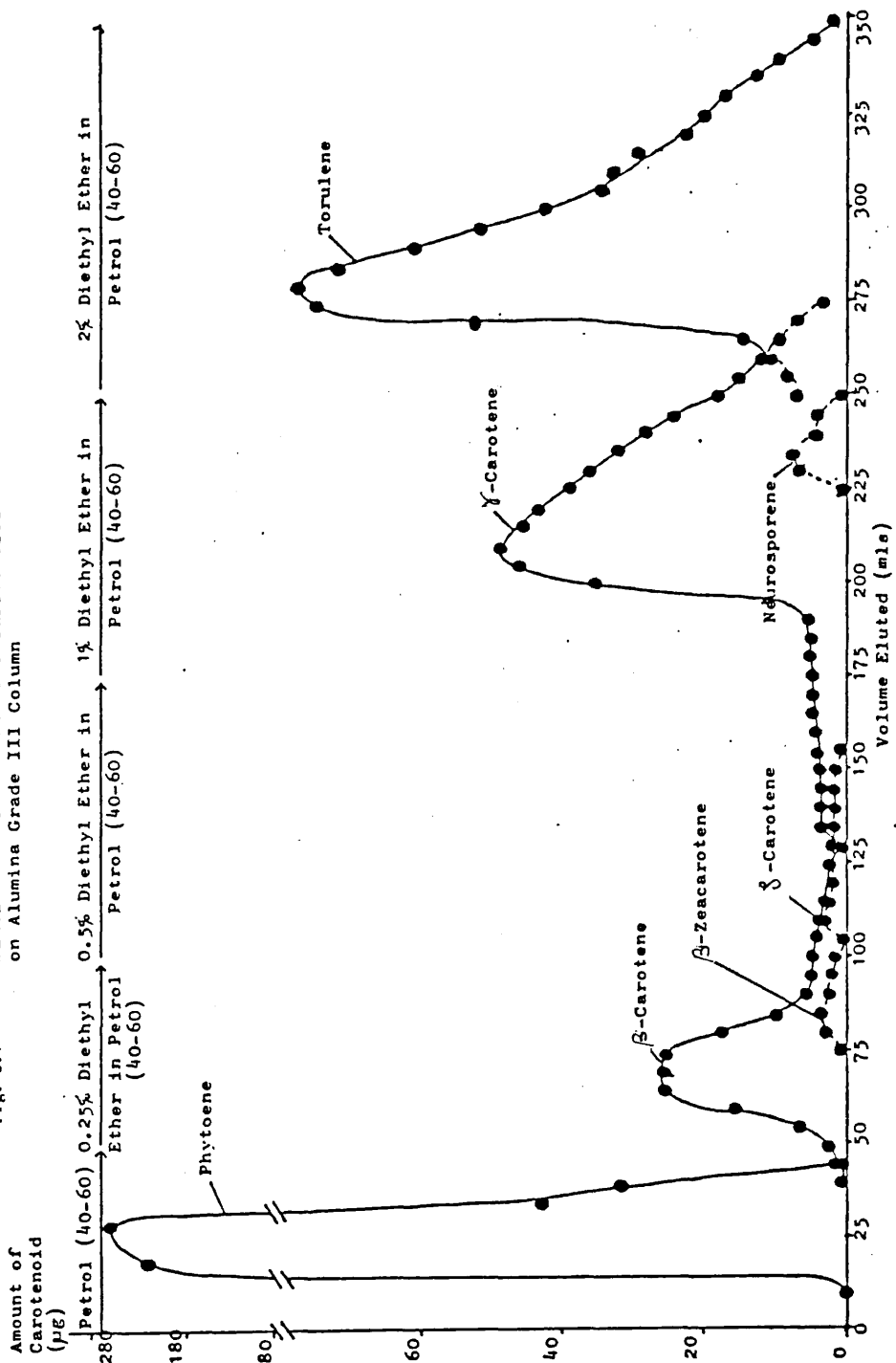




Plate 3.1 Neutral Carotenoids Extract from *G.fujikuroi*  
Mutant SG22 Separating on an Alumina Grade III  
Column

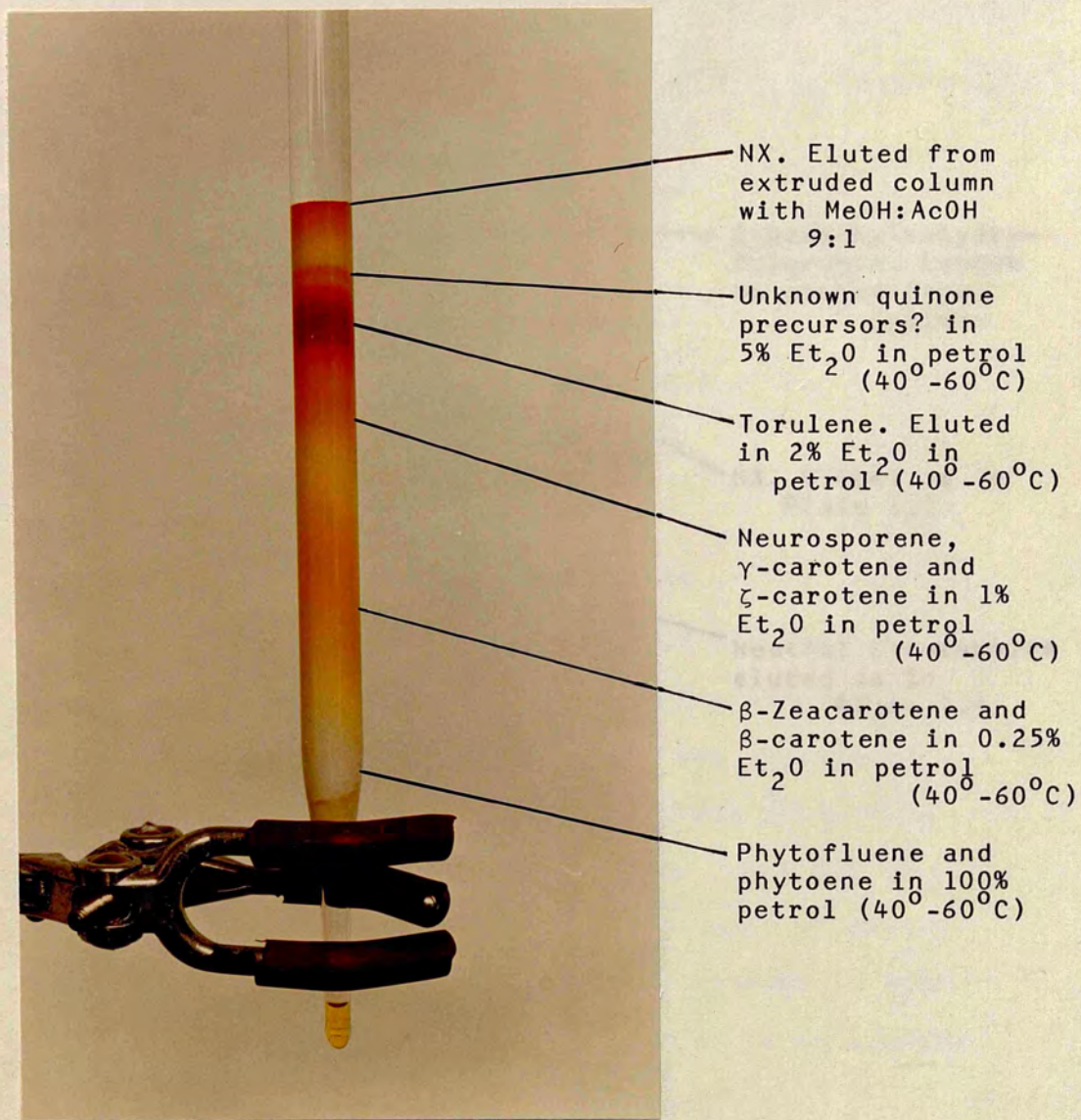
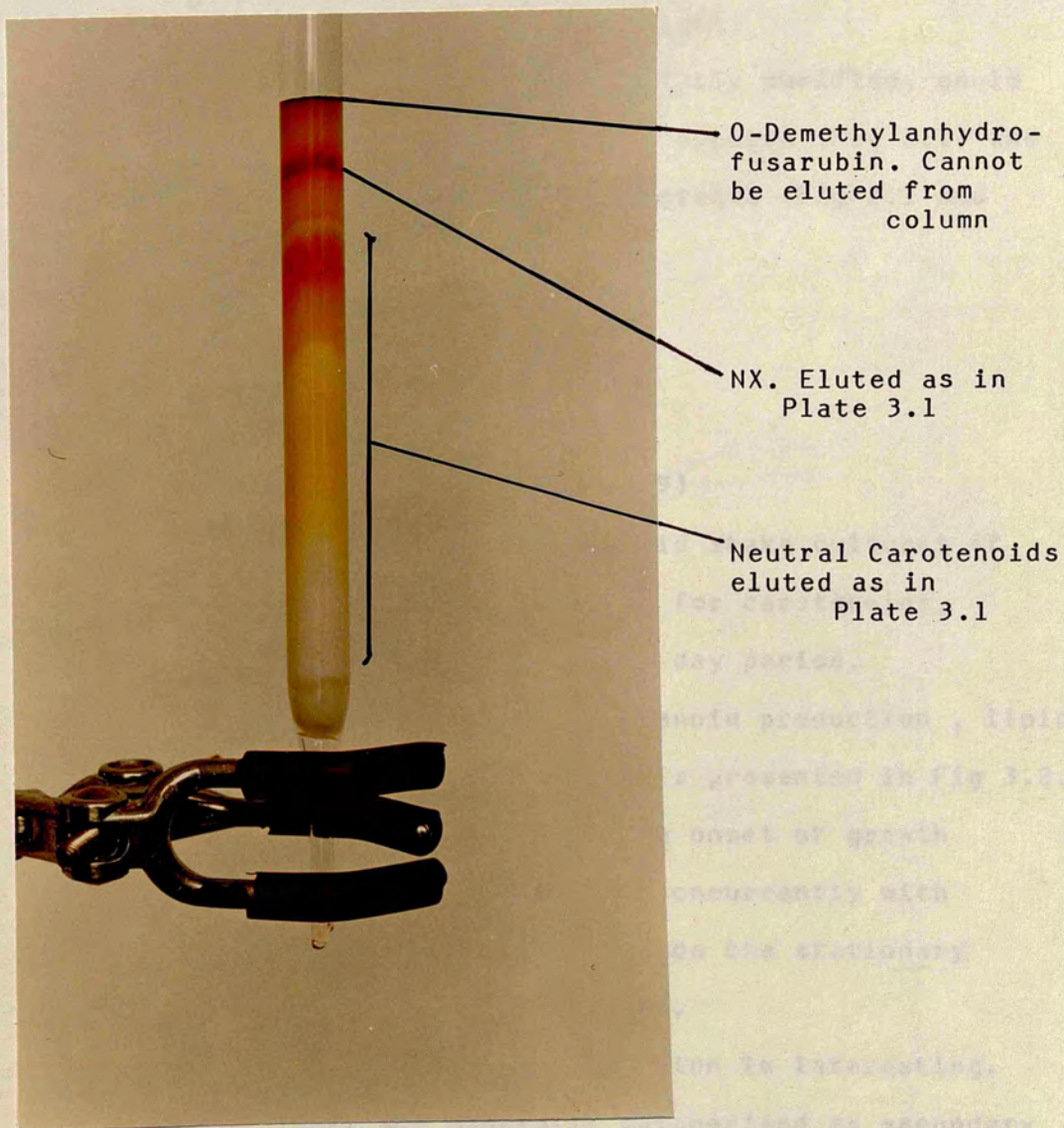




Plate 3.2 Acidic Carotenoid Extract from G.fujikuroi  
Mutant SG22 Separating on an Alumina Grade III  
Column



served to separate the bulk of the acidic and neutral carotenoids. Furthermore, the use of 2 separate columns, effectively halving total lipid extracts on a column, reduced the interference to column elution by the unsaponifiable lipids which are present in relatively large amounts in G.fujikuroi extracts, as much as 30-50% of dry weight.

The carotenoids, once separated and fully purified, could be identified by their characteristic spectra (Fig 2.1) and quantified by their extinction coefficients (Fig 2.1 and 2.2.8).

### 3.3 Results and Discussion

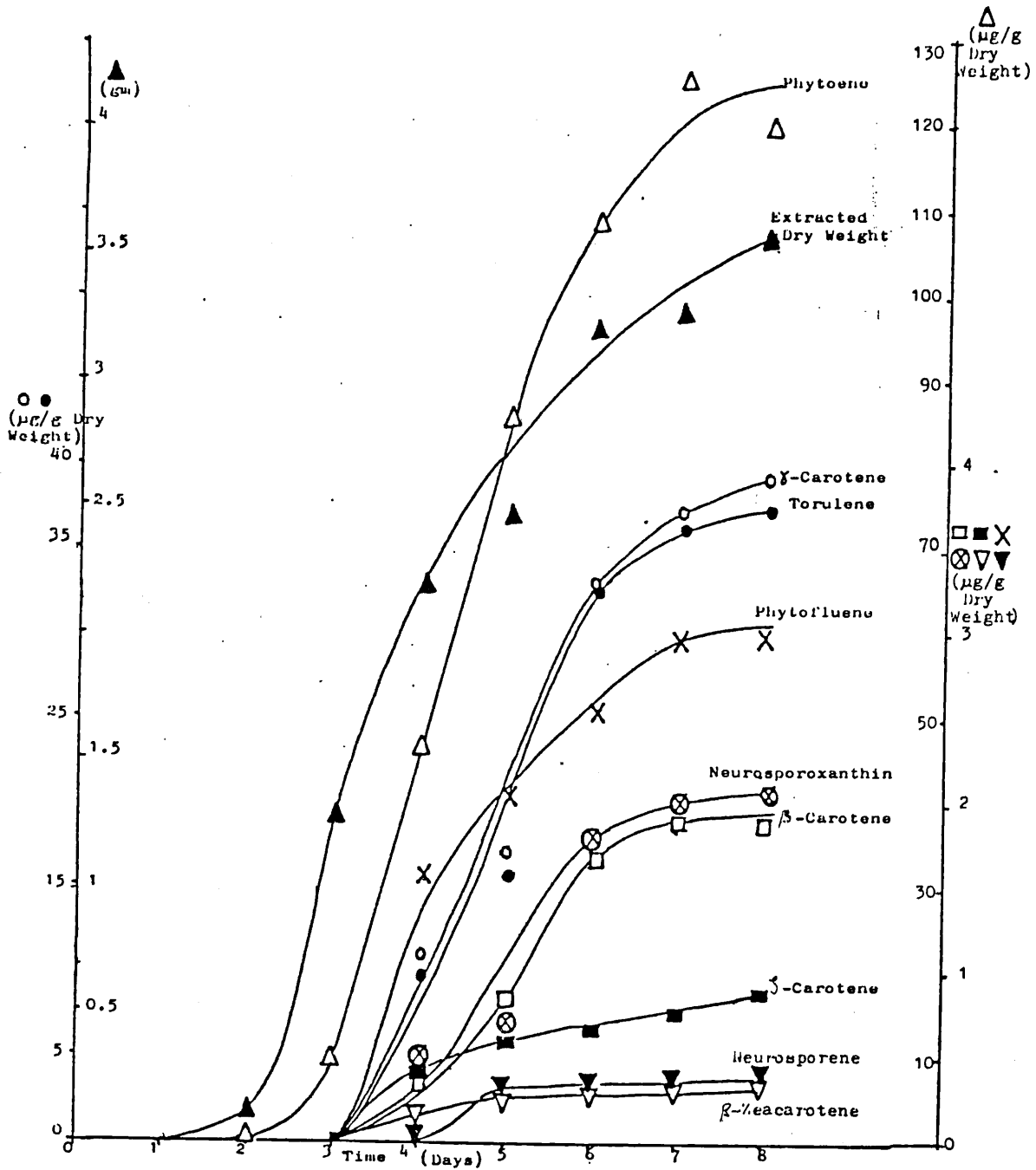
#### 3.3.1 Wild Type G.fujikuroi (CMI 58289)

Wild type G.fujikuroi, grown in liquid shake cultures of AM (2.2.1) in the light, was analysed for carotenoids, ergosterol and other lipids over an 8 day period.

The time course of dry weight, carotenoid production, lipid levels and media pH over this period is presented in Fig 3.2. Carotenoid production commenced at the onset of growth (between 2 and 3 days) and continued concurrently with growth, reaching a maximum level towards the stationary phase of growth (between 6 and 8 days).

The time course of carotenoid production is interesting, Carotenoids in fungi are generally categorized as secondary metabolites, i.e compounds which serve no obvious function in the cells which produce them (Turner, 1975, Bu'Lock, 1975 and reviewed by Campbell, 1984). It has been implied (Goodwin, 1959, Bu'Lock, 1961 and Bu'Lock et al., 1974) that secondary metabolites are produced after growth is complete

Fig 3.2 Time Course of Carotenoid Changes in *Gibberella fujikuroi* Wild Type



i.e available N has been assimilated and remaining, excess C is available for secondary metabolite production. However, in G.fujikuroi carotenoids are synthesized concomitantly with growth, showing that secondary metabolites are not necessarily only produced at stationary phase.

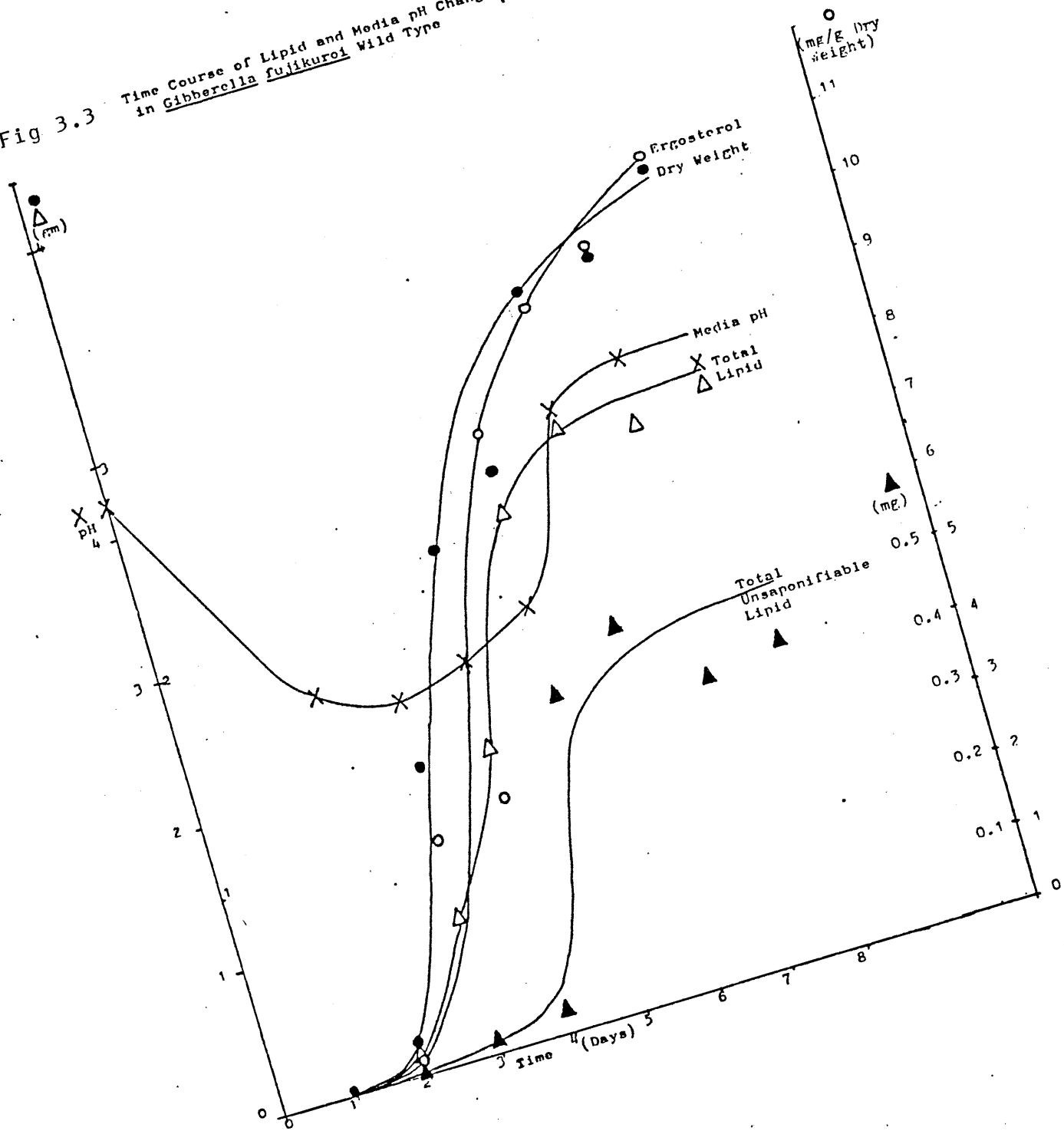
The results also show that 9 carotenoids were isolated, which represent all the major, possible intermediates in carotenogenesis from phytoene to neurospoxanthin (NX), apart from lycopene. An examination of the possible distribution of carotenoids, in different organisms (Fig 1.8) indicates a possible pathway to NX via all the intermediates that are isolated. However,  $\gamma$ -carotene can theoretically be produced from neurosporene via lycopene or  $\beta$ -zeacarotene (1.3.2). The debate as to whether one, or both, routes are present remains inconclusive (1.3.2). The lack of lycopene in G.fujikuroi suggests that the pathway via  $\beta$ -zeacarotene predominates in this organism, although the possibility that lycopene is produced and utilized too rapidly for detection, cannot be ruled out.

The oxidative step(s) between torulene and NX remain unclear as no intermediates were isolated from the wild type.

The time course for lipid production (Fig 3.3) shows an increase with growth of the saponifiable and unsaponifiable lipids and ergosterol levels, reaching a maximum at the end of growth. This is as one would expect for lipids which are either present as energy reserves or as structural components.

The medium pH shows a fall from the initial acidic pH 4.2, to a very acidic level of below pH 2.5 at mid-log growth. It then returns to the original medium pH at the end of the growth period (about 6 days). This fall and rise in pH is

Fig 3.3 Time Course of Lipid and Media pH Changes in *Gibberella fujikuroi* Wild Type



common in fungal cultures (Aisworth, 1966) and is probably due to an initial utilization of N during growth, causing an imbalance in the salts of the media. This balance may be redressed at the end of growth as other media components are utilized and/or fungal metabolites are extruded.

Whether this pH change is relevant to the regulation of the pathway i.e it stimulates, or inhibits, certain metabolic pathways, or whether it is merely a passive reflection of fungal growth, cannot be determined. It may, however, play some role in secretion of metabolites into the medium.

Wild-type G.fujikuroi was also grown in AM liquid shake cultures for 8 days in the light and dark, and carotenoid and lipid levels were determined (Table 3.1). In light-grown tissue the total carotenoid levels are in much greater abundance than in dark-grown mycelia. In the latter the levels are almost negligible. Consequently wild type G.fujikuroi exhibits photoinduced carotenogenesis.

Phytoene is the predominant carotenoid in both light- and dark-grown cultures, with relatively low levels of post-phytoene carotenoids. There are very low amounts of NX and  $\zeta$ -carotene, with higher torulene and  $\gamma$ -carotene levels; these are still quite low. The relative values are similar in both light- and dark-grown cultures, suggesting that light does not affect this distribution. Furthermore, it seems that the medium favours production of these carotenoids, since when grown in DG (3.3.8 and Avalos et al., 1985) and other media (Aitken, personal communication) the carotenoid distribution varies tremendously; low C levels and/or low C:N ratios enhance NX and neutral carotenoid production. Consequently one may speculate that there is a regulatory mechanism,

Table 3.1

*G. fujikuroi* wild-type carotenoid and lipid levels in light and dark grown cultures.

	Light Grown (8 Days)	Dark Grown (8 Days)
Dry Weight (gm)	3.56	2.96
Compounds / gm Dry Weight		
Phytoene	1.2mg	79 $\mu$ g
Phytofluene	3 $\mu$ g	0.8 $\mu$ g
$\zeta$ -Carotene	1 $\mu$ g	0.1 $\mu$ g
Neurosporene	0.15 $\mu$ g	—
$\beta$ -Zeacarotene	0.35 $\mu$ g	0.05 $\mu$ g
$\gamma$ -Carotene	39 $\mu$ g	6 $\mu$ g
$\beta$ -Carotene	2 $\mu$ g	0.1 $\mu$ g
Torulene	37 $\mu$ g	4 $\mu$ g
Neurosporoxanthin	2 $\mu$ g	—
Ergosterol	10.9mg	8.7mg
Total Lipid	0.731g	0.811g
Unsaponifiable Lipid	0.118g	0.111g



linked to the amount of C in the media, with respect to carotenogenesis.

The production of NX appears to be regulated slightly differently from the neutral carotenoids (3.3.8) and high C levels inhibit production from torulene.

The light- and dark-grown lipid levels do not show any large differences and photoinduction of lipids does not appear to be apparent in G.fujikuroi wild type; especially in comparison with the carotenoids, or with the photoinduction of the lipids in A.giganteus which is found to be increased almost 100-fold (El-Jack, personal communication).

### 3.3.2 Mutant Strain SG22

SG22 was grown under similar conditions to the wild type (3.3.1) over 8 days, and the carotenoid and lipid levels were assayed over this period (Fig 3.4 and Fig 3.5 respectively).

The mutant grew in a similar fashion to the wild type, and produced carotenoids concomitantly with the onset of growth. Therefore the mutation has not affected these parameters. However, unlike the wild type strain, all the carotenoids of SG22 do not reach a maximal level at the onset of stationary phase, but instead continue to be produced beyond 6 days at a constant rate. At 8 days the levels continue to rise logarithmically, despite growth having reached a maximum. Consequently this mutant has been affected in its regulation of total carotenoids. The control of NX formation apparent in the wild type, is absent, suggesting that carotenoid levels could continue to rise even later beyond the end of growth. A comparison of the levels in 8 day light-

Fig 3.4 Time Course of Carotenoid Changes in *Gibberella fujikuroi* Mutant SG22

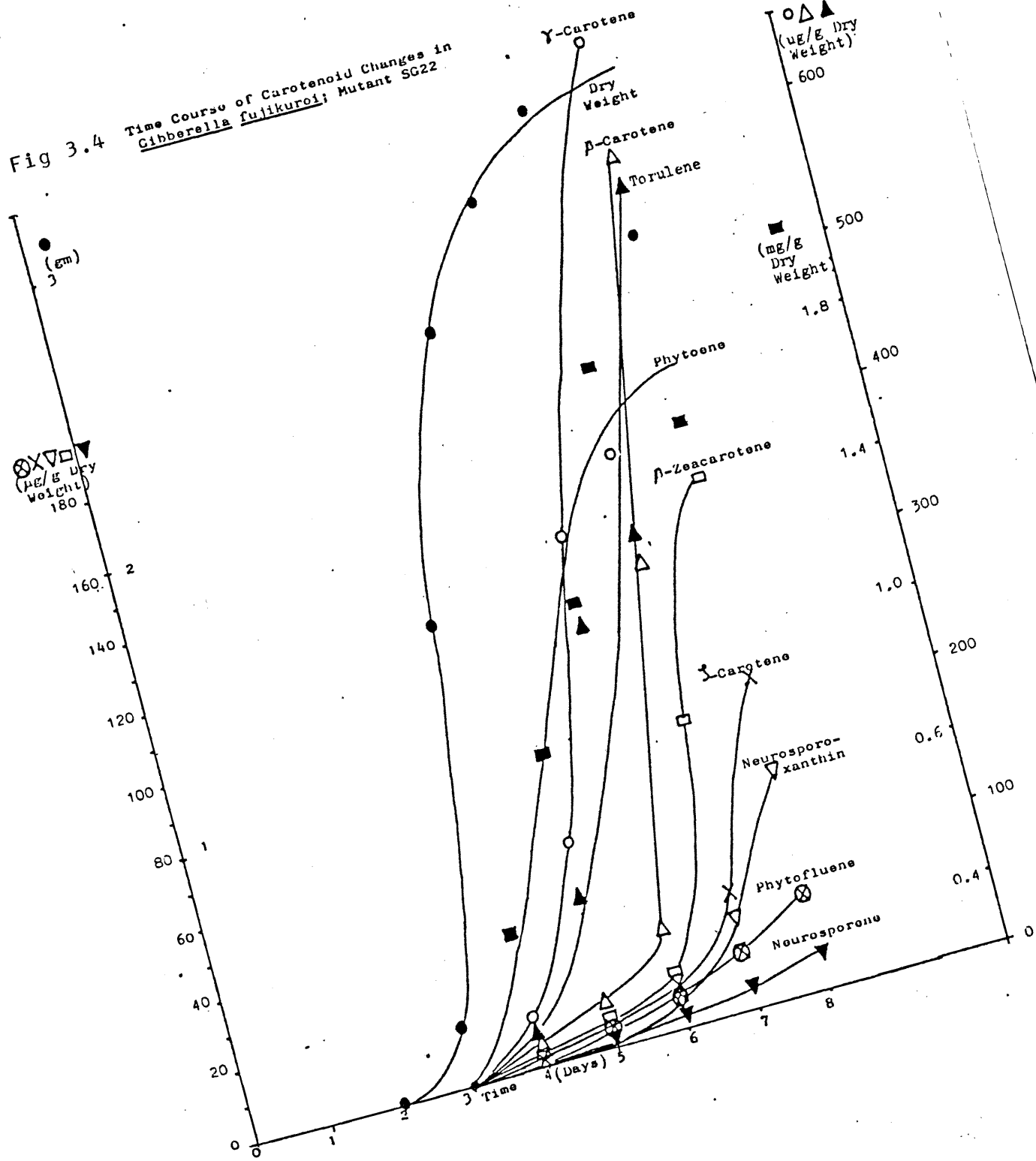
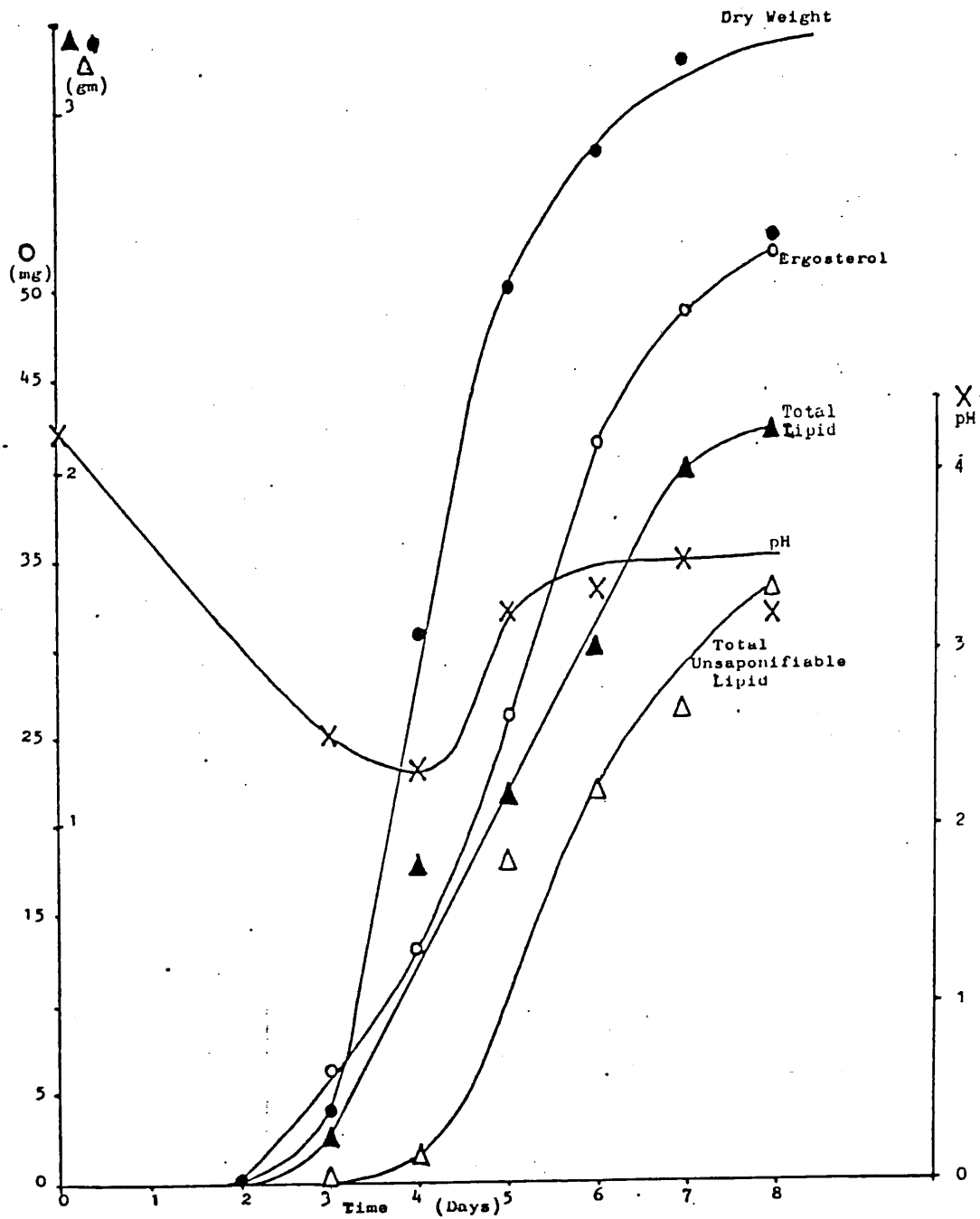


Fig 3.5 Time Course of Lipid and Media pH Changes in *Gibberella fujikuroi*; Mutant SG22



and dark-grown cultures (Table 3.2) with the wild type, shows how marked this regulatory mutation is as vastly increased carotenoid levels are present, with up to a 10-fold increase of some carotenes. Though the relative carotenoid levels are quite similar to those in the wild type, phytoene levels do not appear to be much greater under light-grown conditions in SG22, than in the wild type. Consequently it is possible that the regulatory mechanism affected in this mutation may act only on post-phytoene steps.

The photoregulation seen in the wild type remains in SG22, except that phytoene appears to be slightly greater in dark-grown cultures of SG22 compared to light-grown cultures. This is not expected since a single mutation of wild type G.fujikuroi, to produce SG22 (which statistically this mutation is likely to be) has already been shown to affect the regulation of carotenogenesis so could not effect an additional alteration. Instead, this apparent difference in light- and dark-grown cultures may result from a build-up of phytoene due to the early, terpenoid biosynthesis steps no longer being under regulatory control, combined with post-phytoene enzymes being regulated in dark-grown cultures. Consequently, the apparently similar phytoene levels in light-grown wild type and SG22 may be due to the increase in phytoene production (resulting from increased pre-phytoene enzyme activity; no negative feed-back), offset and hidden by an increased desaturation and cyclization of phytoene to the other carotenoids as a result of this regulatory mutation.

The lipid levels in SG22 (Fig 3.5) are similar to those in the wild type, except for ergosterol which is twice that

Table 3.2

G.fujikuroi mutant SG22  
carotenoid and lipid levels  
in light and dark grown  
cultures.

	Light Grown (8 Days)	Dark Grown (8 Days)
Dry Weight (gm)	2.66	2.36
Compounds / gm Dry Weight		
Phytoene	1.6mg	2.2mg
Phytofluene	37 $\mu$ g	9 $\mu$ g
$\zeta$ -Carotene	120 $\mu$ g	85 $\mu$ g
Neurosporene	20 $\mu$ g	3 $\mu$ g
$\beta$ -Zeacarotene	180 $\mu$ g	128 $\mu$ g
$\gamma$ -Carotene	664 $\mu$ g	104 $\mu$ g
$\beta$ -Carotene	588 $\mu$ g	420 $\mu$ g
Torulene	565 $\mu$ g	90 $\mu$ g
Neurospoxanthin	178 $\mu$ g	16 $\mu$ g
Ergosterol	19.58mg	20.50mg
Total Lipid	0.798g	0.508g
Unsaponifiable Lipid	0.252g	0.157g

found in the wild type. This suggests that the regulatory mechanism that affects the carotenoid levels, affects an early step in the terpenoid pathway, prior to FPP production; this does not eliminate the possibility of additional regulatory points, beyond FPP. The light- and dark-grown levels of ergosterol are identical, and confirm the proposal that the mutation in SG22 affects overall regulation and not photoregulation. The latter suggestion was made by Avalos and workers (1985), who claimed to find photoconstitutive carotenoid levels in SG22. However, it is probable that the levels are only similar between light- and dark-grown SG22 cultures, since the medium on which these workers cultured SG22 (i.e DG), favours higher post-phytoene carotenoid levels (3.3.8) which may mask the presence of any photoinduction.

The pH of the media (Fig 3.5) in SG22 cultures shows a similar fall and rise pattern to that of the wild type cultures.

### 3.3.3 Mutant Strain SG68

SG68 was derived from SG22, (Avalos et al., 1985) i.e. a double mutant of the wild type strain CMI 58289. The time courses for carotenogenesis (Fig 3.6) and lipid formation (Fig 3.7) were determined over 9 days, in AM under light-grown conditions. Comparisons of the light- and dark-grown levels of these metabolites were also made at 8 days (Table 3.3).

The time course of carotenoid production is identical to that of SG22, with similar individual carotenoid levels; although perhaps the later carotenoids are somewhat reduced.

Fig 3.6 Time Course of Carotenoid Changes in *Gibberella fuikuroi*; Mutant SG68

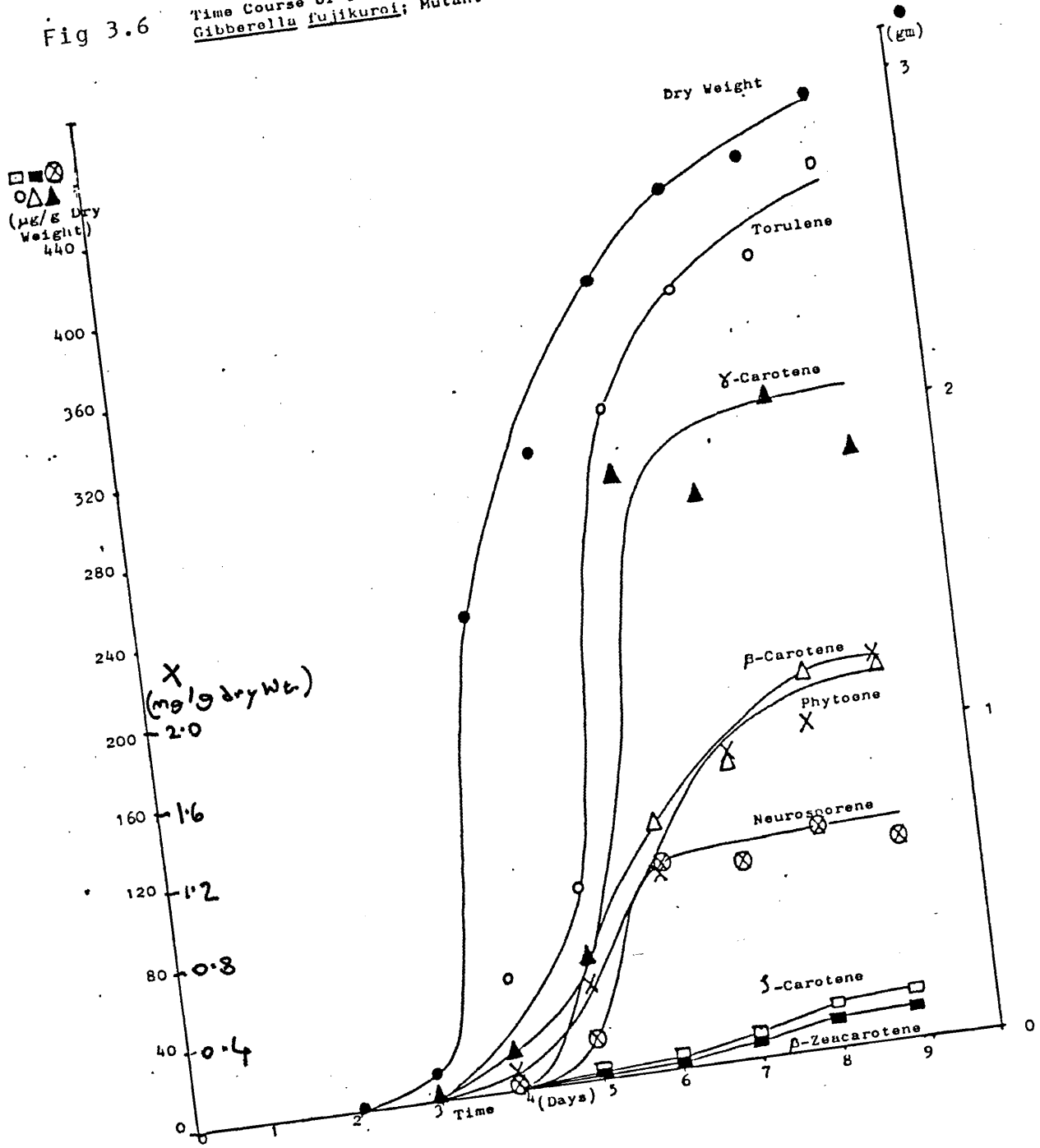


Fig 3.7 Time Course of Lipid and Media pH Changes in *Gibberella fujikuroi*; Mutant SG68

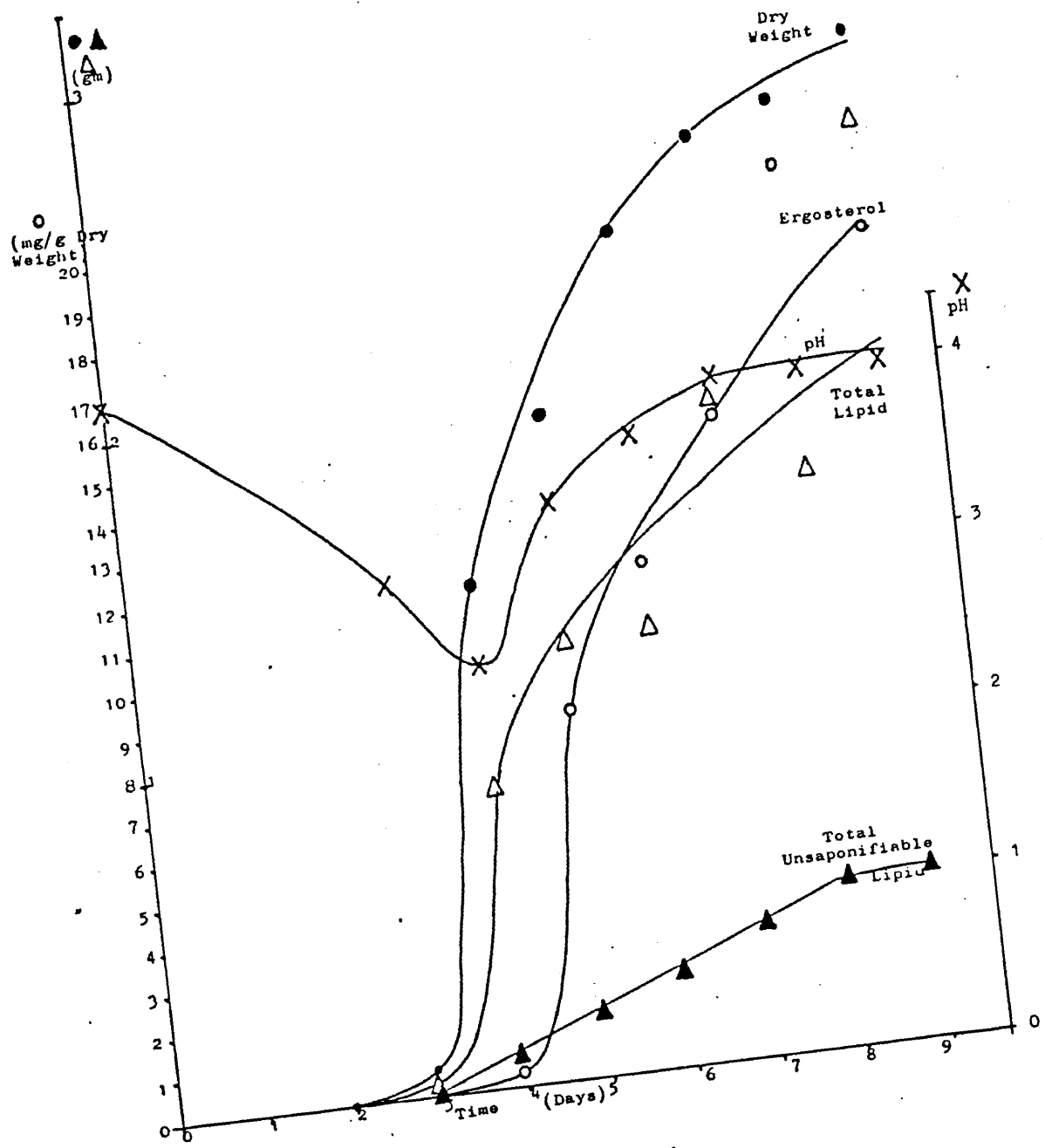




Table 3.3

G. fujikuroi mutant SG68  
carotenoid and lipid levels  
in light and dark grown  
cultures.

	Light Grown (8 Days)	Dark Grown (8 Days)
Dry Weight (gm)	2.78	2.18
Compounds / gm Dry Weight		
Phytoene	1.6mg	2.2mg
Phytofluene	36 $\mu$ g	22 $\mu$ g
$\zeta$ -Carotene	112 $\mu$ g	45 $\mu$ g
Neurosporene	18 $\mu$ g	2 $\mu$ g
$\beta$ -Zeaxanthin	60 $\mu$ g	17 $\mu$ g
$\gamma$ -Carotene	36 $\mu$ g	22 $\mu$ g
$\beta$ -Carotene	188 $\mu$ g	61 $\mu$ g
Torulene	328 $\mu$ g	136 $\mu$ g
Ergosterol	20.72mg	17.76mg
Total Lipid	0.612g	0.550g
Unsaponifiable Lipid	0.180g	0.146g

Another slight difference is that the rate of carotenogenesis increases steadily, but then levels out at the end of log-growth (as shown in the wild type; Fig 3.2), while in SG22 it continued to rise during the stationary phase. The major difference however, is the total absence of NX indicating that the mutation has prevented the step(s) between torulene and NX, causing the former to accumulate. On the premise that SG68 is produced by a single mutation of SG22 (i.e inhibition of torulene metabolism), the anomalies in the carotenoid levels between these two mutants can only be explained by this single mutation. The reduction in the latter levels of carotenoids from  $\gamma$ -carotene to NX, may suggest that NX production activates its precursor formation, either directly on the enzyme(s), or simply that a fully functional pathway is necessary for maximal activity.

The levelling of the carotenogenic rate during stationary growth, suggests that while the mutation in SG22 removes total pathway regulation, NX is necessary in some way for this mutation to be fully realized: it may be involved in a negative feed-back mechanism (compare to P.blakesleeanus photoinduction model, Fig 1.7; Cerda-Olmeda, 1986), the mutation of which must be combined with NX to completely remove all regulation of the pathway. However, much more information must be gathered for such a theory to be even tentative.

The light- and dark-grown data for SG68 shows that photoinduction is still present and the apparently constitutive levels of phytoene are exactly the same as those in SG22.

The lipid levels and time courses, including medium pH, are similar to those found in SG22.

### 3.3.4 Mutant Strain SG43

SG43 was grown for 10 days under the same conditions as the other mutants. Carotenoid and lipid levels were estimated over this time (Fig 3.8).

In this mutant, only phytoene is produced and it is probable that phytoene dehydrogenase has been deleted. From the time course, there are no other differences between SG43 and the wild type with respect to lipid production, medium pH and the rate of phytoene production, except that the total phytoene levels are about half those found in the wild type.

The reduced phytoene level in SG43 compared to the wild type, is seen with 8 day, light- and dark-grown in AM cultures of the strain (Table 3.4). In this mutant, phytoene is seen to be totally photoinducible with no dark-grown constitutive levels, in contrast to the wild type. Again, as this is probably a single mutation (in phytoene dehydrogenase) these other deviations from the wild type must result from the absence of post-phytoene carotenoids. It has already been suggested that there may be some feed-back regulation in carotenogenesis in G.fujikuroi,; which is affected in SG22 and possibly SG68. Consequently it is suggested that certain post-phytoene carotenoids are necessary to activate a pre-phytoene step: the presence of ergosterol and GAs (4.3.5.iii) in dark-grown SG43 suggests that the enzyme in question is phytoene synthetase. Therefore, in this mutant only photoinducible phytoene synthetase is apparent, but in wild type G.fujikuroi there is another phytoene synthetase which is regulated by other factors, possibly unsaturated carotenoids. Whether this means there are 2 distinct enzymes, coded by different genomes, one photoinducible and the other

Fig 3.8 Time Course of Carotenoid, Lipid and Media pH, Changes in *Cibberella fujiikuroi*; Mutant SC43

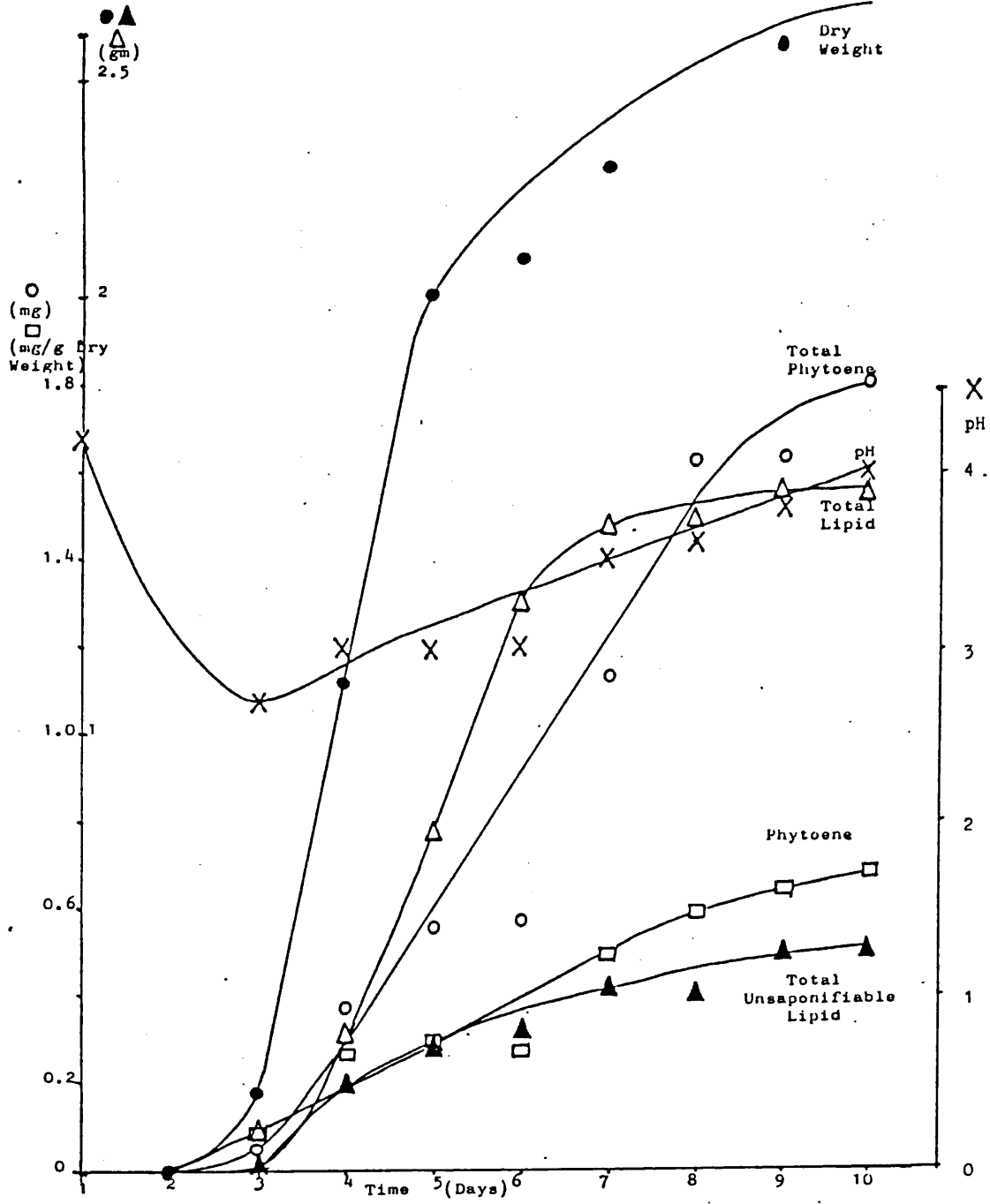


Table 3.4

G. fujikuroi mutant SG43  
carotenoid and lipid levels  
in light and dark grown  
cultures.

	Light Grown (8 Days)	Dark Grown (8 Days)
Dry Weight (gm)	2.69	2.31
Compounds / gm Dry Weight		
Phytoene	0.63mg	—
Ergosterol	9.4mg	8.7mg
Total Lipid	0.574g	0.542g
Unsaponifiable Lipid	0.089g	0.095g

Table 3.5

G. fujikuroi mutant SG78  
carotenoid and lipid levels  
in light and dark grown  
cultures.

	Light Grown (8 Days)	Dark Grown (8 Days)
Dry Weight (gm)	3.04g	2.06g
Compounds / gm Dry Weight		
Phytoene	4.25mg	1.82mg
Ergosterol	20.17mg	18.95mg
Total Lipid	0.785g	0.674g
Unsaponifiable Lipid	0.119g	0.180g

constitutive and chemically regulated, or that there is a single phytoene synthetase gene with regulatory sequences affected by different regulators, is not certain, but the idea of 2 isoenzyme systems in carotenogenesis has been proposed, in for example F.aquaeductuum (Theimer and Rau, 1972).

### 3.3.5 Mutant Strain SG78

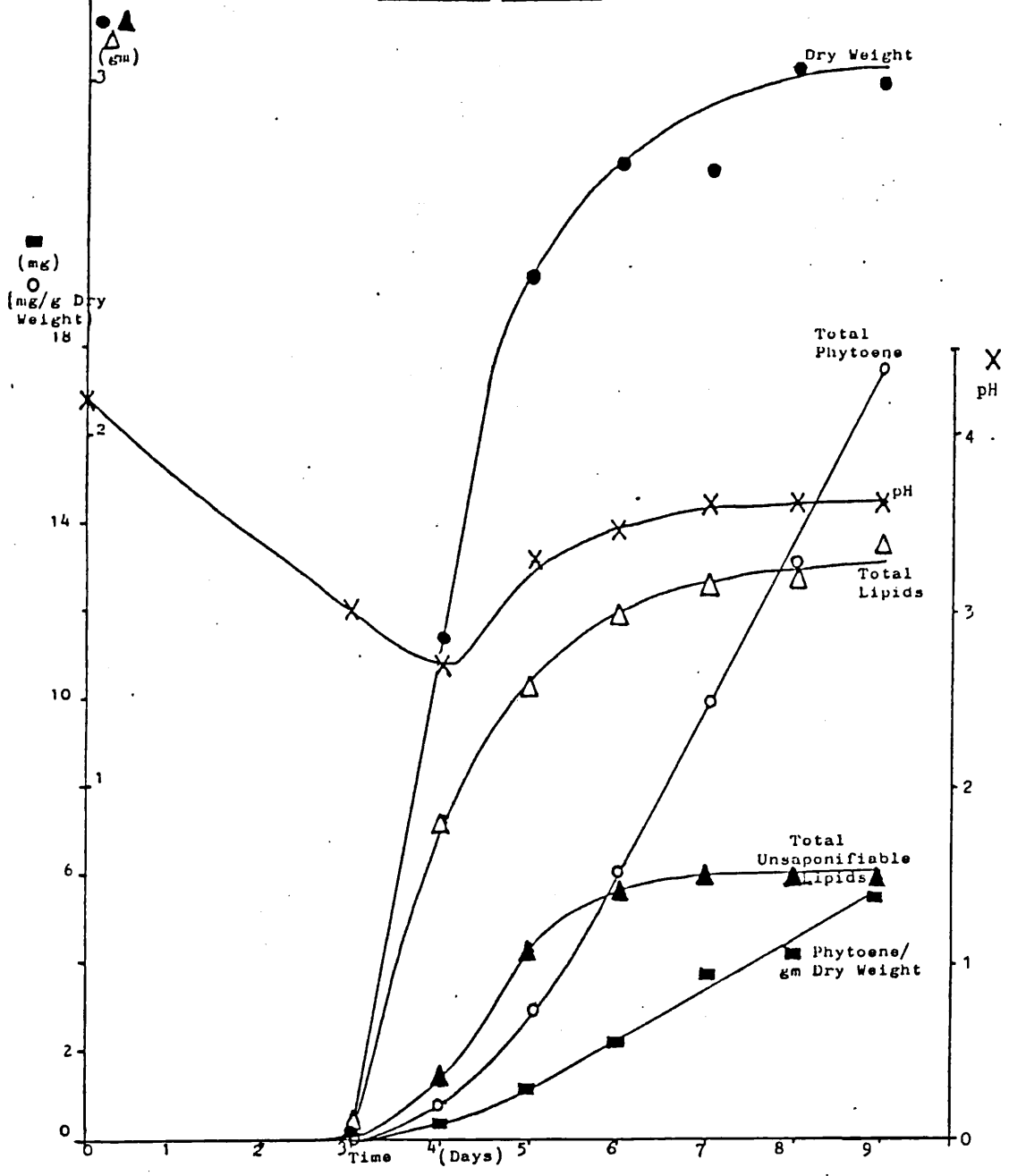
SG78 was analyzed in exactly the same manner as the wild type and other mutants of G.fujikuroi. The carotenoid and lipid contents were measured over time (Fig 3.9) and the light- and dark-grown levels of these were calculated for 8 day old cultures (Table 3.5).

As in SG43, phytoene is the only carotenoid produced by SG78, but the levels are even greater than those in SG22. The rates of phytoene production are the same in SG78 and SG22, as is the time course for lipid formation, media pH changes and the growth rate. Therefore the mutation is likely to be a block at phytoene dehydrogenase alone, thus accumulating large amounts of phytoene; it is comparable to SG43 in this respect.

A consideration of the light- and dark-grown data (Table 3.5), shows that photoinduction is still apparent with respect to phytoene synthetase and there is a high, dark-grown, constitutive level of phytoene, as in SG22. This supports the idea of there being a photoinducible phytoene synthetase as well as a constitutive form.

The light-grown phytoene level is relatively high compared to SG22, but this may be explained by the phytoene dehydrogenase block, allowing a build-up of prenyl units into phytoene, which cannot be converted into other carotenoids. This system is not subject to the absence of post-phytoene

Fig 3.9 Time Course of Carotenoid, Lipid and Media pH Changes in *Gibberella fujikuroi*; Mutant SC78



carotenoids causing a reduced phytoene synthesis, as in SG43, because this regulatory mechanism has been affected in SG22 and so it is inoperative in the double mutant, SG78.

### 3.3.6 Mutant Strain SG48

SG48 was described (Avalos et al., 1985) as a temperature dependent mutant in which pigmentation (due to carotenoids) was pale orange at 23°C when dark-grown and orange when at 30°C in the dark, or at either temperature in the light. In the present study SG48 was grown on DG plates over a range of temperatures between 23°C and 30°C, in the light and dark (Plate 3.3).

The results are not fully conclusive, but they do suggest a temperature dependent level of carotenoids in dark-grown cultures which seems to be overcome by light.

One interpretation of the data is that a particular carotenogenic enzyme has been mutated to be temperature dependent for activity. This effect is masked in light-grown cultures by high photoinduced levels; a constitutive level of carotenogenesis being present in the dark. Such a mutation may prove useful, especially if the affected enzyme is photoinduced. But more detailed investigation of this mutant is necessary.

### 3.3.7 Neurospora crassa ACC 10816

The apparent absence of lycopene in G.fujikuroi and mutants poses an interesting question as to whether this is due to  $\gamma$ -carotene, being formed from neurosporene entirely via  $\beta$ -zeacarotene (in which case cyclization and dehydrogenation enzymes are relatively specific), or whether lycopene is



formed and metabolized too rapidly for detection in 24 h  
(3.3.1 and 3.3.2).

*N. crassa* belongs to the same taxonomic group as *Fujikuroi*  
(the *Prenomyces* test and since it is a fungus, the photo-

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agreement. Since *Fujikuroi* and *N. crassa* are such

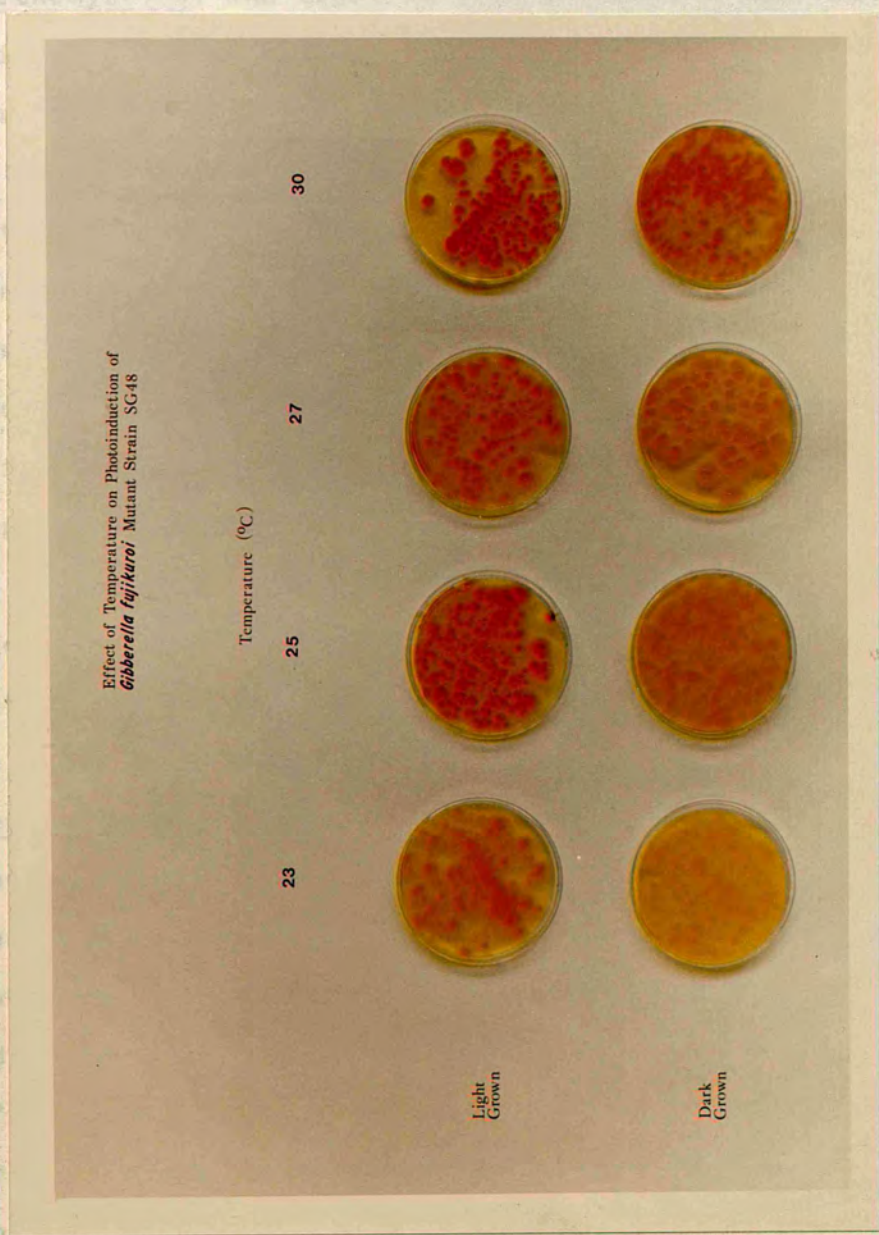
differences, there will be no question of questioning the

absence of lycopersin in *Fujikuroi* but that the growth

conditions may favour a particular pathway, not enzyme

specificity or absence.

Plate 3.3



formed and metabolized too rapidly for detection in vivo (3.3.1 and 1.3.2).

N.crassa belongs to the same taxonomic series as G.fujikuroi (the Prenomyces) and since it also produces NX the carotenogenic abilities of these organisms may be considered similar. Consequently, N.crassa was grown in an analogous manner to G.fujikuroi (2.2.1.ii) and the carotenoids extracted and levels measured after 8 days growth. This was performed so as to isolate lycopene and confirm that this carotenoid is absent from G.fujikuroi and that the analysis technique is not insufficient. Furthermore, it allowed re-establishment of the point made by Rau (1976) with N.crassa, that apparent carotenoid levels vary tremendously either due to different growth conditions or methods of analysis

The results, together with those for the carotenoid content of N.crassa obtained by a number of other researchers (Table 3.6), show that a number of workers did find lycopene in N.crassa, with low levels or even no  $\beta$ -zeacarotene i.e the reverse of what was found in G.fujikuroi (3.3.1). Lycopene was also found in N.cra<sup>s</sup>a in these studies, suggesting that the technique would allow detection of lycopene, should it be present.

The figures also show the variability in the levels of the different carotenoids produced by differing conditions, with none of the studies of N.crassa carotenoid levels being in agreement. Since cultural conditions may cause such differences, these must be considered when questioning the absence of lycopene in G.fujikuroi in that the growth conditions may favour a particular pathway, not enzyme specificity or absence.

Table 3.6 Carotenoid content of *N. crassa* compared to that found by different investigators (cited by Rau, 1976)

	1	2	3	4	5	6	7
	(µg/g dry weight)						
Total	755	*	*	*	449	92	825
Phytoene	219	*	*	465	242	31	450
Neutral carotenoids	200	129	20	167	107	19	260
Neurosporo-xanthin	336	15	244	*	100	42	95
	(% of neutral carotenoids)						
3,4-Dehydro-lycopene	9	2	24	1	6	19	33
Lycopene	13	*	15	6	6	14	10
Torulene	—	12	5	1	1	3	5
Neurosporene	16	22	10	9	14	18	6
ζ-Carotene	34	5	18	32	26	10	9
γ-Carotene	17	32	26	22	27	27	18
β-Carotene	4	16	—	13	4	*	9
β-Zeacarotene	*	*	—	6	2	1	—
Phytofluene	7	11	*	11	15	8	10

\* Data not given

— Not detected

1 Zalokar 1954

2 Liaaen-Jensen 1965

3 Harding et al. 1969

4 Davies 1973

5 Mitzka and Rau (cited in Rau 1976) wild-type

6 " " "slime"

7 *N. crassa* extraction for comparison with other workers and to confirm method can detect lycopene.

### 3.3.8 The Effects of DG and AM media

Wild type G.fujikuroi was grown, under light, on AM and DG agar plates for 8 days and the mycelia extracted for their carotenoid contents.

AM Growth strongly favoured phytoene production over other carotenoids, whereas DG-grown cultures produced predominantly NX (Table 3.7).

The major differences between the 2 media are their total C levels and their C:N ratios. It appears a high C level and/or a high C:N ratio greatly favours phytoene production and reduces other neutral carotenoid levels and inhibits most of NX production, whereas a lower C and/or C:N level reduces phytoene production but increases NX biosynthesis.

Generally a high C level, or high C:N ratio is considered to favour metabolism (Bu'Lock, 1961 and Bu'Lock et al., 1974); after N has been assimilated the excess C is 'shunted' into secondary metabolic pathways. Consequently, AM-grown cultures should have a high level of secondary metabolism compared to that in DG-grown cultures. Indeed this is the case for GAs (4.3) with higher GA levels from strains of G.fujikuroi grown on AM, than the levels obtained from DG-grown strains. However, this is not the case for post-phytoene carotenoids. In fact, the levels of NX are greater in DG-grown cultures compared to that in AM-grown cultures. Therefore high C levels appear to inhibit post-phytoene, carotenogenesis, in particular NX, thus regulating a certain amount of secondary metabolism. Phytoene levels on the other hand, do behave as non-C-inhibited secondary metabolites, mimicking the GA results (4.3).

Generally, AM should be a good media in which to study

Table 3.7

Carotenoid content of G. fujikuroi  
wild-type grown on AM and DG plates.

	AM (8 Days)	DG (8 Days)
Carotenoid / gm Dry Weight		
Phytoene	1.2mg	162 $\mu$ g
Phytofluene	3 $\mu$ g	—
$\zeta$ -Carotene	1 $\mu$ g	—
Neurosporene	—	—
$\beta$ -Zeaxanthene	—	—
$\gamma$ -Carotene	69 $\mu$ g	9 $\mu$ g
$\beta$ -Carotene	4 $\mu$ g	2 $\mu$ g
Torulene	59 $\mu$ g	7 $\mu$ g
Neurospoxanthin	150 $\mu$ g	4 $\mu$ g

phytoene synthesis (and GAs) in G.fujikuroi and the mutants.

### 3.3.9 Separation of 15-cis and all-trans Phytoene

Phytoene extracts from AM, light-grown SG78 cultures were applied to a grade III, aluminium oxide column. The elution profile (Fig 3.10) indicates that the cis isomer of phytoene predominates in G.fujikuroi; 98% 15-cis phytoene and 2% all-trans phytoene. This is in accordance with the usual amounts of these isomers in fungi (generally 1% to 5% all-trans phytoene; 1.1.4 and 1.3.1).

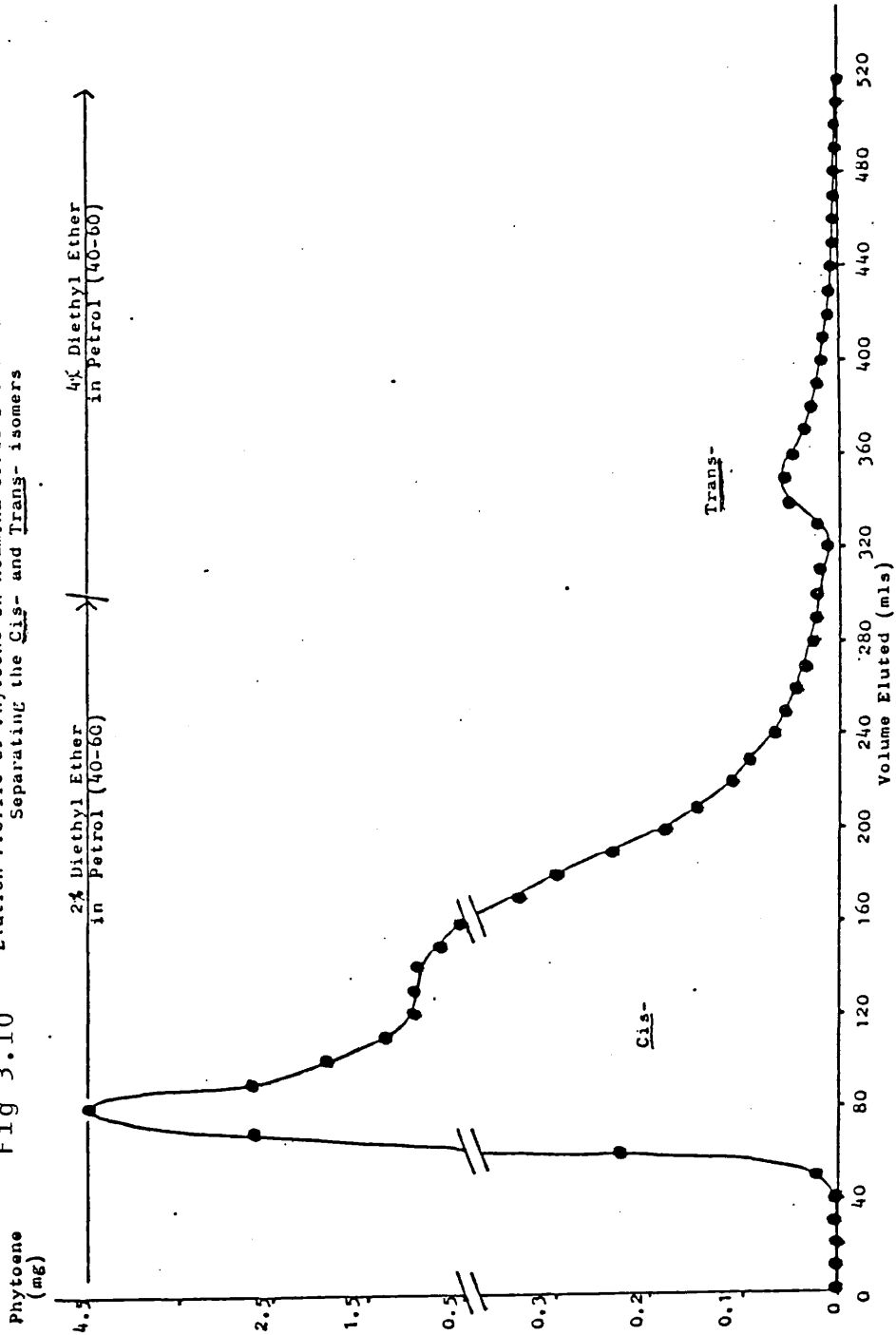
### 3.3.10 O-Demethylanhydrofusarubin

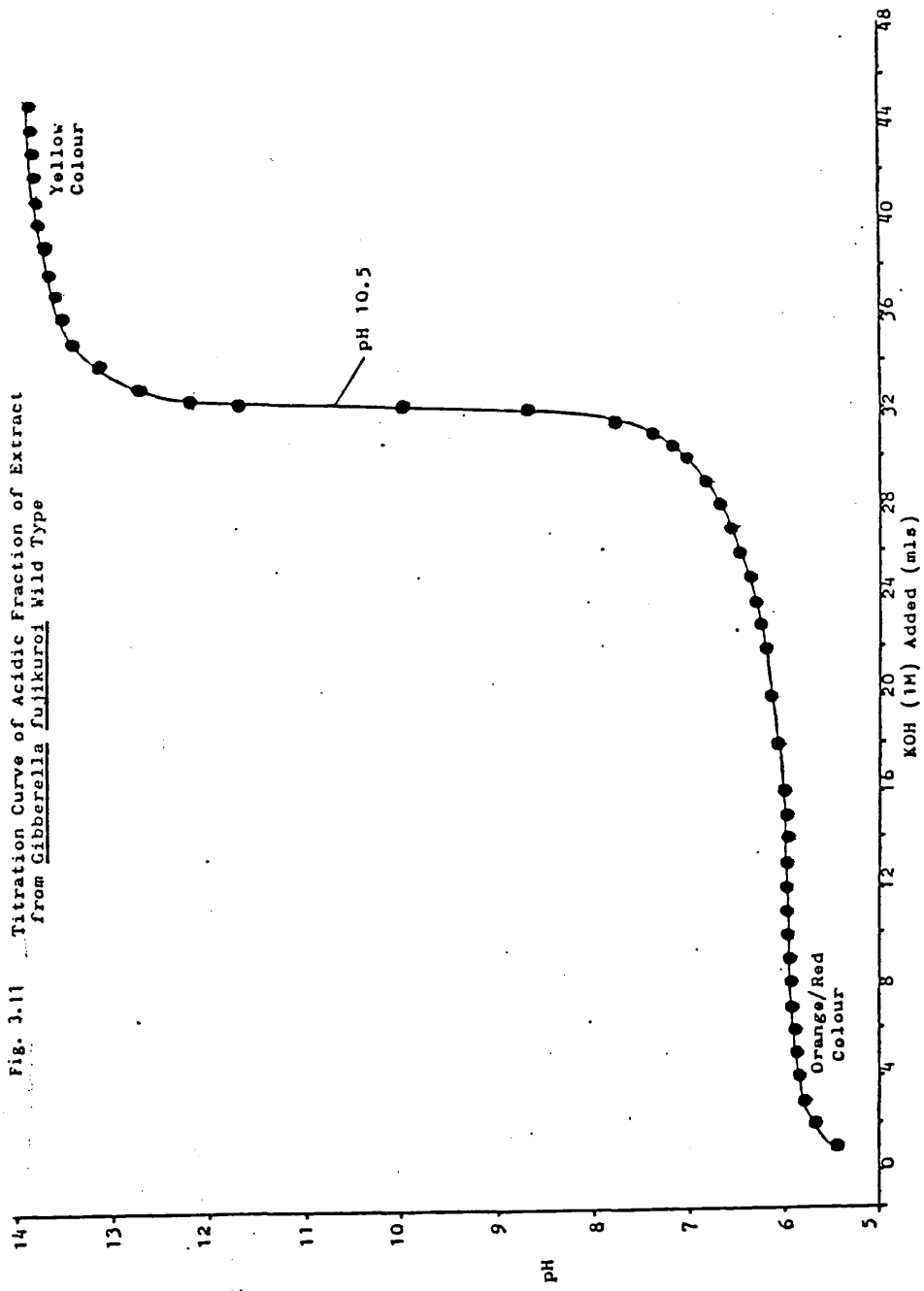
In all cultures of G.fujikuroi grown in AM, in the light or dark, a deep red, non-carotenoid pigment was produced in the mycelia and excreted into the media towards the end of growth. This pigment was polar and was extracted in the acidic fraction of the total lipid (2.2.6) and remained at the top of an aluminium oxide, grade III column, even with highly polar elutents (Plate 3.2).

The pigment production was found to increase with an increase in C, or C:N levels in the media, suggesting it to be another secondary metabolite (Bu'Lock et al., 1974 and Aitken, personal communication).

The pigment was seen to have a number of physical and chemical characteristics common to a quinoid compound. These include a high polarity and a pH dependent colour change. The pH titration plot shows a pKa of about 10.5 with alkali conditions producing a yellow colour and acid inducing a change to orange/red (Fig 3.11). These properties result from the phenolic groups and associated conjugation

Fig 3.10 Elution Profile of Phytocene on Alumina Grade I Column  
Separating the Cis- and Trans- isomers







possible in a quinone.

A sample of the pigment, which at very high concentrations crystallized from the acidic, methanol extracts, was subjected to mass spectrometry. A  $M^+$  of 274 suggested a molecular weight of 274, which matched that of 0-demethylanhydrofusarubin, one of the quinones known to be produced by cultures of G.fujikuroi (Cross et al., 1970).

There were suggestions that  $Zn^{2+}$  is necessary for the production of this quinone (Hanson, personal communication), and consequently wild-type G.fujikuroi and strain SG22 were grown in AM shake cultures over a range of  $Zn^{2+}$  concentrations (0-4mg/l).

Under the premise that at 0mg/l of  $Zn^{2+}$  there would be no quinone production, it may be possible to reveal the carotenoid pigments which are usually masked by the red quinone in AM-grown mycelia. An inhibitor of coloured carotenoid synthesis, DPA (1.2.3.v) was used in conjunction with varying  $Zn^{2+}$  levels in these strains (Plate 3.4 and Plate 3.5).

The effect of  $Zn^{2+}$  concentration is quite marked in both strains and it is seen to be necessary for quinone synthesis such that at zero  $Zn^{2+}$  (and low DPA levels) the quinone is not synthesised and the carotenoid pigments are revealed producing orange mycelia. Therefore the  $Zn^{2+}$  appears to be essential to the polyketide pathway (1.9.2), perhaps as a cofactor in an essential enzymic step.

In the absence of  $Zn^{2+}$  the dramatic effect DPA (at 20-40 $\mu$ M) has inhibiting carotenoid desaturation can be seen in both strains, producing a colourless mycelia. At concentrations of DPA above 80 $\mu$ M, mycelial growth was inhibited, indicating that the inhibitor affects more essential functions at these

Plate 3.4





Plate 3.5

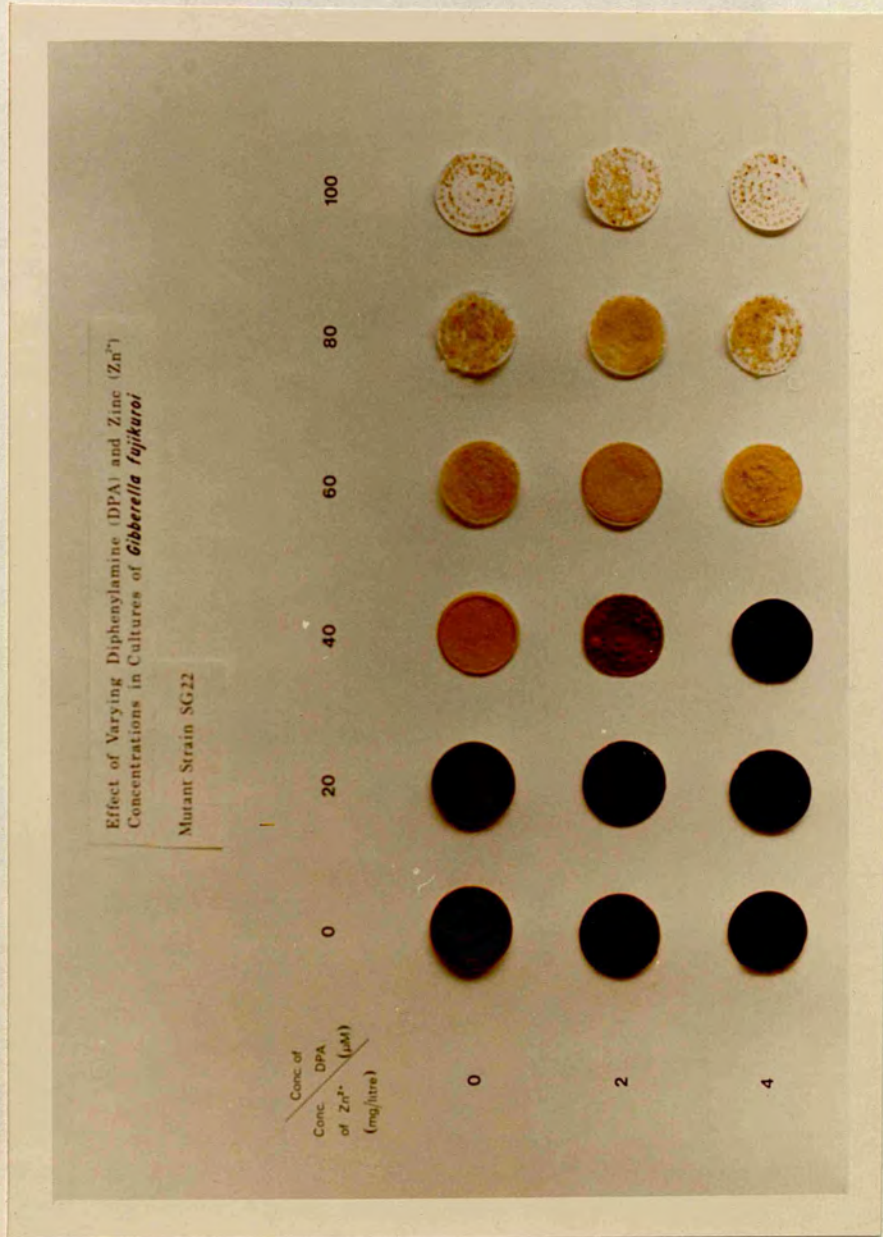
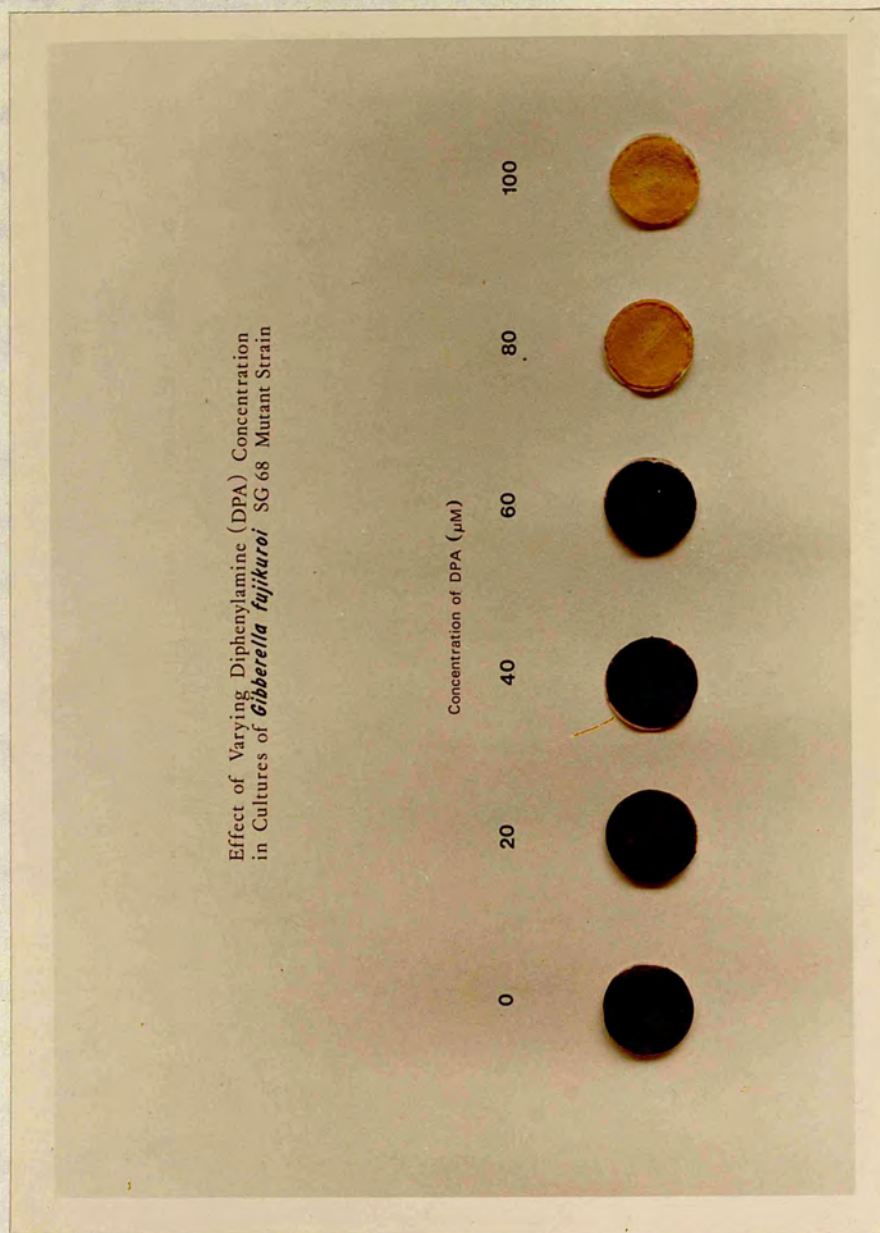




Plate 3.6



concentrations.

It was noticed that 60-80 $\mu$ M DPA inhibits O-demethylanhydrofusarubin produced in media containing 4mg/l Zn<sup>2+</sup> (effectively AM) in both the wild type and SG22 strains and also in SG68 (Plate 3.6). Therefore, some step within the polyketide pathway is inhibited by DPA and this may prove useful in polyketide pathway studies.

### 3.3.11 Summary and Conclusions

The separation technique developed for the carotenoids, allowed characterization of the carotenoid-producing mutants and a pathway for carotenogenesis in G.fujikuroi to be proposed, together with some regulatory theories conforming for carotenoid biosynthesis. The absence of lycopene in all strains, implies that a specific dehydrogenase and cyclase may exist for the formation of  $\gamma$ -carotene from neurosporene; although lycopene may be formed as a transient intermediate.

The studies also showed that G.fujikuroi carotenogenesis is photoinduced, with very low constitutive levels in dark-grown cultures. The most interesting conclusion which can be drawn from these data and the analyses of the mutants, is the possibility of 2 different phytoene synthetases, regulated by different mechanisms; (i) a photoregulated form exclusive to SG43, and (ii) an additional form regulated by some other mechanism which is also present in light-grown SG78 while exclusive to dark-grown SG78. The possibility of 2 isoenzymic forms is an interesting aspect to pursue, since the mutations allow a unique opportunity to investigate each form independently.

Lipid levels in G.fujikuroi did not display any photo-regulatory phenomena, although ergosterol was subject to other regulatory mechanisms in conjunction to the carotenoids (especially in SG22), indicating that the terpenoid pathway as a whole possesses some common, early regulation point.

A red pigment, produced by G.fujikuroi under certain conditions, was tentatively assigned to be 0-demethylanhydrofusarubin, a naphthaquinone produced by the polyketide pathway. This was shown to require  $Zn^{2+}$  for its biosynthesis.

On the whole, the carotenoid mutants of G.fujikuroi display some interesting phenomena which cannot be easily explained by single-step blocks. This emphasizes the inter-relatedness of the total terpenoid pathway and of the overall regulation. It also indicates that caution is necessary when studying individual steps, mutation points or intermediates within an extensive pathway where single events may have a multitude of effects throughout that pathway.

Nevertheless, the mutations do afford some valuable information towards understanding the terpenoid biosynthetic pathway in G.fujikuroi and further offer a number of possibilities to studying certain phenomena and strains with which to pursue these areas: such as SG43 and SG78 towards examining the 2 possible forms of phytoene synthetase.

CHAPTER IV

RESULTS

The Gibberellin Contents of  
Wild Type and Mutants of  
Gibberella fujikuroi

"Knowledge comes, but wisdom lingers"

A.L.Tennyson

#### 4.1 Introduction and Aims

G.fujikuroi is one of the only fungi known to produce GAs; there are reports of the fungus Sphaceloma manihoticola producing appreciable levels of a number of GAs (Rademacher et al., 1982). The levels of GAs reported to be produced by G.fujikuroi far exceed those produced by any higher plants (1.4). However, the GAs do not serve any apparent function in G.fujikuroi and the biosynthetic pathway appears to be slightly different to that found in higher plants (especially in the later stages of the biosynthesis; 1.5.3) in which the GAs serve a number of essential hormonal roles. Despite these differences, the organism, along with the available mutants within the terpenoid pathway (1.10.1), serves as an excellent model for studies within GA biosynthesis.

The mutants available for characterization were mainly isolated in the carotenogenic pathway and GA analysis had not been exhaustive in the initial investigations (Avalos et al., 1985). Consequently, it was important to analyze the GA content of each mutant more thoroughly, to ascertain how the mutations within the carotenogenic pathway had affected GA, and more generally, terpenoid biosynthesis. For example, SG22, shown to be a 'super producer' of carotenoids and sterols (3.3.2) may also produce greater amounts of GAs compared to the wild type strain. This being the case, the regulatory mechanism affected within SG22 must be involved in the whole terpenoid pathway, indicating the existence of a general terpenogenic, regulatory mechanism.

It is also of interest to determine what effects a block within the carotenoid pathway may have on the GA levels i.e. to discover whether such mutations in one pathway affect a



related terpenoid pathway.

An important regulatory mechanism of carotenogenesis in G.fujikuroi is photoinduction (3.3.1 and discussed in 1.2.3.iii). Previous studies towards photoregulation of GAs have been relatively limited, with a few reports of light-elevated GA levels (1.4.3.iii). Furthermore, the initial characterizations of the G.fujikuroi mutants (Avalos et al., 1985 and personal communication) have suggested that the total GA levels are the same in light- and dark-grown cultures of all the strains. One of the major aims of these current studies was to determine the effects of light on GA biosynthesis and establish whether there is any terpenoid photoinduction along with carotenoid photo<sup>n</sup>duction.

The regulatory effects of growth media on GA levels has been investigated to a certain extent in G.fujikuroi (1.4.3.i). Generally it was concluded that high C:N levels favour GA production while lower C:N levels limit GA synthesis; reflecting their secondary metabolite nature. Such media regulation was studied in the G.fujikuroi mutants, especially the 2 media used in the carotenoid studies (3.3.8), i.e AM and DG, to further establish whether any such regulation may be connected to the carotenoid, and indeed the terpenoid pathway in general.

Those strains which were to be characterized with respect to their GA levels included 2 wild type strains (IMI 58289 and ACC 917) along with the mutants SG22, SG43, SG75, SG76 and SG78 (1.10.1); a number of strains have been derived from ACC 917 for industrial production of GAs by ICI plc (Lawrence, personal communication).

#### 4.2 Additional Methods

In order to analyze the GA levels in the different mutants it was necessary to first develop an HPLC system capable of measuring the predominant GAs produced by cultures. The method established (2.2.13) gave a typical separation of 5 major GAs (Fig 4.1) identified by co-chromatography with authentic standards.

GAs were quantified by dissolving a known amount of standard GA in a suitable volume of medium, extracting this medium (2.2.12) and applying the extract to HPLC. The resulting peak area was measured and related to the original amount:

$$1 \text{ unit area} = 1.667 \mu\text{g/ml GA in a } 20 \mu\text{l sample}$$

where:

$$1 \text{ unit area} = 1 \text{ cm}^2 \text{ at } 1.28 \text{ range UV detector and } 10 \text{ mV deflection; } 1 \text{ cm/min chart recorder setting}$$

(2.2.13)

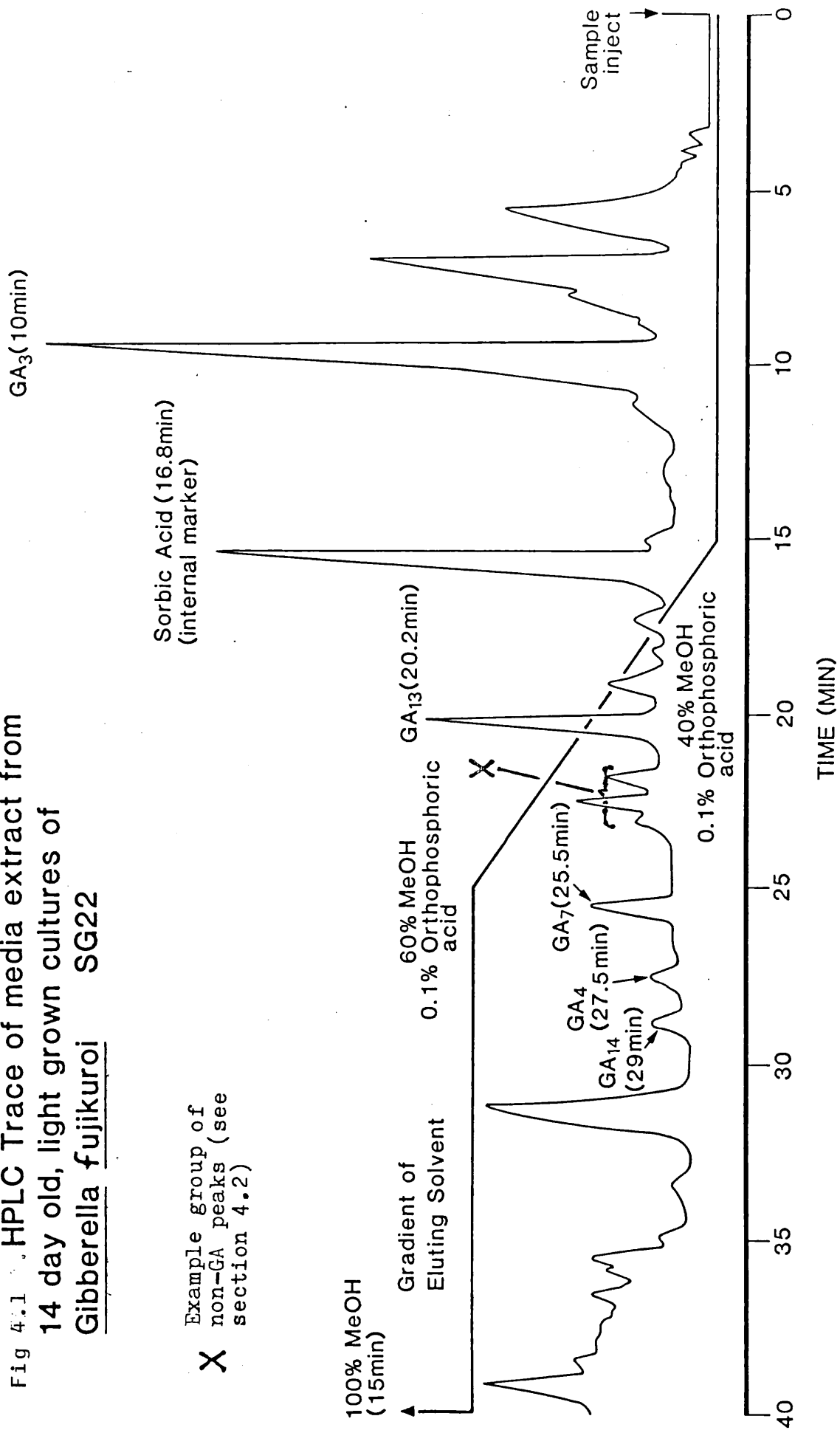
The relationship was tested over a range of concentrations for GA<sub>3</sub> and found to be linear. Due to the low availability of GA<sub>4</sub>, GA<sub>7</sub>, GA<sub>13</sub>, GA<sub>14</sub>, it was assumed that the relationship between peak area and GA<sub>3</sub> levels was identical in the other GAs measured. Since the UV detector was set at 206nm and therefore sensitive to common groups in these GAs, this assumption is probably valid.

The method of GA extraction (2.2.12) was tested for recovery efficiency by comparing the extracted standards with a pure solution of standard placed directly on to HPLC. From these results recovery was found to be at least 99%.

A number of other peaks were detected, which were not GAs, and generally common to most extracts (Fig 4.1). Those peaks prior to GA<sub>3</sub> were also detected in extracts of fresh, sterile media (both DG and AM) and so were determined to be

Fig 4.1 HPLC Trace of media extract from  
 14 day old, light grown cultures of  
Gibberella fujikuroi SG22

X  
 Example group of  
 non-GA peaks (see  
 section 4.2)



media constituents. Other later peaks could not be identified, such as for the group of peaks labelled 'X' in Fig 4.1, but it was found, those samples where light-grown GA levels were much greater than dark-grown GA levels (e.g SG22, Table 4.4), that these peaks remained similar in light- and dark-grown cultures. Consequently these peaks were assumed to be non-GAs, but some other metabolite, detectable at 206nm.

#### 4.3 Results and Discussion

The wild type strain IMI 58289 was analyzed for GA production over time under light- and dark-grown conditions, in AM and DG media. The same measurements were made for SG22, to compare the wild type GA levels with those of this 'super producer' of carotenoids, and for SG76 to see the effects of an early, terpenoid pathway block.

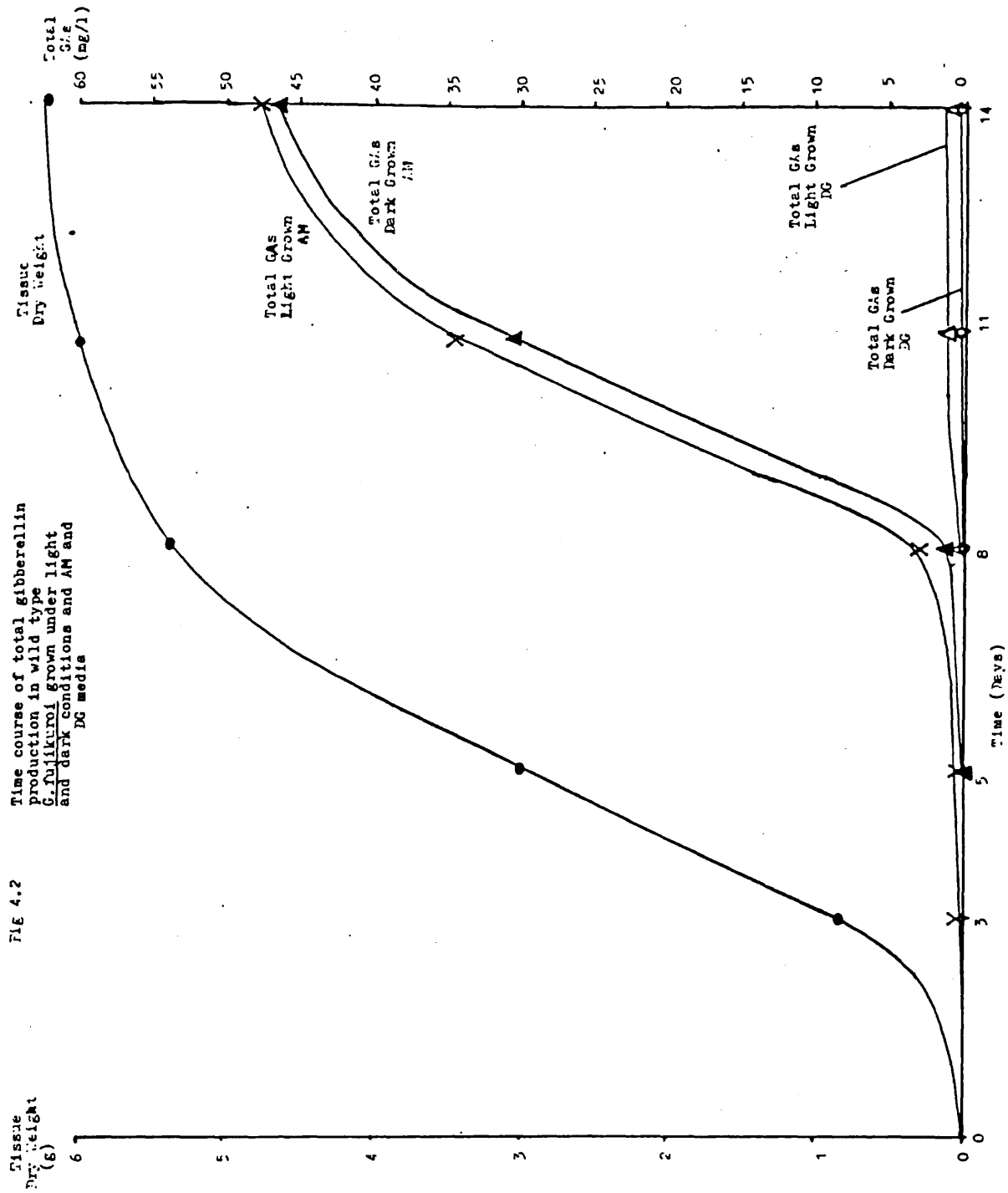
A number of mutants were then analyzed in a similar manner for GA levels, though only one age of growth was examined.

##### 4.3.1 Wild Type IMI 58289

IMI 58289 was grown over 14 days in AM and DG liquid shake cultures, in light- and dark-grown conditions. At 3,5,8,11 and 14 days, culture media were extracted for GA analyses.

From the time course ( Fig 4.2 ) it is apparent that GA biosynthesis in DG media is extremely low and relatively negligible compared to AM-grown, GA biosynthesis in wild type G.fujikuroi. This is to be expected in a low C:N ratio medium, which generally reduces secondary metabolite biosynthesis (1.4.3.i).

The GA levels in AM-grown wild type, though low up to 8 days,



increase at the onset of stationary growth, reaching a level which is almost 40 fold greater than that in DG medium (Table 4.1) in both light- and dark-grown conditions. This induction of GA biosynthesis at the end of log-growth reflects a secondary metabolite property, production coming from the excess C remaining in cultures after all the N has been assimilated for tissue growth.

Light- and dark-grown GA levels do not show any apparent differences in this strain, although there are slightly greater light-grown GA levels (Table 4.1). However, in DG medium at later stages of growth and in AM at 8 days growth where GA levels are quite low, the relative differences indicate a light induced GA level. It may be argued that such differences are only apparent because of the low, total GA levels and in fact the differences may not be significant, since the total amounts are so small. This point is refuted from other results with SG22, where elevated GA levels compared to the wild type, indicate these differences are of similar magnitude (4.3.2 and Table 4.2).

The light- and dark-grown differences are apparent in each of the 5 GAs (Table 4.1). However, the levels are relatively low, especially before 11 days, so again any differences cannot be considered conclusive. These low levels also prevent comparison of the individual GAs, although in most cases GA<sub>3</sub> appears to be predominant and at very late stages of growth GA<sub>13</sub> may be considered the next most common: GA<sub>4</sub>, GA<sub>7</sub> and GA<sub>14</sub> are found in similar quantities.

#### 4.3.2 Mutant Strain SG22

SG22 was grown in an analogous manner to the wild type strain

Table 4.1 The major gibberellin levels produced over time in wild type *G. fujikuroi*, grown under light and dark conditions and in AM and DG media.

Conditions of Growth			GA <sub>3</sub>	GA <sub>13</sub>	GA <sub>7</sub> (mg/l)	GA <sub>4</sub>	GA <sub>14</sub>
Age	Media	Light					
3 Days	AM	Light	0.08	0.01	t	t	t
		Dark	0.01	t	t	t	t
	DG	Light	t	t	t	t	t
		Dark	t	t	t	t	t
5 Days	AM	Light	0.46	0.11	0.02	0.08	0.02
		Dark	0.08	0.06	t	t	t
	DG	Light	0.16	0.01	0.01	t	t
		Dark	0.13	0.01	t	t	t
8 Days	AM	Light	1.76	0.04	0.11	0.56	0.53
		Dark	0.24	0.01	0.03	0.14	0.33
	DG	Light	0.12	t	0.01	0.04	0.01
		Dark	0.17	t	t	t	t
11 Days	AM	Light	26.5	5.33	0.30	0.29	0.81
		Dark	21.2	3.95	0.21	0.30	0.50
	DG	Light	0.09	0.01	0.01	0.03	0.02
		Dark	0.03	t	t	t	t
14 Days	AM	Light	38.7	6.70	0.50	0.40	0.90
		Dark	34.9	6.90	1.70	0.60	1.00
	DG	Light	0.03	0.05	0.06	0.02	0.04
		Dark	0.02	t	0.05	t	t

t=trace levels < 2µg/l

Tissue dry weights (see Fig 4.2) similar, at any given time, under the different growth conditions

(4.3.1) and the media were analyzed for GA levels from DG and AM, light- and dark-grown cultures at 3,5,8,11 and 14 days (Fig 4.3 and Table 4.2).

As for the wild type, DG medium was found to be a poor GA producer compared to that of AM-grown cultures. Also in the DG medium there were no detectable differences between the light- and dark-grown strains, although there may be a slight increase in GAs in the light.

The total levels of GAs in DG compared to those found in the wild type under similar growth conditions, do appear to be greater in SG22. However, in light- and dark-grown cultures, the relatively low levels of GAs make it difficult to be certain that the apparent GA-level differences are significant.

SG22 grown in AM medium on the other hand, produces sufficient GA levels to enable meaningful differences to be detected, both under different growth conditions and compared to the wild type.

The most immediate difference of the GA levels between SG22 and the wild type grown in AM media, is that the total levels are much greater in SG22 than those in the wild type; this is the case at all stages of growth although the differences are not all of the same order (Table 4.1 and Table 4.2). The difference appears to be the rate of GA production, which results in GAs being produced during log-growth, especially in light-grown tissues where maximal GA levels appear to be reached by 11 days (Fig 4.3).

This rate of production is also seen to be greater in light-grown SG22 compared to GA synthesis in dark-grown cultures. In dark-grown AM, GA production begins around 8 days, as in



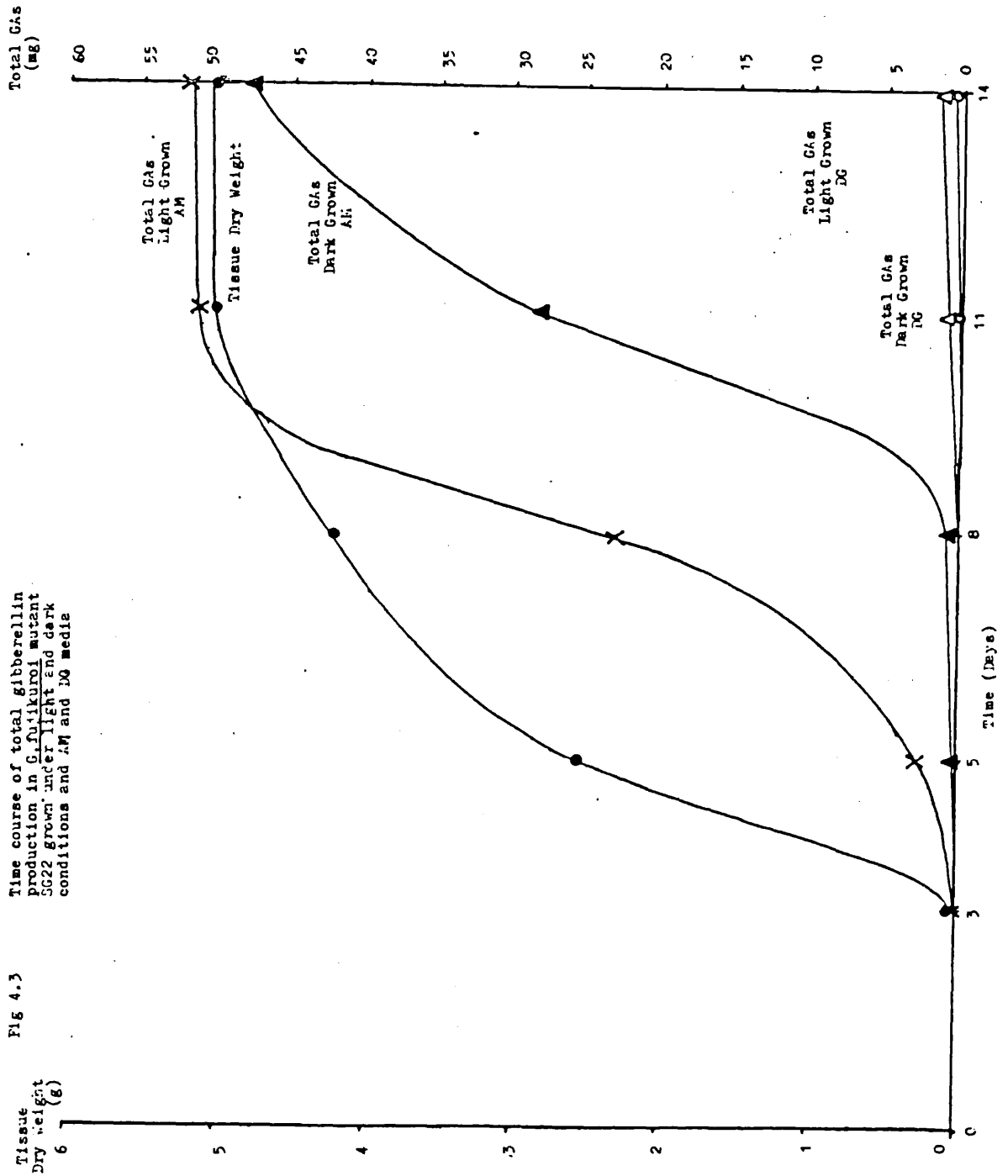


Table 4.2 The major gibberellin levels produced over time in *G. fujikuroi* mutant SG22 grown under light and dark conditions and in AM and DG media.

Conditions of Growth			GA <sub>3</sub>	GA <sub>13</sub>	GA <sub>7</sub>	GA <sub>4</sub>	GA <sub>14</sub>
Age	Media	Light	(mg/l)				
3 Days	AM	Light	0.03	t	t	t	t
		Dark	t	t	t	t	t
	DG	Light	t	t	t	t	t
		Dark	t	t	t	t	t
5 Days	AM	Light	2.03	0.05	0.07	0.03	0.12
		Dark	0.13	0.03	0.03	0.18	0.10
	DG	Light	t	t	t	t	t
		Dark	t	t	t	t	t
8 Days	AM	Light	15.8	4.67	5.13	0.27	7.70
		Dark	0.33	0.04	0.18	0.08	0.21
	DG	Light	0.03	0.09	0.04	t	t
		Dark	0.02	0.08	t	t	t
11 Days	AM	Light	35.9	6.50	3.62	0.80	0.33
		Dark	20.5	4.13	3.27	0.67	0.33
	DG	Light	0.10	0.19	0.90	0.25	0.20
		Dark	0.65	0.56	0.05	0.32	0.19
14 Days	AM	Light	39.8	7.35	3.30	0.83	1.50
		Dark	33.9	9.30	2.30	1.50	0.83
	DG	Light	0.19	0.29	0.20	0.59	0.57
		Dark	0.12	0.12	0.08	0.67	0.80

t=trace levels < 2µg/l  
Tissue dry weights (see Fig 4.3)  
similar, at any given time, under  
the different growth conditions

the wild type, whereas in light-grown AM, SG22 synthesizes GAs concomitantly with the onset of growth. In addition, the rate of GA production beyond 8 days in dark-grown, AM, SG22 is greater than either light- or dark-grown, AM, wild type. So there is an increase in GA biosynthesis in both light- and dark-grown cultures of SG22 compared to the wild type; the light-grown, AM, SG22 rate being greater than that of the dark-grown cultures.

Confirmation that it is the rate that has been affected rather than total production, is shown in that the total GA levels in SG22 and the wild type, in light- or dark-grown, AM conditions, are similar or approaching equivalent amounts at 14 days.

Apparently therefore, SG22 is a 'super producer' of GAs, as it is of carotenoids and sterols (3.3.2), and the regulation that has been affected in this mutant involves the total terpenoid pathway. One may speculate that there is some common regulatory point, or even points, in the pathway, before FPP, which normally regulates terpenogenesis and it is this which has been deleted in SG22. Whether each pathway (i.e sterol, GA and carotenoid) has its own regulatory mechanism acting at an early, common point, or early, individual points, or whether only one pathway has feedback regulation which in turn affect the other pathways indirectly, cannot be firmly established. However, the experimental evidence suggests that it is the regulation of the carotenoid pathway that has been affected in SG22, and this indirectly affects other pathways e.g. increasing the rate of GA biosynthesis. This is because there is a major difference between the 'over-production' of carotenoids and the increased rates

of GA and sterol biosynthesis in SG22. While the regulation 'switch-off' (or activation) causes an unregulated carotenogenesis with no apparent maximal level being reached (3.3.2), in sterol (3.3.2) and GA biosynthesis levels still reach a maximum, which for the GAs are only slightly greater than those in the wild type at 14 days. Therefore, the GA and sterol pathways are still subject to some regulation while carotenogenesis is not. This suggests an increased activity of a common, early terpenoid pathway step, increasing total terpenoid production, but while carotenogenesis appears to be un-regulated, the GA and sterol production pathways are still subject to total regulation.

The differences in GA levels of light- and dark-grown, SG22 cultures, especially in AM medium, appear to indicate that light increases the rate of GA biosynthesis before the end of growth and replaces the mechanism that initiates dark-grown GA biosynthesis. This is probably not the result of the mutation but, in line with the previous conclusions that the GA pathway is indirectly altered by effects of carotenogenic regulation, that the increased GA levels in SG22 compared to the wild type cause the light- and dark-grown GA differences to be more apparent. In the wild type these differences are not seen due to low overall GA levels, which on reaching amounts that may be considered significant (beyond 8 days), it is so late in growth that dark-grown synthesis is also substantial: note that the difference between light- and dark-grown, AM, GA levels in the wild type, is apparent at e.g. 8 days but the low levels made it difficult to be sure of photoinduction in this case (Table 4.1 and 4.3.1).

#### 4.3.3 Mutant Strain SG76

The carotenoidless mutant SG76 was grown in AM and DG liquid shake cultures, in light and dark conditions, and the media analyzed at 3,5,8,11 and 14 days for GA content (Fig 4.4 and Table 4.3).

The time course shows similar results to those found in SG22 (Fig 4.3). GA production is low in DG with slightly elevated levels in light-grown tissue compared to those in dark-grown cultures, and, as for SG22, the levels are greater than in the wild type under identical conditions. However, they are also slightly greater in SG76 than in SG22, in DG medium.

In AM media, GA production is higher compared to DG and compared to wild type grown on AM. Furthermore, at 14 days the GA levels are even greater than those in SG22 under similar conditions. The photoinduction phenomenon in SG22 is also seen in AM in SG76 i.e light-grown mycelia produces GAs from an earlier stage of growth and at a greater rate than dark-grown mycelial GA biosynthesis, in which production is only apparent from 8 days. Unlike SG22 however, the total GA levels at 14 days in AM, light- and dark-grown cultures for SG76 are not similar.

Since the mutation in SG76 is probably a structural mutation in phytoene synthetase (6.2.5; Avalos et al., 1985 and Avalos and Cerda-Olmeda, 1986), one could argue that the elevated GA levels compared to those in SG22 (from which SG76 is derived), result from a carotenogenic block allowing an increase of GA pathway substrate (such as GGPP) into GA biosynthesis, thus supporting the proposed point of mutation.

The light- and dark-grown GA level differences may be explained in the same way as for SG22 (4.3.2) i.e. SG76 has increased terpenoid biosynthesis, due to removal of some

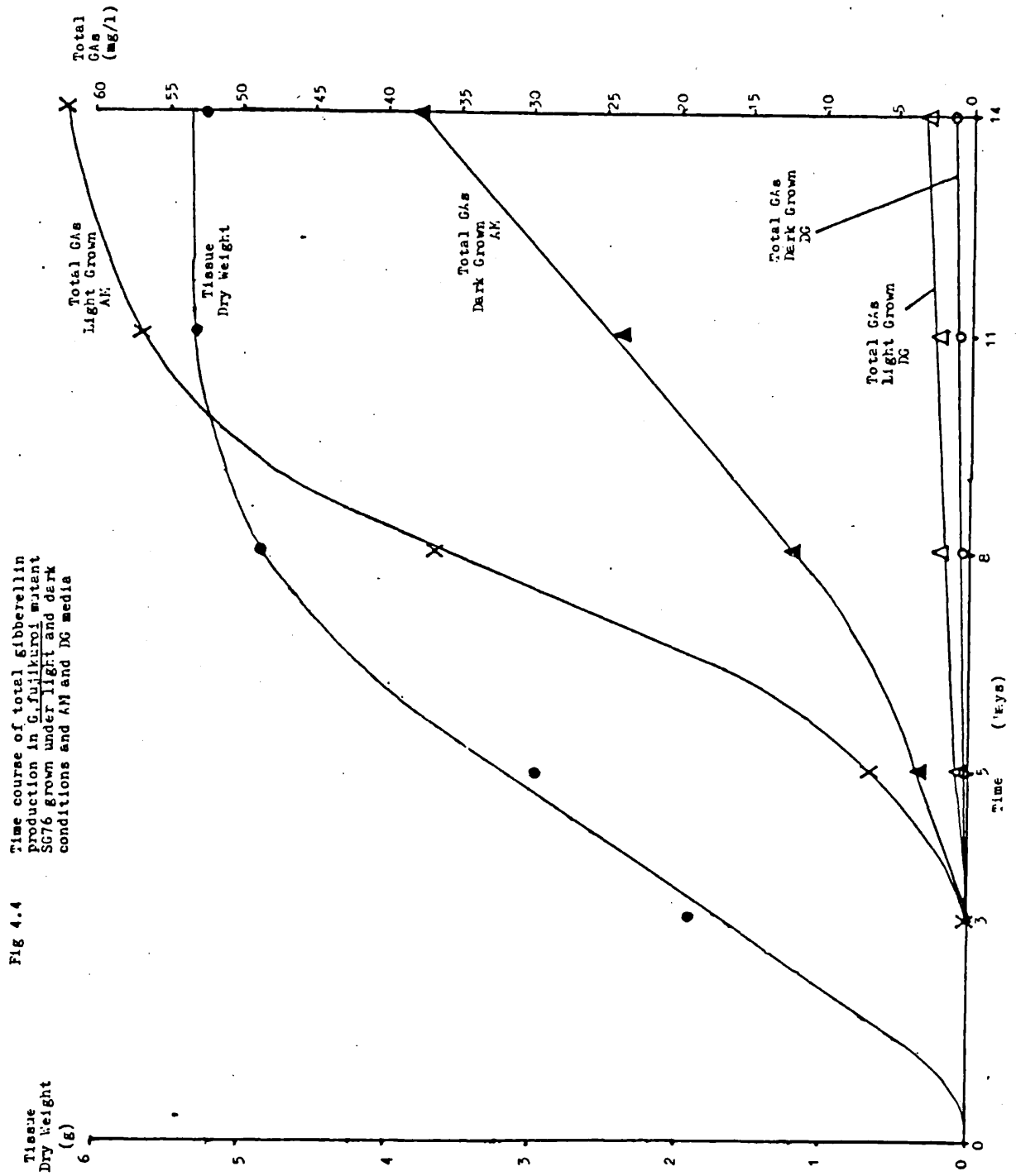


FIG 4.4 Time course of total gibberellin production in *G. fulvikerol* mutant SC76 grown under light and dark conditions and AM and DG media

Table 4.3 The major gibberellin levels produced over time in G.fujikuroi mutant SG76 grown under light and dark conditions and in AM and DG media.

Conditions of Growth			GA <sub>3</sub>	GA <sub>13</sub>	GA <sub>7</sub> (mg/l)	GA <sub>4</sub>	GA <sub>14</sub>
Age	Media	Light					
3 Days	AM	Light	0.08	t	t	t	t
		Dark	0.02	t	t	t	t
	DG	Light	0.01	t	t	t	t
		Dark	t	t	t	t	t
5 Days	AM	Light	6.00	0.06	0.27	0.16	0.21
		Dark	3.33	0.03	0.02	0.02	t
	DG	Light	0.20	0.17	0.19	0.17	0.17
		Dark	0.20	0.13	0.07	0.03	0.03
8 Days	AM	Light	10.0	9.83	9.92	4.45	2.50
		Dark	4.80	2.85	1.87	1.20	1.33
	DG	Light	1.00	0.48	0.32	0.53	0.64
		Dark	0.33	0.29	0.10	0.05	0.43
11 Days	AM	Light	29.4	10.2	11.2	3.30	1.00
		Dark	9.45	7.65	2.20	3.20	0.05
	DG	Light	1.00	0.39	0.10	0.20	0.30
		Dark	0.20	0.30	0.21	0.02	0.03
14 Days	AM	Light	33.6	12.7	13.3	2.00	0.40
		Dark	17.1	9.10	3.33	4.67	0.40
	DG	Light	1.12	0.30	0.08	0.02	0.30
		Dark	0.30	0.38	0.13	0.03	0.30

t=trace levels < 2µg/l

Tissue dry weights (see Fig 4.4) similar, at any given time, under the different growth conditions

regulatory mechanism in SG22, which operates at a common, early terpenoid pathway step(s). These increased levels 'exaggerate' light- and dark-grown, GA level differences which actually are present in the wild type (4.3.1). The photoinduction of GA biosynthesis can be replaced by an induction mechanism which is initiated at the end of log-growth, either due to the stage of growth itself and/or the assimilation of medium N which appears to induce secondary metabolism. Unlike SG22 however, dark-grown GA synthesis does not reach equivalent light-grown levels at 14 days. This is unexpected from simple mutation explanations, but one possibility relates to growth. While tissue dry weights are similar in light- and dark-grown cultures and with SG22 and the wild type, it is seen from growth on agar plates (Plate 1.1) that there is a distinct morphological difference between SG76 mycelium and that of the other strains. Perhaps the mutation, or another has effected this change which in turn has effected dark-grown GA biosynthesis: it would be of interest to discover if the levels eventually became equivalent between light- and dark-grown cultures, as their rates suggest they may.

#### 4.3.4 Mycelial GA Levels

The mycelia from the mutants SG22 and SG76 along with wild type IMI 58289, were extracted and analyzed for GA content. Tissue from 3,5,8,11 and 14 days was examined in each case, from DG and AM cultures. In each case the GA levels were too low to be detected ( $< 1\mu\text{g}/20\text{g}$  fresh weight mycelium).

These results led to speculation as to the means by which GAs are extruded from the fungal tissues. It is possible



that the relatively low pH of the medium, favours GA diffusion from the mycelium, but the apparent absence of GAs in the latter suggest some sort of active transport mechanism.

To understand such a system, it is necessary first to consider the possible sites of GA biosynthesis within the fungus to appreciate from where they may be transported. Unfortunately studies of intracellular sites of GA biosynthesis are extremely limited in G.fujikuroi (Stoddart, 1983). However, presuming that the pathway has similar, subcellular affiliations to those determined for the higher plants, one can presume an analogous situation. It has been suggested that while pre-kaurene steps are cytosolic, the proceeding mixed function oxygenase and hydroxylation steps are microsomal, i.e. bound to the endoplasmic reticulum (ER). Therefore, GA biosynthesis is an integrated function of the cytoplasm and the contained (or containing) membrane systems, the later transformations occurring on membranes having a direct interface with the cytoplasm (1.8 and reviewed by Stoddart, 1983). It is further proposed that the later steps may culminate in the formation of secretion vesicles which aid transport from the cells (Stoddart, 1983).

Kaurene and kaurenol have also been detected in media extracts in appreciable quantities (Cross et al., 1963). It could be argued that their appearance may be an indirect result of vesicle formation, for GA secretion, carrying contaminating levels of these metabolites which are synthesized in the approximate region of potential vesicle formation; the presence of the neutral kaurene in the medium also argues against the theory of GA movement by a pH dependent gradient since this would not favour kaurene diffusion.

It has also been suggested that GAs are secreted into vacuoles and glycosylated, to protect them from the action of hydrolytic enzymes (Ohrlogge et al., 1980). The involvement of glycosylation in secretion mechanisms has been observed for a number of enzymes e.g in A.niger (Speake et al., 1979) and in other eukaryotic systems (e.g Hickman and Kornfield, 1978 and Housely et al., 1980) and proposed to confer essential structural stability for translocation (Orishi et al., 1979). This work involved the use of tunicamycin, a glycosylation inhibitor (Lehle and Turner, 1976). It has been used in attempts to inhibit G.fujikuroi GA secretion (Aitken, personal communication). Results so far are disappointing and inconclusive, but further studies may lead to some insight into the GA secretion mechanism in G.fujikuroi.

#### 4.3.5 GA Levels in G.fujikuroi Strains

The GA levels were determined in DG and AM media, under light and dark conditions at 8 days growth in the mutant strains SG4, SG43, SG78, and SG75 along with the wild type strain ACC 917. The results were compared, along with those from SG22, SG76 and wild type IMI 58289 under similar growth conditions (Table 4.4).

##### 4.3.5.i Wild Type Strains CMI 58289 and ACC 917

The 2 wild type strains both produce similar levels of GAs which are low compared to the other mutants (Table 4.4).

Since the effects of media and light on GA production have already been discussed for strain CMI 58289 (4.3.1) and since strain ACC 917 shows a similar pattern the results will not

Table 4.4 Gibberellin levels in the media of 8 day old *Gibberella fujikuroi* strains grown in different media in the light and dark.

Gibberella fujikuroi STRAIN	MEDIA CONDITIONS	Gibberellins (mg/l)					
		GA <sub>3</sub>	GA <sub>13</sub>	GA <sub>7</sub>	GA <sub>4</sub>	GA <sub>14</sub>	Total
WILD TYPE IMI 58289	AM Light	1.76	0.04	0.11	0.56	0.53	3.0
	Dark	0.24	0.01	0.03	0.14	0.33	0.8
	DG Light	1.12	t	0.08	0.04	0.12	1.4
	Dark	0.17	t	t	t	t	0.2
WILD TYPE ACC 917	AM Light	1.00	0.14	1.20	t	0.23	2.57
	Dark	0.21	0.04	t	t	0.33	0.58
	DG Light	0.11	t	t	t	t	0.11
	Dark	t	t	t	t	t	t
SG43	AM Light	7.0	2.1	0.87	0.80	2.5	13.3
	Dark	0.63	0.23	0.84	0.83	0.22	2.8
	DG Light	1.3	0.63	1.47	1.5	0.63	5.0
	Dark	0.48	0.17	0.09	1.2	0.46	2.4
SG4	AM Light	9.8	3.85	3.8	0.67	5.5	23.9
	Dark	4.13	0.17	1.54	0.07	1.07	7.0
	DG Light	0.09	t	t	t	t	0.1
	Dark	t	t	t	t	t	t
SG22	AM Light	15.84	1.2	3.08	0.11	2.7	22.9
	Dark	0.32	0.13	0.75	t	0.53	1.7
	DG Light	1.12	0.10	0.18	2.0	0.03	3.4
	Dark	3.7	0.07	0.03	0.03	0.03	3.9
SG78	AM Light	22.0	4.67	5.13	0.27	7.7	39.8
	Dark	0.33	0.04	0.18	0.08	0.21	0.8
	DG Light	1.92	0.12	0.12	1.20	0.04	1.10
	Dark	1.17	0.08	0.06	0.03	0.04	1.20
SG76	AM Light	10.0	9.83	9.92	4.45	2.5	36.7
	Dark	4.8	2.8	1.87	1.2	1.33	12.0
	DG Light	3.3	2.9	1.3	0.5	4.3	12.3
	Dark	1.0	0.48	0.32	0.53	0.84	3.0
SG75	AM Light	10.3	3.33	2.1	3.38	3.15	22.3
	Dark	3.18	0.70	0.15	0.03	0.33	4.4
	DG Light	3.33	0.57	0.17	0.83	0.50	5.4
	Dark	1.8	0.20	0.15	0.13	0.07	2.4

Tissue dry weights for each strain under each condition are relatively similar being  $4 \pm 0.5g$

t = trace amount  
 $< 2\mu g/l$

be discussed here. The major observations are that AM medium favours GA production compared to DG-grown cultures; at 8 days GA production is photoinduced i.e greater in light-grown cultures than in dark-grown of either wild type and that GA<sub>3</sub> is predominant.

#### 4.3.5.ii Mutant Strain SG4

This mutant does not produce carotenoids (Avalos et al., 1985 and Avalos and Cerda-Olmeda, 1986) and appears to be blocked at phytoene synthetase, thus accumulating PPPP (5. From the GA figures for SG4 (Table 4.4) it appears that this mutational block in the carotenoid pathway has effected a redistribution of prenyl units into the GA pathway and produced the elevated levels compared to the wild type.

SG4 shows similar light- and dark-grown GA level differences to IMI 58289, except the amounts involved are larger. Therefore, the mutation does appear to be an enzymic block.

The relative amounts of each individual GA however, have not increased accordingly compared to those seen in the wild type. There may be complex regulatory mechanisms within the late GA pathway which affect GA distribution when amounts are elevated to these higher levels.

Another slightly unusual aspect of the GA levels in SG4 is the low levels seen in DG media which are lower than those in the wild type. This may be due to the relative ages of the cultures. Although the strains are very close in their tissue dry weights at 8 days, there may well be a difference in the maturity of culture, such that a less mature SG4 in DG media may produce less GAs than a more mature culture of wild type. Since the levels of the GAs in both cases are so low,

a slight difference in maturity may cause an apparently marked effect. It is more difficult to explain these observations as an actual phenomenon, especially in view of the other mutant figures, but it should be noted that the mutant was first described as a non-GA producer (Avalos, personal communication) being analysed on DG medium.

#### 4.3.5.iii Mutant Strain SG43

SG43 is a phytoene accumulating mutant (3.3.4) derived from wild type IMI 58289. It produces similar GA level patterns to that found in SG4 under all conditions of growth (Table 4.4). There is a greater production of GAs in AM-grown cultures compared to the levels found in DG media and light-grown cultures produce more GAs than dark-grown at 8 days.

The media differences have already been observed in other strains (4.3.1-4.3.3) and result from the higher C:N ratio favouring secondary metabolism.

The light- and dark-grown differences in the GA levels seen in wild type IMI 58289 (4.3.5.i) are also seen in mutant SG43 which, as in SG4, involve higher GA levels which in turn exaggerates these differences.

The elevated GA levels in this mutant, as in SG4, may result from a mutational block in the terpenoid pathway which allows an increased GA pathway activity by means of an increased precursor availability. The total GA levels, though much greater than the wild type, are slightly less than those found in SG4. This may be because the mutational block in SG43 is later in the pathway than it is in SG4, so it is possible that more isoprene units are available to the GA pathway in the latter, compared to SG4. That this is the

reason for the different GA levels between SG4 and SG43 may also be deduced from the total terpenoid pathway figures. The difference between these 2 mutants is the enzyme step phytoene synthetase, which from the carotenoid results produces 0.63mg phytoene/g dry weight in SG43 (3.3.4). Effective removal of this enzyme in SG4 sees a rise in GA levels of about 10mg/l which approximates to 0.60mg/g dry weight i.e. an equivalent GA level rise to a phytoene level fall, thus supporting the proposed mechanism.

#### 4.3.5.iv Mutant Strain SG22

The GA levels of the mutant SG22 have already been discussed under different growth conditions (4.3.2) and are included in Table 4.4 for comparison with the other strains.

The levels compared to the wild type, SG4 and SG43 indicate that while in these mutants, compared to the wild type, the explanation for the elevated GA levels is a terpenoid pathway block causing an influx of precursors into GA biosynthesis, in SG22 the pathway is fully functional and elevated GA levels (and carotenoids and sterols) are purely due to the inactivation (or activation) of a regulatory mechanism affecting the whole terpenoid pathway.

#### 4.3.5.v Mutant Strain SG78

GA levels in SG78 have similar differences in media, and light- and dark-grown cultures, to those in the strains already discussed, and so no further explanation is necessary for these phenomena.

The GA levels themselves are greatly elevated (even higher than SG22), especially in light-grown, AM media. Since SG78

is blocked in phytoene synthetase (3.3.5) it is probable that these raised levels, compared to SG22 from which it was derived, result from this mutational block causing an influx of prenyl units into the GA pathway. This bears direct analogy with the mutant SG43 and the wild type 'parent', except that the total GA levels involved are greater in the case of SG22 and SG78.

#### 4.3.5.vi Mutant Strains SG75 and SG76

Both SG75 and SG76 are blocked in phytoene synthetase, accumulating PPPP (6.2.5). By the previous analogy of comparing total GA levels in SG4 and SG43 (4.3.5.iii), the mutants SG75 and SG76 should both accumulate more GAs than SG78, since the latter is blocked later in the carotenogenic pathway, so less GA biosynthesis may result from excess GGPP (precursor) availability. However, this is not the case and in both instances the total GA levels are somewhat less than in SG78. So another explanation is required in order to fit in with the models already proposed for each mutant i.e. pathway blocks and subsequent redistribution of isoprenes into different parts of the terpenoid pathway.

One possibility is that the elevated GA levels have had an effect on GA biosynthesis regulation and distribution of the different GAs. This is particularly seen in SG76 where total GA levels are quite similar to those in SG78, under AM, light-grown conditions, except that the individual GAs have different levels, suggesting that a qualitative change in the GAs has occurred. This is further supported by the elevated, dark-grown, AM and DG media, GA levels (in light and dark), compared to the same conditions in the

mutant SG78. In these instances the raised levels show the pattern expected for a simple block in carotenogenesis, but since the levels are not high i.e. not as great as those seen in light-grown, AM cultures, the GA pathway is not affected in the proposed regulatory mechanism.

This is also noticeable in SG75, and similar explanations as those for SG76 can be given. However, the light-grown AM conditions result in a total GA level which is slightly less than that in SG76 (and SG78) which is a further complication to the proposed models. It may be argued that a more complex regulation within the GA pathway is in operation and once the GA levels and total biosynthesis is affected in any way, such mechanisms are initiated.

Another explanation for the atypical GA distribution, induced within the mutants SG75 and SG76, especially the lower than predicted total GA levels in SG75, is that of an enzyme complex being affected. Since phytoene synthetase and kaurene synthetase both require GGPP as a substrate, it is possible that these 2 enzymic steps may be involved in a complex; thus allowing mutual regulation which may be necessary at such a biosynthetic branch point (1.8). If this is the case, then perhaps the mutation in SG75 and SG76 i.e. at phytoene synthetase has, in some way, impaired the efficiency of kaurene synthetase, thus reducing the predicted high GA level in these mutants, especially SG75.

In any case, the GA biosynthetic pathway may well be subject to more complex regulatory phenomena than can be inferred from these simple mutations and the proposed models of explanation.

The light- and dark-grown differences in the GA levels, and



those between the different media, show similar patterns in all the strains so far discussed and so demand similar explanations.

#### 4.4 Summary and Conclusions

The technique developed for analysis of GAs allows a suitable recovery and detection of the different GAs at levels as low as 4µg/l.

5 major GAs were detected in all the strains tested, GA<sub>3</sub>, GA<sub>4</sub>, GA<sub>7</sub>, GA<sub>13</sub> and GA<sub>14</sub>. These were also found to be the major GAs for G.fujikuroi by another group (Hanson, personal communication).

The peaks on HPLC (Fig 4.1) that could not be identified as GAs, were determined not to be GAs by their behaviour under different growth conditions, which did not match those of the identified GAs. However, whether within the HPLC separation some GAs are hidden by these non-GA components, or are eluted at 100% MeOH with a large number of other less polar compounds, cannot be determined. This may well be the case since other GAs have been detected at trace levels in G.fujikuroi filtrates (1.4.1). However, the present study indicates that when the GA levels are high i.e late in growth, there are no major, unidentified peaks eluted from the media extracts, so any other GAs may be trace.

Media appear to make a significant difference to the total GA levels produced, irrespective of the strain tested. This is probably the result of C:N ratios, such that high ratios induce GA biosynthesis due to the presence of excess C after N assimilation, at the end of log-growth. This C is thus available for secondary metabolite synthesis which will not

occur at lower C levels i.e. DG medium.

The most interesting observation is the effect of light on GA levels. It appears that while GA biosynthesis is activated when a strain reaches the stationary phase of growth, light can replace this, or precede, the induction such that GA synthesis begins during log-growth. It is suggested that that this photoinduction is not a result of any of the mutations but it is common to all the strains examined. It is only in the wild type G.fujikuroi that the total GA levels are so low, in early stages of growth, that the light and dark differences are not obvious. When GA levels are elevated, as an indirect result of a mutation, the differences are more apparent. The phenomenon is further complicated by the activation of GA biosynthesis at the onset of stationary phase growth, and the resulting levelling of light- and dark-grown GA levels to similar maxima, late in stationary phase growth. That these maxima are reached suggests that the elevated, light-grown synthesis is almost an indirect result, or side effect, of a photoinduction of an early, common terpenoid pathway step. The different carotenoid levels of light- and dark-grown cultures never approach similar levels (3.3.2) so the whole pathway, and possibly post-GGPP steps, are photoinduced. The GA pathway is still subject to total regulation i.e. light- and dark grown- levels both reach similar maxima, thus photoinduction is not an inherent regulatory mechanism in GA biosynthesis: it would be interesting to see if, within a time course, there is an initial surge of light-grown ergosterol levels, which also reach equivalent maximum levels between light- and dark-grown cultures. This would suggest a similar mechanism as is

proposed for the GAs and a common terpenoid pathway.

On the whole, the GA levels within the different mutants seem to reflect the predicted result of an enzymic block within the terpenoid pathway, causing a redistribution of individual terpenoid synthesizing activities. Furthermore, the resulting GA levels in each of the mutants and strains tested seem to correlate with the sterol and carotenoid figures (Chapter III). For example, SG22 is affected in total carotenoid and sterol regulation and is also affected in total GA level production so that the mutation is explicable in that it has affected the whole terpenoid pathway. Also a block, such as that in the mutant SG4, compared to SG43, causes a fall in phytoene synthesis which is matched by an equivalent rise in the total GA levels (4.3.5.iii).

However, as the results for the mutants SG75 and SG76 exemplify, one cannot always propose simple pathway blocks and resulting 'flux' changes to explain observed phenomena. Therefore, when considering such large and complex pathways, which are subject to many possible forms of regulation, it is important to appreciate that a number of unknown, regulatory mechanisms may be operative which may prevent straight forward interpretation of results.

CHAPTER V

RESULTS

The Development and Properties  
of In Vitro Systems from the  
Wild Type and Mutants of  
Gibberella fujikuroi

"By indirections find directions out"

W.Shakespeare

## 5.1 Introduction and Aims

In vitro or cell-free systems can provide a number of valuable experimental techniques for metabolic and regulatory studies. In developing such systems it becomes possible to isolate biosynthetic pathways and, in some cases, individual enzyme-catalysed steps for assay. Consequently, one can determine the enzyme activities within these pathways and possibly their regulation; which in an otherwise in vivo system may be limited by interference of other pathways or endogenous regulators. Thus with a cell-free system it may be possible to examine certain enzyme activities in isolation.

However, the use of in vitro systems for analysis of enzyme activities also has a number of drawbacks, since the cell-free systems may possess artifactual properties which must be considered in order to avoid erroneous conclusions from the experimental data.

An initial problem is that of obtaining an enzymically active cell-free system. The technique for developing an in vitro system, varies tremendously depending on the system used and the workers using it.

An important step is cell-breakage for which a number of methods can be employed, including: the French Press; acetone powder preparations; bead beating; enzymic lysis; osmotic shock; sieving freeze-dried tissue and liquid N<sub>2</sub> with subsequent grinding. Any technique which breaks the cell-wall is sufficient, but in doing this one may subject the intracellular enzymes to harsh conditions which may disrupt and damage their natural activities. Furthermore, in damaging the cells one destroys any compartmentation present in vivo. This in turn may result in altered pathway activities by effects on certain regulatory mechanisms and by the possible

release of degradative enzymes from the lysosome e.g. proteolytic enzymes, phosphatases and lipases, all of which may severely disrupt normally functional systems.

In developing a cell-free system, one must also obtain suitable substrates in order to assay pathway activities. It is common to use a spectrophotometric assay if possible, but if not, radiolabelled precursors enable analysis by means of levels of incorporation. Certain precursors will be easier to obtain radiolabelled than others, either commercially or by chemical and biochemical syntheses. The system used must be able to utilize the substrate e.g. a lipid-soluble substrate would not be appropriate in an aqueous environment. Preferably, the substrate should be exclusive to the pathway to be investigated in order to avoid interference from competing enzymes.

Finally, with respect to using radiolabelled, in vitro systems, when examining the activity of an individual step within a pathway, unless it is in isolation, the activity will be affected by the preceding or following steps. This may result in an apparent activity which does not reflect the enzyme itself but the group of enzymes. Consequently, in vitro systems are more commonly used to examine individual steps in isolation, not within a whole pathway, unless total pathway activity is to be assessed. Furthermore, in studying whole pathways it may prove useful to use cell-free systems for comparative analysis of relative activity levels e.g. the activity under different conditions or in the presence of regulators as opposed to total activities from different systems which may be more subject to individual variations.

If these problems can be overcome, however, and a suitable cell-free system is developed, the in vitro system is an

invaluable tool for biochemical analysis of enzyme activities and whole pathway activities, together with associated regulatory mechanisms.

The aims of this part of the project were to develop an in vitro system from G.fujikuroi exhibiting enzymic activities of the terpenoid biosynthetic pathway. The in vivo data (Chapters III and IV) involved analyses of each individual terpenoid pathway and the levels of intermediates were extrapolated towards the total activity of the pathways. The disadvantage to such conclusions is that they do not necessarily reflect the individual enzyme activities, nor indeed the activity of the whole pathway and the interrelatedness of the GA, sterol and carotenoid pathway activities: these cannot be accurately established by merely examining product levels.

Another aspect of cell-free systems which will be exploited in this investigation, is that they suggest the subcellular location of an enzyme, or group of enzymes, within a cell i.e. one may determine whether the activity is cytosolic or microsomal: determining enzyme location may be complicated if organelles are disrupted in preparation of a cell-free system.

A number of in vitro systems have been established in different systems, including fungi, and used to investigate the terpenoid pathway. However, they are generally specific for a particular area of the pathway, e.g. the carotenoids (1.3), the sterols (1.7) and the GAs (1.5). In G.fujikuroi an in vitro system has been developed to examine the GA pathway (reviewed by Hedden, 1983) but not in the carotenoid or sterol pathways. The aim in this study was to obtain in

vitro activities of the sterol, carotenoid and GA pathways.

Usually the soluble, cytosolic enzymes are obtained in an active form since their soluble form suggests that they may be removed from a cellular system and retain activity in an artificial environment. This being the case, one may be able to incorporate chosen substrate into the prenyl pyrophosphates at least, since these steps are cytosolic in several organisms (see 1.1.1). The substrate in the present study is [2-<sup>14</sup>C]-MVA, a water soluble compound which is the first committed terpenoid precursor.

Difficulties are often found in obtaining the microsomal or membrane bound enzymes in an active cell-free system. These enzymes may be dependent on associated lipids or proteins in the membrane for integral structure and function, and cellular disruption may well inactivate these proteins. On the other hand, the preparation technique may produce membrane fractions which retain active enzyme complexes capable of carrying out their normal activities. It may also be possible to remove an enzyme from the associated membrane by the preparative technique employed. This is especially likely in the case of the peripheral proteins. Such extrinsic enzymes may be involved in linking cytosol substrates with membrane products. An examination of the terpenoid pathway (see 1.1) suggests, on the basis of intermediate polarities, the points in the pathway where such enzymes may operate. Squalene, kaurene and phytoene synthetases all use polar substrates to produce apolar products, and therefore may be located at the cytosol/membrane interface. If these enzymes are like peripheral proteins they may possess the ability to remain active in a soluble system, due to their relationship with the cytosol.



Consequently, it may be possible to obtain these enzymes in a soluble, cell-free system. Though this has been seen to be the case for phytoene synthetase in P.blakesleeanus (Bramley and Davies, 1975), squalene synthetase in other systems is generally membrane bound (see 1.1.3), although kaurene synthetase is found to be cytosolic (see 1.5.1).

Complete cell-free systems have been developed in other organisms for sterol biosynthesis (1.7), GA biosynthesis (1.5) and carotenogenesis (1.3).

Generally, since tightly bound membrane enzymes are difficult to obtain in an active cell-free system, it may be difficult to obtain activity into the acidic carotenoids (reviewed by Bramley, 1985) such as NX.

## 5.2 Additional Methods

The in vitro system utilized (2.2.15) was an adapted method of Bramley (1973).

[2-<sup>14</sup>C]-MVA was used as substrate, and the buffer and co-factors used were analogous to those in a number of other systems (reviewed by Bramley, 1985). DTT was used due to its general thiol-protecting properties (Cleland, 1963); it has been shown that IPP isomerase from tomato fruit plastids is stabilized by DTT (Spurgeon et al., 1984) and the same enzyme from yeast (Arganoff et al., 1960) and pig liver (Shah et al., 1965) is stimulated by thiol protecting reagents.

Fluoride was used to inhibit phosphatase activity (see 5.3.1) as indicated by Upper and West (1967).

The assay systems used were mainly TLC systems (outlined in Tables 2.2.2, 2.2.3a and b), and intermediates identified by cochromatography and by spark chamber localization (2.2.19).

## 5.3 Results and Discussion

### 5.3.1 Alkaline Phosphatase Activities in G.fujikuroi

#### 5.3.1.i Standard Phosphatase Activity

A standard calibration curve of para-nitrophenol (pNP; 0-0.5 $\mu$ M) against absorbance at 405nm (determined to be the  $A_{\max}$  for pNP) was constructed (Fig 5.1), and this was used in the assay of commercial alkaline phosphatase activity in 0.4M Tris-HCl buffer pH 8.0 and 0.4M potassium phosphate buffer pH 7.5, with or without F (2.2.16). The formation of pNP with time, under these conditions, was determined (Fig 5.2). and the enzymic activities calculated (Table 5.1).

The results suggest that phosphate buffer inhibits phosphatase activity, compared with that found in Tris buffer. This probably occurs by inorganic phosphate competing with the phosphatase active site, and thus inhibiting activity. It has been shown that inorganic phosphate stimulates carotenogenesis in B.trispora (Dholakia and Modi, 1984), and this is probably due to the phosphate inhibiting phosphatase breakdown of the prenyl pyrophosphate intermediates in carotenogenesis: these workers suggest that this phenomenon is a natural regulatory mechanism in secondary metabolism.

Fluoride, a known inhibitor of phosphatase (Upper and West, 1967) also reduces phosphatase activity in Tris-buffer. However, in phosphate buffer this inhibition effect is absent. Therefore, although both phosphate buffer and F inhibit phosphatase activity individually, the effects are not additive suggesting similar modes of action. Fluoride also stimulates carotenogenesis in a similar manner to inorganic phosphate in B.trispora (Dholakia and Modi, 1984).

Fig 5.1 Standard Calibration  
Curve of pNP

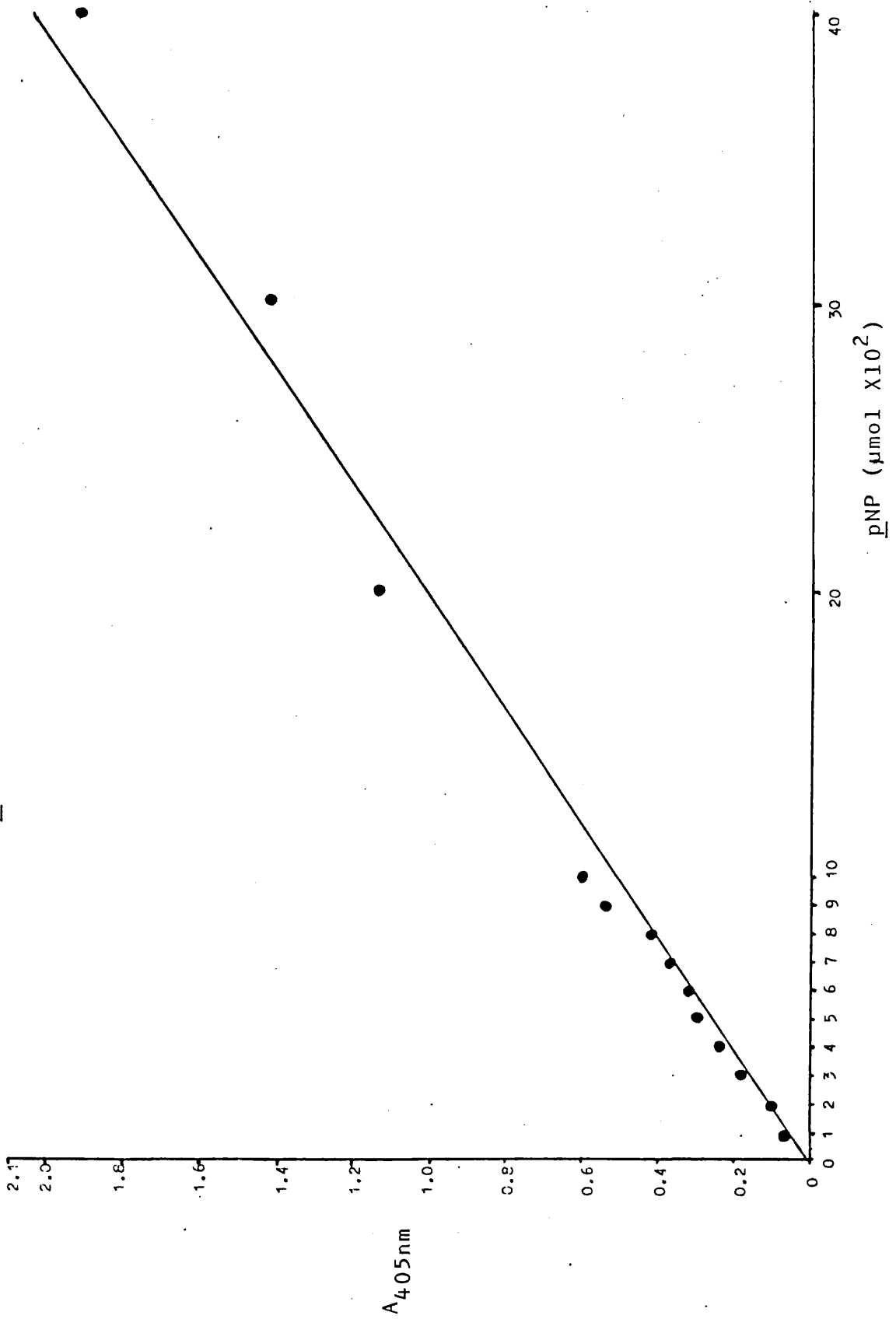


Fig 5.2 Amounts of pNP formed from PNPP, over time, by Standard Alkaline Phosphatase in Tris-HCl pH 8.0 and Potassium Phosphate pH7.5 Buffers, with and without F

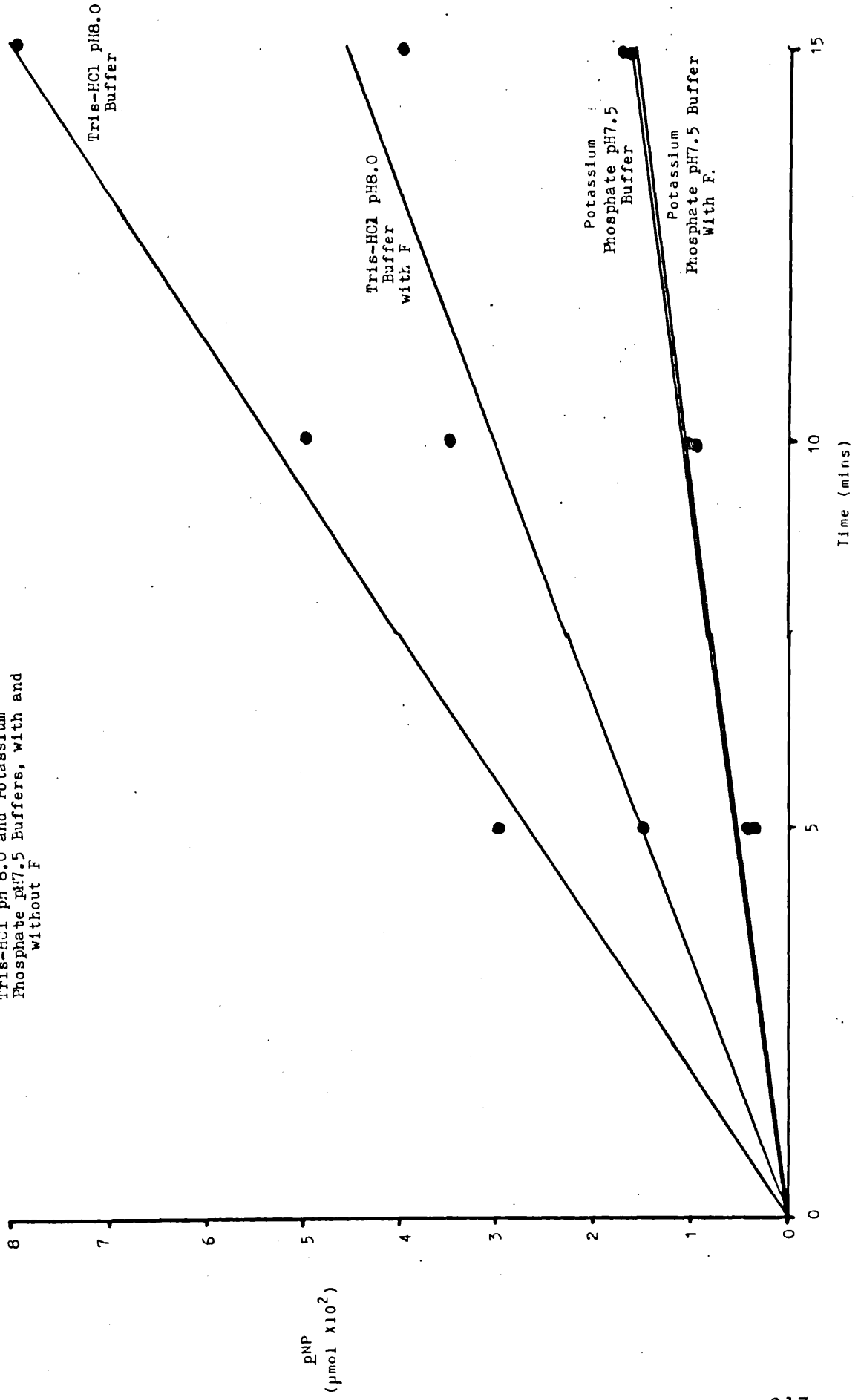


Table 5.1 Rates of pNP Formation from pNPP by Commercial, Alkaline Phosphatase in Different Buffers with and without F

Assay Conditions	Rate of pNP Formation ( $\times 10^3$ $\mu\text{mol}/\text{min}$ )
Tris-HCl pH8.0 Buffer containing 5mM DTT	5.33
" plus 10mM Fluoride	3.00
Potassium Phosphate pH7.5 Buffer containing 5mM DTT	1.23
" plus 10mM Fluoride	1.17

Another explanation is that the pH of each buffer has an effect. The higher pH may favour phosphatase activity over that at the lower pH, so that at pH 7.5 the inhibitory mechanism of F is prevented. However, both F and inorganic phosphate at pH 7.2, stimulate carotenogenesis in B.trispora (Dholakia and Modi, 1984), so suggesting that their modes of action are in fact, similar.

#### 5.3.1.ii In Vitro Phosphatase Activities

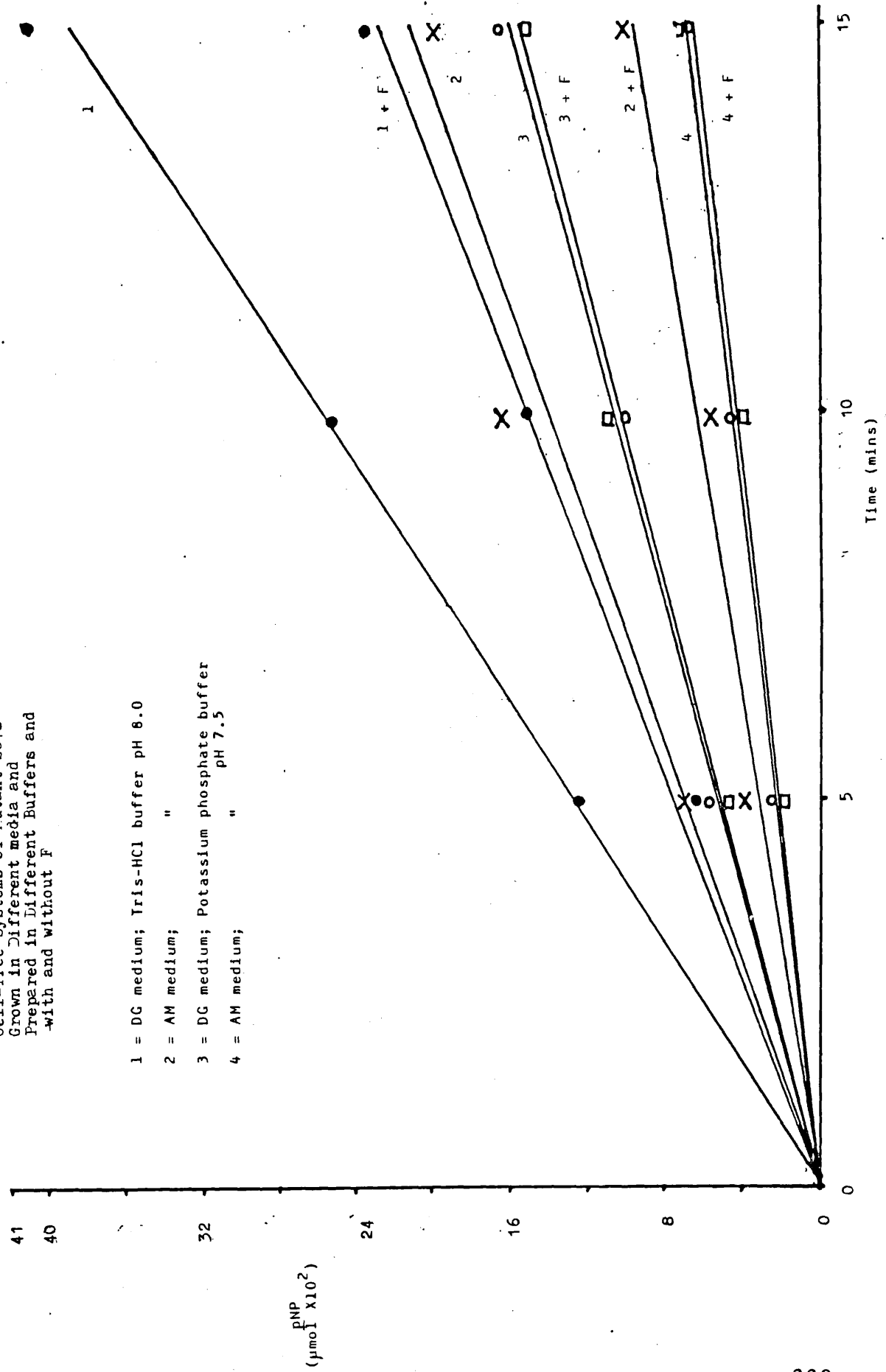
Having established the effects of buffers and F on commercial alkaline phosphatase activity, the endogenous phosphatase activities, in S<sub>10</sub> preparations (2.2.14) of SG78 in Tris and phosphate buffers, with and without F (2.2.16), were determined (Fig 5.3 and Table 5.2).

Phosphatase activity is present in both preparations, and this may reduce total incorporation into the terpenoids (see 5.3.2). The activity is higher in DG-grown mycelia than that from AM cultures. This may reflect a generally higher metabolic activity in DG-grown tissue (the mycelial dry weights are approximately the same in both tissues), since this medium has higher N levels than AM, which in turn has a high C:N ratio. The latter may be specific towards secondary, rather than general mycelial metabolism, which in turn may include phosphatase activity, as seen in DG cultures. If inorganic phosphate is an inhibitor of secondary metabolism (suggested by Dholaki and Modi, 1984), one may be seeing a regulatory mechanism by which altered phosphatase levels regulate terpenoid biosynthesis.

Within the differences of phosphatase activity between DG- and AM-grown mycelia, it appears that the effects of F and the different buffers, are equivalent from each tissue and

Fig 5.3 Amounts of pNP formed from pNPP over time by Endogenous Phosphatase in Cell-Free Systems of Mutant SG78 Grown in Different media and Prepared in Different Buffers and with and without F

Fig 5.3



- 1 = DG medium; Tris-HCl buffer pH 8.0
- 2 = AM medium; "
- 3 = DG medium; Potassium phosphate buffer pH 7.5
- 4 = AM medium; "

Table 5.2 Rates of pNP formation from pNPP by Endogenous Phosphatase in Cell-Free Systems from SG78, Grown in Different Media and Assayed Under Various Conditions

Assay Conditions*	Rates of pNP Formation ( $\times 10^3$ $\mu\text{mol}/\text{min}$ )
1	22.1
1 + F	16.5
2	15.0
2 + F	7.2
3	10.0
3 + F	10.0
4	4.4
4 + F	4.4

\* 1 = DG Medium; Tris-HCl buffer pH 8.0

2 = AM Medium; "

3 = DG Medium; Potassium phosphate buffer pH 7.5

4 = AM Medium; "



identical to those found with commercial phosphatase (5.3.1.i), i.e phosphate buffer reduces phosphatase and F inhibits the latter only in Tris buffer.

From these data, AM-grown tissue may be more suitable for in vitro terpenoid assays (along with the increased in vivo terpenoid levels: Chapters III and IV). Also, while phosphate buffer reduces phosphatase activity, the pH of the buffer may not favour in vitro activity of the terpenoid pathway enzymes, as suggested for P.blakesleeanus (Bramley, 1973). This is, in fact, the case for G.fujikuroi in which Tris-buffered incubations produce greater terpenoid incorporation levels than in phosphate-buffered incubations (5.3.2). Consequently, if F has no effect on the in vitro terpenoid enzymes (5.3.2), then in cell-free systems of G.fujikuroi for terpenoid activities, 0.4M Tris-HCl buffer pH 8.0 containing F (10mM) and DTT (5mM) should be used.

### 5.3.2 Effects of F and Buffers on In Vitro Synthesis from AM- and DG-grown Cultures

Using 5 day, SG78 mycelia, S<sub>10</sub> preparations were assayed for incorporations of [2-<sup>14</sup>C]-MVA into terpenoids. 5 Day cultures were chosen since, from in vivo results (3.3.5), it appears that carotenogenesis and sterol biosynthesis are high. GA biosynthesis is not active although there may be some trace activity in SG78 (see 4.3).

The incubations were performed as described previously (2.2.15) and incorporation into kaurene, squalene and phytoene determined, together with a total incorporation into the prenyl alcohols and oxidative products of kaurene (i.e. 'origin').

From the incorporation levels (Table 5.3), it can be seen

Table 5.3 Incorporation Levels of [2-<sup>14</sup>C]-MVA into the Terpenoid Intermediates in Cell-Free Systems of Mutant SG78 Grown in Different Media and Prepared in Different Buffers with and without the Addition of F

Metabolite	Growth and Assay Conditions								
	AM Medium				DG Medium				
	A		B		A		B		
+F	-F	+F	-F	+F	-F	+F	-F		
dpm X 10 <sup>-3</sup>									
Kaurene	0.6	0.6	0.3	0.1	40	10	29	15.3	
Squalene	55	32.7	12.8	12	35	3	9.6	4.5	
Phytoene	74.6	55.7	9.9	8	22	13.7	5	4.6	
Origin *	33	80	10	9.5	15	200.7	35	30	
Total **	190	200	35	34	280	250.5	100	95	
Protein	10.5mg/ml				20.0 mg/ml				

\* Contains prenyl alcohols and oxidative products of kaurene (see 5.3.4)

\*\* Total lipid counts, after extraction, before separation

A = Tris-HCl buffer pH 8.0

B = Potassium phosphate buffer pH 7.5

that phosphate-buffered incubations have lower levels of total and individual incorporations than those in Tris-buffered conditions. This is especially apparent in AM-grown mycelia, where total activity is 10-fold greater compared to phosphate-buffered incubations. It is likely that this reduced activity is a result of the buffer components themselves, and while phosphate has been shown to inhibit phosphatase activity (5.3.1), it appears that the pH of the buffer is having this effect; pH 8.0 is more favourable to the terpenoid pathway enzymes than pH 7.5.

The effects of F support those found in the inhibitory action on phosphatases in different media (5.3.1). In Tris buffer, with both DG- and AM-grown cultures, F increases incorporation into the terpenoid pathway intermediates i.e kaurene, squalene and phytoene, while reducing the incorporation into the 'origin' (see 5.3.4 for components). This would be expected for a phosphatase inhibitor. It should be noted, however, that the prenyl alcohol containing fraction ('origin') also contains the kaurene oxidation products which should, theoretically, also have increased incorporation in conjunction with kaurene. This may indeed be the case, but the changes in the prenyl alcohol levels 'mask' those in the kaurene oxidative products; especially if, in this age of culture, kaurene oxidation is low (implied in Chapter IV and 5.3.3): the prenyl alcohols are shown conclusively to have reduced incorporation levels due to F addition (5.3.4).

Generally, total levels of incorporation are greater in DG-grown, cell-free systems than in AM-grown tissue preparations. However, this does not necessarily imply efficient in vitro terpenoid synthesis, since the total levels include the

prenyl alcohols.

In comparing AM- and DG-grown systems, squalene and phytoene levels are greater in the former (especially in Tris buffer), suggesting higher terpenoid activity in AM-grown tissue; also indicated by the in vivo results (see Chapters III and IV). In addition, the higher levels of incorporation in the 'origin' fraction from DG may reflect the prenyl alcohol levels, since phosphatase activity is higher in this medium than that in AM-grown cultures, even in the presence of F (see 5.3.1.iii). It is also possible that these increased levels are due to the kaurene oxidative products which are also in the 'origin' fraction (5.3.4). This is further suggested by the unusually high kaurene levels seen in DG, compared to those seen in AM-grown tissue. However, it has been shown (see Chapter IV) that DG-grown cultures produce low GA levels compared to AM cultures which makes high levels of kaurene oxidative products unlikely, although it also makes the high kaurene levels difficult to explain.

The observations may result from the fact that high activity of the GA pathway in AM tissue, means a large post-kaurene enzymic activity, thus producing apparently low levels of kaurene itself. In DG-grown cultures, however, kaurene increases because the oxidative steps are relatively low in activity. This suggests that within DG-grown strains, kaurene is the major product of the GA pathway and that the media exerts an inhibitory effect on kaurene oxidase.

These results assume a fully active kaurene oxidase under the in vitro conditions. It has been shown (see 1.5.1) that NADPH is an essential cofactor for kaurene oxidase and it is assumed that enough endogenous NADPH is present, in these

incubations, to allow full activity of the oxidase. This assumption is supported by the observation that addition of NADPH to in vitro incubations has no effect on the incorporations into kaurenoic acid (5.3.4).

In phosphate-buffered incubations, a similar pattern between DG tissue and AM tissue to that seen in Tris buffer was found, except that the individual incorporation levels are slightly lower. This may be due to the lower pH of the phosphate buffer (pH 7.5) reducing the enzyme activities.

The protein levels of the SG78, S<sub>10</sub> preparations are twice as high in DG-grown mycelia as those in AM cultures. This may reflect the higher N levels in DG medium, thus allowing a larger amount of protein synthesis, and general metabolism in DG-grown cultures. A higher phosphatase activity is present in DG-grown tissue compared to AM cultures (see 5.3.1.ii). Consequently, the high levels of incorporation into the terpenoids in AM-grown mycelium, compared to that in DG-grown tissue, are even greater when calculated as specific activities. Apparently, therefore, AM medium strongly favours terpenoid biosynthesis, and it does so over and above the activities of other metabolic pathways in such cultures.

### 5.3.3 Effects of Age of Culture and Light- and Dark-Growth Conditions on In Vitro Terpenoid Synthesis of Wild Type and SG22 Strains of G.fujikuroi

Wild type G.fujikuroi and SG22 were grown in AM liquid-shake cultures in white light (48 Wm<sup>-2</sup>) and dark-grown conditions for 3,5,8,11 and 14 days. At each age, mycelial preparations (2.2.14) were assayed for in vitro activity of the terpenoid pathway (2.2.15).

The incorporation levels of [<sup>14</sup>C] were measured for kaurene,

squalene,  $\beta$ -carotene, phytofluene, phytoene and the other carotenoids ( $\zeta$ -carotene, neurosporene,  $\beta$ -zeacarotene,  $\gamma$ -carotene and torulene) and an 'origin' band, containing the kaurene oxidative products, kaurenal, kaurenol and kauren-  
oic acid together with the prenyl alcohols. These were assay-  
ed by applying incubation extracts (2.2.17) to TLC system H  
followed by system G (see Table 2.2.2 and 2.2.18) and measur-  
ing incorporation by radioassay (2.2.20).

#### 5.3.3.i Wild Type IMI58289

The results for the wild type incubations are tabulated in  
Table 5.4a and b, specific activities in Fig 5.4a and b.

The majority of activity, under all conditions, was in  
squalene, followed by the 'origin' fraction, with low levels  
of incorporation in the carotenoids and kaurene. These  
incorporation levels reflect the in vivo data, since relatively  
low carotenoid (3.3.1) and GA levels (4.3.1) were found,  
especially compared to the regulatory mutants (i.e SG22 and  
derivatives).

In light-grown wild type the highest activity in all metabo-  
lites was found at 3 days, and decreased on consecutive days  
until 14 days where activity was almost zero. However, the  
protein levels in the  $S_{10}$  preparations show a concurrent fall  
with age of culture. Consequently, the specific activities  
(Fig 5.4a) show that total activity and 'origin' activities  
are maximal at 5 days, while squalene specific activity is  
greatest at 3 and 5 days. The specific activity of kaurene  
and carotenoid synthesis is maximal at 11 days, while caroteno-  
genic activity is optimal between 3 and 8 days.

A similar pattern of incorporations was found in dark-grown,  
wild type cell-free systems (Table 5.4b). In most metabolites,

Table 5.4a Incorporation Levels of [2-<sup>14</sup>C]-MVA into Terpenoid Intermediates in Cell-Free Systems of Wild-Type *G.fujikuroi* Grown on AM media in the Light, Measured over Time.

Metabolite	Time (Days)				
	3	5	8	11	14
Kaurene	0.60	0.05	0.09	0.22	-
Squalene	149.8	44.6	0.65	0.02	-
Other Carotenoids*	1.50	0.28	0.36	-	-
β-Carotene	0.07	0.01	0.02	-	-
Phytoene	0.01	-	-	-	-
Phytofluene	0.01	-	-	-	-
Origin**	28.6	27.2	2.20	1.30	-
Total***	220.1	148.2	5.3	0.04	-
Protein (mg/ml)	29.5	9.5	5.9	4	1

\* Carotenoids other than NX, β-carotene, phytoene and phytofluene, found in *G.fujikuroi* (see 3.3.1)

\*\* Contains prenyl alcohols and oxidative products of kaurene (see 5.3.4)

\*\*\* Total lipid counts, before separation, after extraction

Table 5.4b

Incorporation Levels of  $[2-^{14}\text{C}]$ -MVA into Terpenoid Intermediates in Cell-Free Systems of Wild-Type *G.fujikuroi* Grown on AM media in the Dark, Measured Over Time

Metabolite	Time (Days)				
	3	5	8	11	14
	dpm $\times 10^{-3}$				
Kaurene	-	-	0.09	0.06	-
Squalene	169	68.5	0.77	0.15	-
Other Carotenoids*	0.44	0.64	0.18	0.07	-
$\beta$ -Carotene	-	-	-	-	-
Phytoene	-	-	-	-	-
Phytofluene	-	-	-	-	-
Origin**	33756	127.6	3.86	1.5	-
Total***	246.1	234.8	4.94	5.7	0.3
Protein (mg/ml)	27.5	25	4.6	4.5	0.95

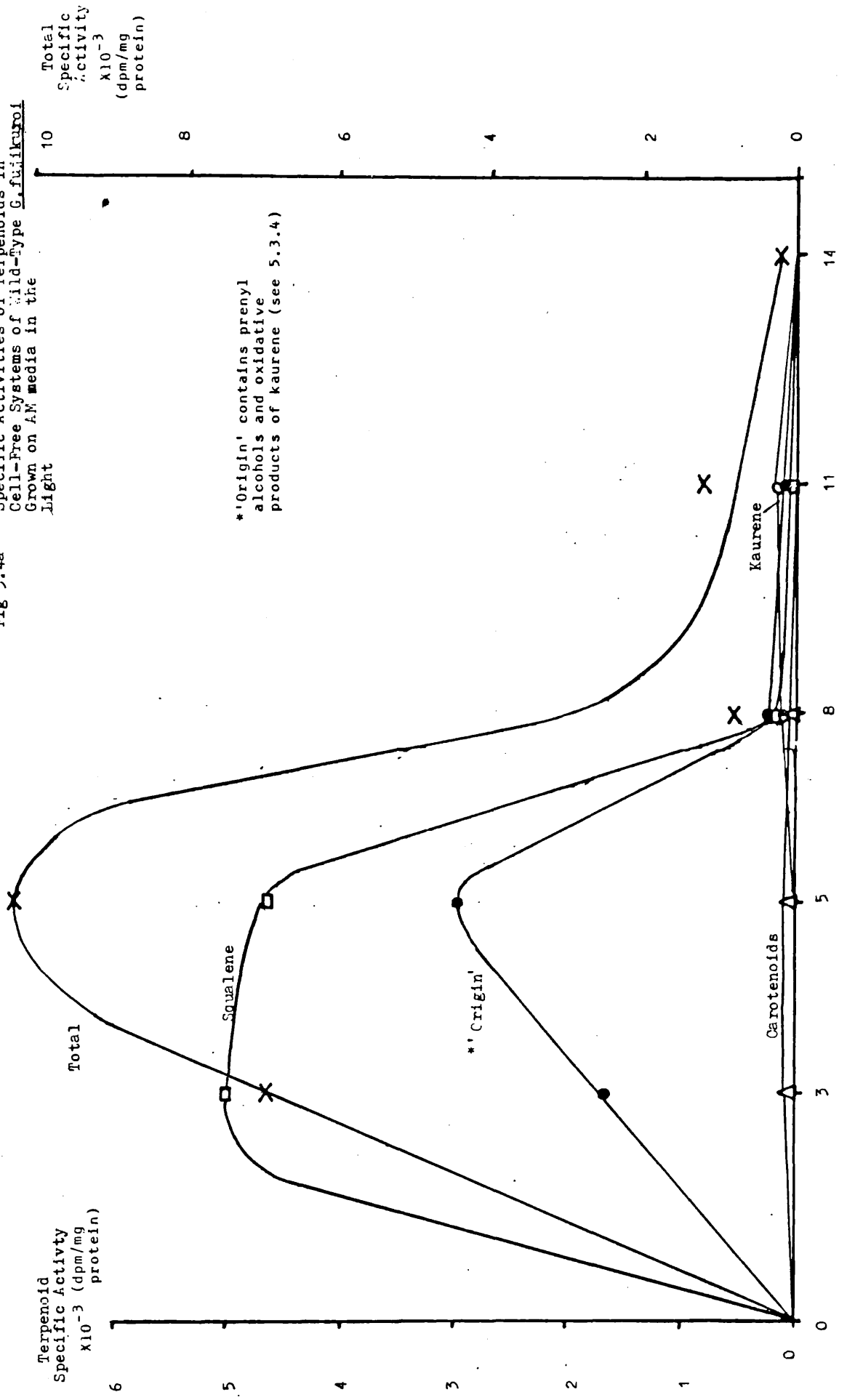
\* Carotenoids other than NX,  $\beta$ -carotene, phtoene and phytofluene, found in *G.fujikuroi* (see 3.3.1)

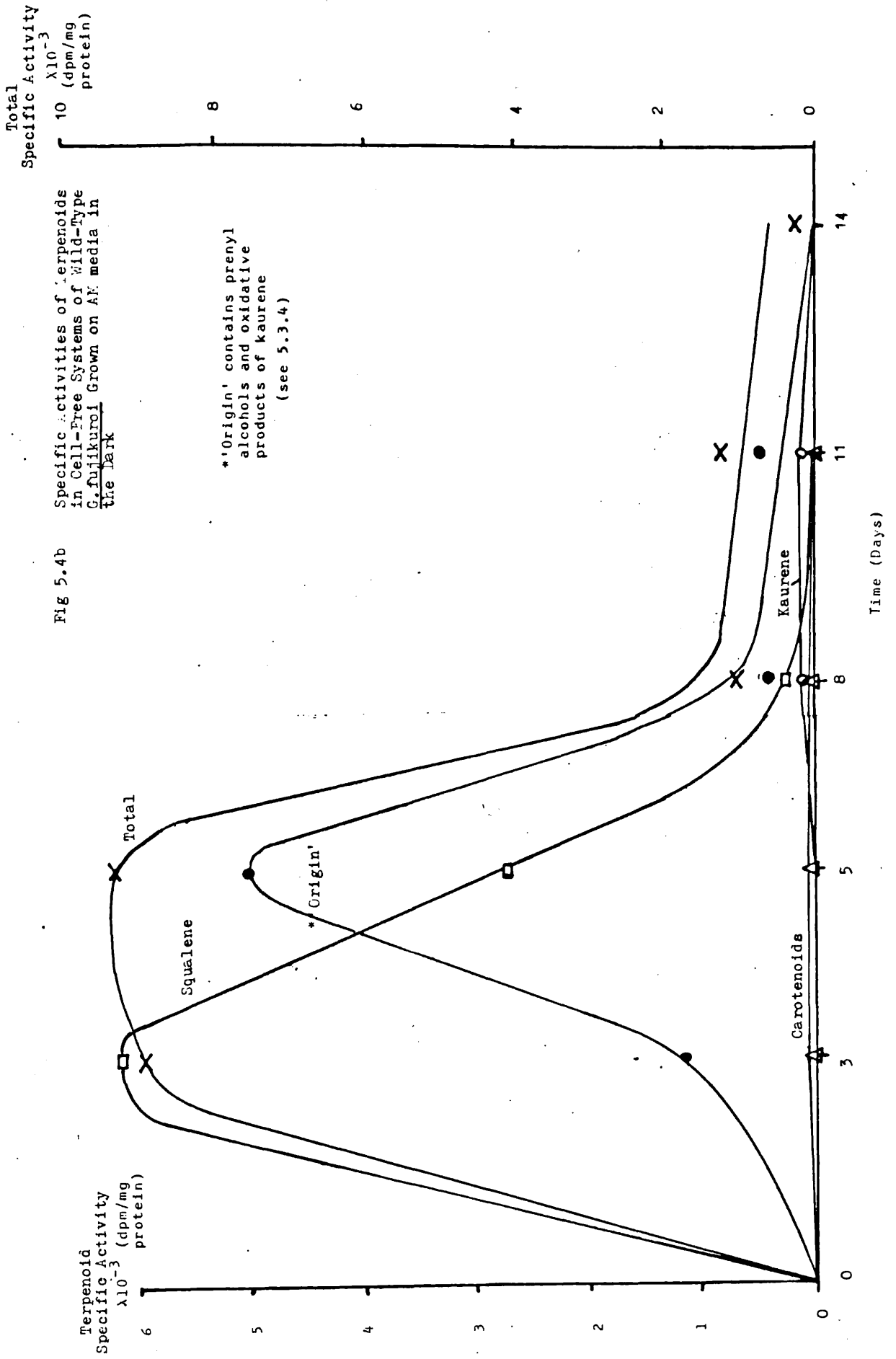
\*\* Contains prenyl alcohols and oxidative products of kaurene (see 5.3.4)

\*\*\* Total lipid counts, before separation, after extraction



Fig 5.4a Specific Activities of Terpenoids in Cell-Free Systems of Wild-Type *G. fujikuroi* Grown on AM media in the Light





incorporation was highest at 3 days and zero at 14 days; although the 'origin' and carotenoid levels appear to peak at 5 days. The analogous protein levels decrease from an initial maximum level at 3 days to that seen in light-grown tissue suggests that maximal activity is generally shifted to later stages in growth (Fig 5.4b). The patterns of syntheses are similar to those in the light-grown cultures, with squalene activity maximal at 3 days, 'origin' at 5 days, carotenoids at 5-8 days and kaurene at 8-11 days. As in light-grown conditions the highest incorporations are in squalene and the 'origin' metabolites, compared to kaurene and the carotenoids.

The light- and dark-grown in vitro results (3.3.1 and 4.3.1). The carotenoids, for example, while greater in light-grown tissue show similar light-grown specific activities compared to dark-grown preparations. Kaurene incorporation levels appear to be slightly greater in light-grown tissue compared to dark-grown, reflecting the in vivo GA data to a certain extent, but the levels are too low to be conclusive. Generally, the differences between light- and dark-grown cultures for in vivo terpenoid formation are not apparent in the in vitro activities, with the light- and dark-grown specific activities being similar.

These results may indicate that the photoinduction phenomenon observed in vivo is not due to de novo protein synthesis, as shown for other systems (1.2.3.iii), but due to an activation or inactivation of existing enzymes, which may have occurred during the preparation of the cell-free systems, which in turn could have activated the enzymes of dark-grown cultures. However, an alternative explanation concerns the value of

cell-free systems to reflect in vivo data. Incorporation levels in the wild type are relatively low and may not be sufficient to demonstrate differences of light- and dark-grown cultures. This may result from reduced enzymic activities within the in vitro system, or interference from other pathways whose activity is much greater than the pathway being studied, effectively removing substrate from the latter. This may be the case for squalene and the prenyl alcohols, whose high incorporation levels may affect those seen in the carotenoids and kaurene, and therefore differences between light- and dark-grown cultures cannot be determined.

The time courses imply that maximal activity of carotenogenesis is at about 5 days, which does agree with the in vivo data.

Since sterols are not produced in this cell-free system, incorporations into squalene can be used to reflect the sterol pathway. They are high at 3 days as would be expected for the production as a cellular structural component such as ergosterol.

Although incorporation levels into kaurene are low throughout growth, the specific activities suggest that synthesis is greatest late in growth (8-11 days). This reflects the in vivo data for GA synthesis (4.3.1) which shows GAs to be induced towards the stationary phase of growth. However, since kaurene oxidase may be active, as implied by the identification of [ $^{14}\text{C}$ ] -labelled kaurenol, kaurenal and kaurenoic acid (5.3.4), the kaurene incorporation may be affected by a combination of kaurene synthetase and kaurene oxidase activities, which if regulated differently, may produce misleading incorporations with respect to total activity

of the GA pathway.

The decrease in protein levels with age from an initial 3 day maximum was unexpected; as one might expect the greatest protein values at mid-log. growth, when the growth rate is maximal. However, it could be argued that protein synthesis is greatest at early growth when intermediary metabolites are required to the greatest extent. After this time, protein synthesis is reduced and the protein levels fall with a concomitant increase in tissue levels such that enzyme concentrations fall while still carrying out necessary cellular functions, until 14 days when mycelial tissue is old and protein levels are low due to cellular breakdown.

#### 5.3.3.ii Mutant Strain SG22

Incorporations of [ $^{14}\text{C}$ ] into the terpenoids from cell-free systems of SG22 at different ages of culture were determined (Table 5.5a and b). The specific activities of squalene and kaurene (representing the sterol and GA pathways respectively) and the carotenoids and the 'origin' fraction (see 5.3.4), were assayed over time (Fig 5.5a and b).

The results show similar patterns to those seen in the wild type cell-free systems (5.3.3.i).

In both light- and dark-grown cultures, total incorporation levels are greatest at 5 days, while total specific activity is maximal between 5 and 8 days. It is noticeable that the total activities are much greater in SG22 than those found in the wild type: maximum specific activity in SG22 is twice that found in wild type. Furthermore, enzymic activity is still high at 11 and 14 days compared to wild type.

The protein levels in SG22 also show a similar pattern, with time, to that in the wild type, being greatest at early stages

Table 5.5a

Incorporation Levels of  $[2-^{14}\text{C}]$ -MVA  
into Terpenoid Intermediates in Cell-Free  
Systems of Mutant SG22 Grown on AM media  
in the Light, Measured over Time

Metabolite	Time (Days)				
	3	5	8	11	14
	dpm $\times 10^{-3}$				
Kaurene	0.14	0.50	61.5	11.7	0.02
Squalene	31.9	43.8	0.52	0.18	0.75
Other Carotenoids*	2.42	14.1	3.73	0.66	0.18
$\beta$ -Carotene	0.02	1.36	2.45	0.11	-
Phytoene	0.01	0.10	0.35	0.05	-
Phytofluene	0.01	0.14	0.32	0.04	-
Origin**	33.7	155.6	14.0	11.0	1.80
Total***	96.6	343.9	209.9	83.7	4.94
Protein (mg/ml)	7.2	22.5	9.3	5.35	1.7

\* Carotenoids other than NX,  $\beta$ -Carotene, phytoene and phytofluene, found in G.fujikuroi (see 3.3.1)

\*\* Contains prenyl alcohols and oxidative products of kaurene (see 5.3.4)

\*\*\* Total lipid counts, before separation, after extraction

Table 5.5b

Incorporation Levels of  $[2-^{14}\text{C}]$ -MVA into Terpenoid Intermediates in Cell-Free Systems of Mutant SG22 Grown on AM media in the Dark, Measured over Time.

Metabolite	Time (Days)				
	3	5	8	11	14
	dpm $\times 10^{-3}$				
Kaurene	1.17	4.86	117.7	8.89	0.01
Squalene	77.5	169.1	6.03	0.44	0.08
Other Carotenoids*	9.0	36.9	8.7	4.3	0.63
$\beta$ -Carotene	0.54	5.74	3.34	0.24	0.01
Phytoene	0.23	0.32	0.26	0.05	-
Phytofluene	0.17	0.65	0.29	0.04	-
Origin**	163.4	44.0	30.0	27.0	7.50
Total***	284.0	307.4	173.1	54.0	12.3
Protein (mg/ml)	26	14.5	10	8.1	2

\* Carotenoids other than NX,  $\beta$ -Carotene, phytoene and phytofluene, found in G.fujikuroi (see 3.3.1)

\*\* Contains prenyl alcohols and oxidative products of kaurene (see 5.3.4)

\*\*\* Total lipid counts, before separation after extraction

Fig 5.5a Specific Activities of Terpenoids in Cell-Free Systems of Mutant SG22 Grown on AM Media in the Light

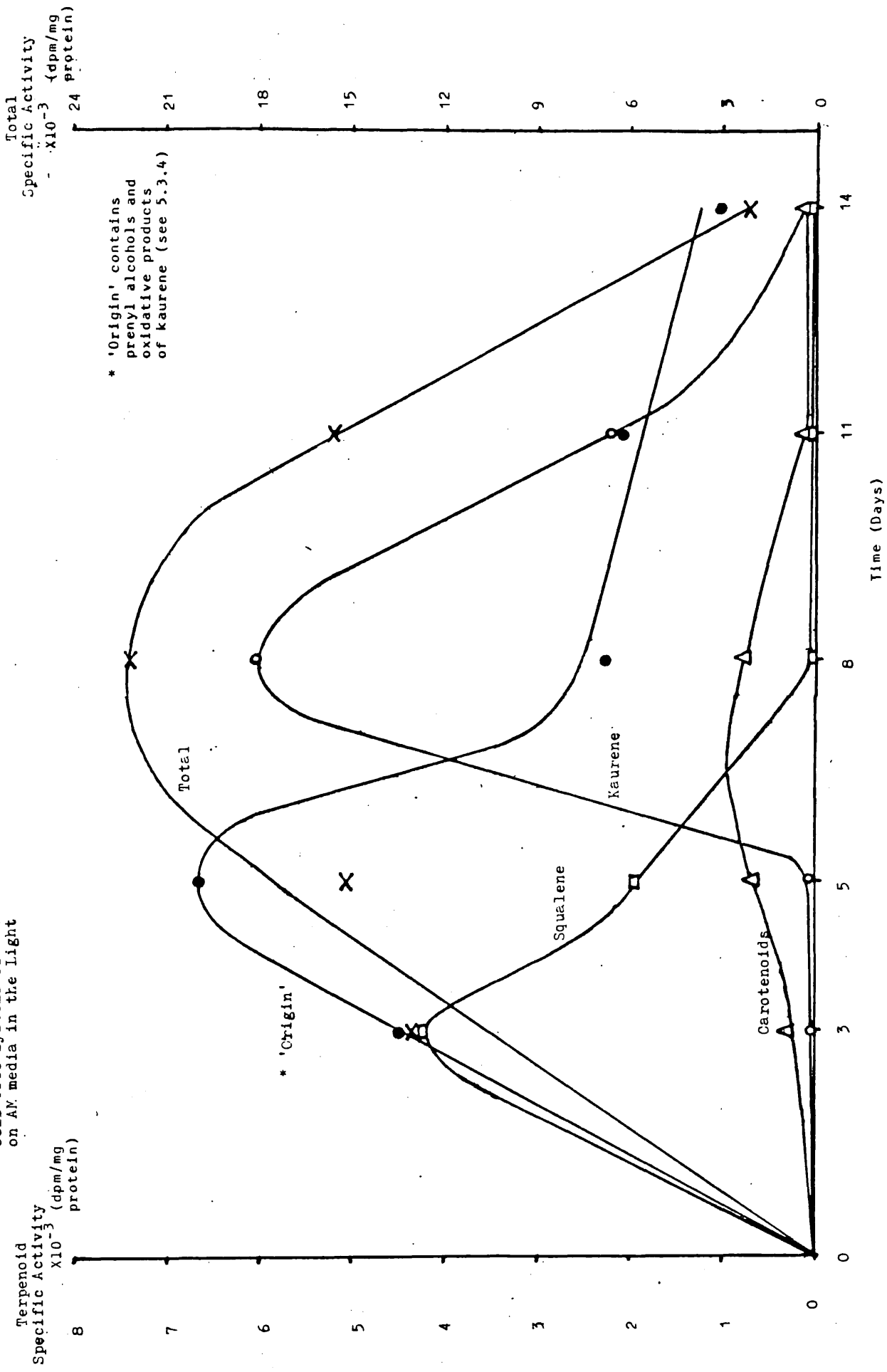
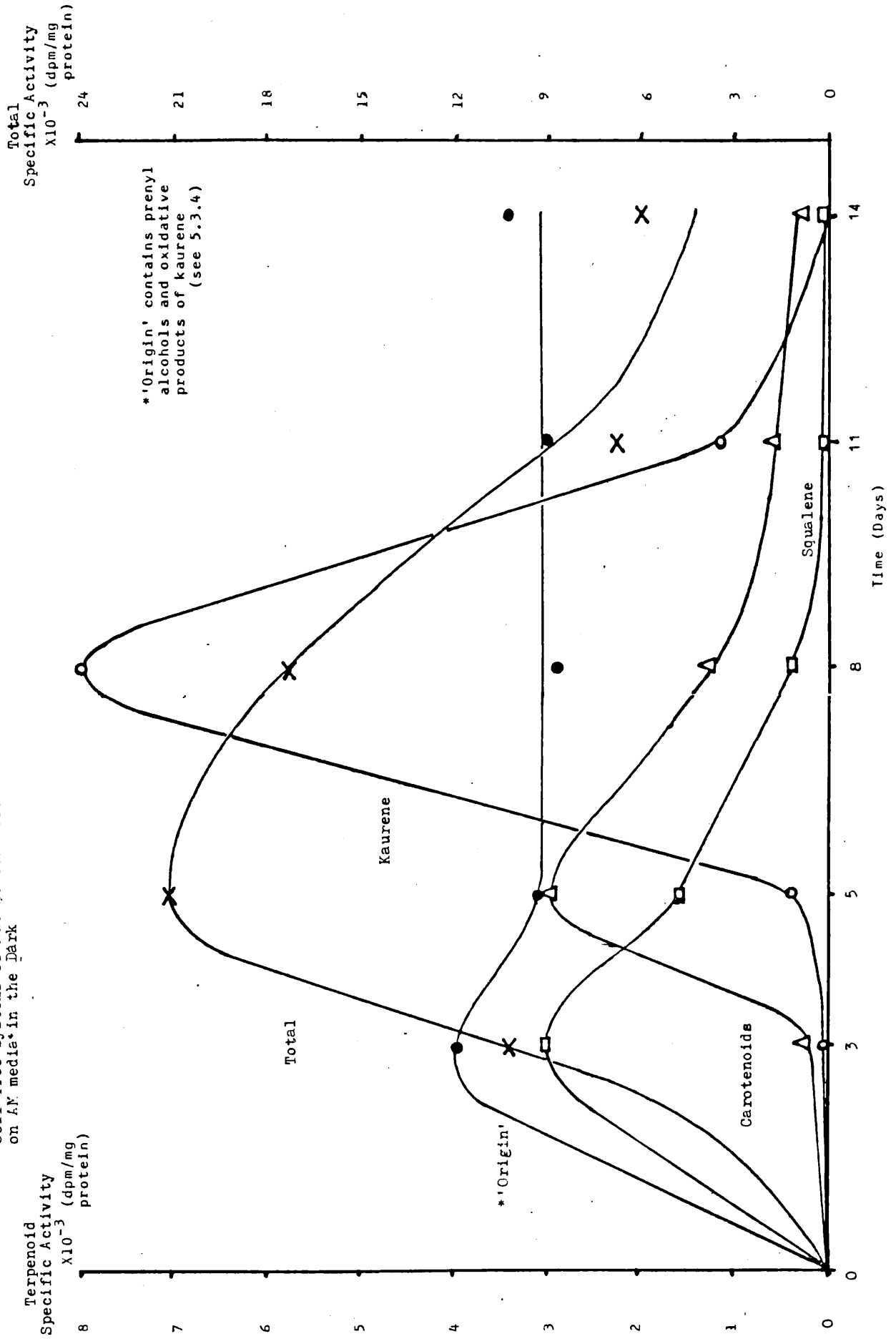




Fig 5.5b Specific Activities of Terpenoids in Cell-Free Systems of Mutant SG22 Grown on AN media in the Dark



of growth, falling as the mycelium ages. However, in the wild type highest levels are at 3 days as they are in dark-grown SG22. In contrast, light-grown SG22 has maximal protein levels at 5 days. Perhaps the light-grown culture is slightly delayed in growth, and even though the inoculum in light- and dark-grown cultures was the same, a slight difference may result in a slower growth and a 'shift' in the maximal protein levels for light-grown SG22.

It should be noted that protein levels are analogous in SG22 to those in the wild type, but the incorporation levels are greater in SG22. Generally, one can conclude that the mutation in SG22 has increased enzyme activity in the terpenoid pathway above that of other biosynthetic pathways.

A comparison of incorporations in SG22 and the wild type, shows greater levels of all terpenoid intermediates in SG22, and while the 'origin' has the greatest incorporation, followed by kaurene, squalene and then the carotenoids (which is similar to the wild type pattern), the levels do not change with age of culture in the same way as the wild type, such that different distributions predominate at different times.

From the time course of specific activities (Fig 5.5a and b), squalene is seen to have greatest incorporation at 3 days in light and dark i.e. as in the wild type, but with lower incorporations than the wild type. Since total incorporation has increased, a redistribution of activity within the terpenoid pathway, away from the sterol pathway may occur. This is seen in specific activities of kaurene which are greatest between 8 and 11 days, as in the wild type, but are again much higher. The specific activity of carotenoids in light- and dark-grown cultures also shows a similar, 5 day

period for maximal activity, but again the total levels are raised compared to the wild type: this is also the case for the 'origin' specific activity levels compared to the wild type.

Apparently, therefore, the terpenoid pathway in SG22 has an elevated rate of synthesis compared to the wild type. This activity is higher throughout the pathway, except apparently for sterol biosynthesis, where there is a decrease. This is probably more a result of the increased activity in the other pathways as opposed to a lower enzyme activity in the sterol pathway. The raised activity in SG22 compared to the wild type is also implied from the in vivo data (3.3.2 and 4.3.2), providing further evidence for the proposal that the mutation in SG22 has released inhibition, or activation, of the whole terpenoid pathway and that in vitro systems can reflect in vivo data and cellular enzyme activities.

However, in comparing the light- and dark-grown in vitro results, the analogy with the in vivo results is very poor, and there is no apparent elevation of light-grown GA and carotenoid levels. This anomaly is also seen in the wild type (5.3.3.i). It may be argued that this situation is a true reflection of activity in vivo, and dark-grown enzymes for terpenoid biosynthesis are present in an inactive form, and that creating a cell-free system removes this inhibition. The argument between activation of proteins already present, or the induction of de novo protein synthesis by light, has been discussed (1.2.3.iii). Experiments in different systems tend to favour the latter as opposed to enzyme activation. Furthermore, since the preparation of an  $S_{10}$  fraction does not remove soluble proteins, it is difficult to explain how inactivation may occur in this process. Another point against

protein activation in photoinduction of carotenogenesis in G.fujikuroi, is that in the mutant SG43, where there is no production of phytoene in darkness (3.3.4), cell-free incubations did not produce [ $^{14}\text{C}$ ]-phytoene either (6.2.3). If preparing  $S_{10}$  fractions in the wild type and SG22 releases a dark-grown regulatory mechanism it should also do so in SG43. Therefore the dark-grown in vitro incorporations reflect constitutive carotenogenesis apparent in vivo. That the levels are similar to the light-grown incorporations may also indicate that the enzymes that are photoregulated are earlier than MVA kinase (see 1.1.1), since MVA was the radio-labelled substrate for the incubations. It has been shown in R.minuta that HMG-CoA reductase is photoinduced (Tada and Shiroishi, 1982).

Alternatively, while total incorporation levels may reflect the total activity of a whole pathway, individual intermediates cannot necessarily portray the activity of the pathway in which they are involved i.e photoregulation of individual enzyme steps may be masked by activities of other enzymes. In addition, the pathway may not necessarily be fully active or complete in a cell-free system. Therefore, certain important regulatory and interrelated enzymic and metabolic relations are absent e.g the lack of ergosterol, GA and NX production suggesting latter parts of these pathways are non-functional. It has been suggested (Cerdeira-Olmeda, 1986) that the end-product carotenoid in P.blakesleeanus,  $\beta$ -carotene, is necessary for photoregulation. However, this is unlikely to be the case in G.fujikuroi, since NX is absent in SG43, SG78 and SG68 but there is still in vivo photoinduction of carotenogenesis.

In conclusion, the in vitro data show that in both strains the terpenoid pathway activity is greatest in the sterols at early growth, during log. growth in the carotenoids (5 days) when carotenogenesis is greatest (3.3.2) and in the GAs at 8-11 days when GA biosynthesis is maximal (4.3.5). What is interesting is the apparently short periods during which all these activities are maximal i.e appearing active over a 3 day span and generally optimal for only 1 day. This is unusual since in each pathway the levels of sterols, GAs and carotenoids (from in vivo data, Chapters III and IV) remain high beyond maximal levels of specific activities; in SG22, GAs and carotenoids continue to increase long after their in vitro activities are absent. This phenomenon has also been seen in carotenogenesis in A.giganteus (El-Jack, personal communication). One may conclude that there is a specific period of enzyme synthesis (just before pathway metabolites), after which turnover of the protein and pathway products is low allowing metabolite levels to remain high. The decrease in specific activities in each case, may actually be an artifact of the in vitro system. That each pathway appears to be activated consecutively leads to the possibility that the induction of one, e.g. carotenogenesis or GA biosynthesis, removes incorporation of an already active pathway, e.g. sterol biosynthesis or carotenogenesis respectively, since the pathway substrates to these become utilized by the induced pathway.

If this is so, it further suggests the possibility of a strict regulation of the pathways within terpenoid formation such that sterols are activated, then inactivated as carotenogenesis is initiated which in turn is down-regulated with

the onset of GA biosynthesis: enzyme activities are relative, such that a very active pathway has the highest rate of synthesis, but this does not preclude activity in other pathways, albeit at a reduced rate.

In addition, one may consider the 'origin' band, while containing kaurene oxidase products, also contains the prenyl alcohols and in the wild type incorporation is maximal at 5 days, reflecting general terpenoid biosynthesis, as would be expected for these metabolites. But the activity levels are low and cannot reflect any additional relationships. However, in SG22, the total terpenoid activity is increased compared to the wild type, which is reflected in incorporations in the 'origin'. These continuously high levels are difficult to interpret with respect to changing activities in each pathway. However, the elevated levels in SG22 allow one to notice that while each pathway is strictly regulated, activity in the 'origin' fraction remains high even in early growth when kaurene oxidation should be absent (4.3.2) and so the 'origin' may be considered as solely prenyl alcohols. This suggests that the prenyl alcohols are not subject to the regulation apparent in the other parts of the pathway. Therefore the prenyl pyrophosphate stage of the terpenoid pathway may have a constant activity (though higher than that in the wild type), and the individual pathways are regulated by growth at steps later than prenyl transferase(s). Since specific enzyme activities are measured with respect to kaurene, squalene and phytoene, these respective synthetases seem prime candidates for such regulation, as has been speculated (1.8). It should be noted that changes in prenyl alcohols may also be affected by regulation of the phosphatases involved in their production, which may complicate

any conclusions.

5.3.4 Kaurenal, Kaurenol, Kaurenoic Acid and Alcohol Derivatives of the Prenyl Pyrophosphates in G.fujikuroi Cell-Free Systems: Characterization of the 'Origin' from TLC system H

Lipid extracts of incubations were normally applied to TLC system H (Table 2.2.2) for separation of carotenoids, squalene and GA intermediates (e.g. 5.3.2 and 5.3.3). The origin band of this TLC contained high levels of incorporation (10-50% total levels, depending on conditions). Although a certain amount of this may be due to breakdown of the labelled intermediates, the remaining incorporations are due to those terpenoid metabolites which are too polar for separation by the initial solvent system.

Two approaches were taken to determine the components of this origin fraction. The first considered the possible terpenoid metabolites which may be located therein, and attempts were made to isolate these from the incubations.

Of the carotenoids in G.fujikuroi, NX may be located at the origin, due to its polarity. Incubations of 5 day-old SG22 were extracted and separated in the same manner as in vivo acidic extracts (2.2.7). Using unlabelled NX, no incorporation of [ $^{14}\text{C}$ ] into NX was detected.

Acidic extracts of SG22 (10 days) cell-free incubations were subjected to HPLC with appropriate GA standards and [ $^{14}\text{C}$ ] incorporation into the GAs was determined (2.2.13). No [ $^{14}\text{C}$ ]-GAs were detected, indicating that the in vitro system was incapable of full GA pathway activity. Kaurene activity has been shown to be present in G.fujikuroi cell-free systems (5.3.2 and 5.3.3), and from the HPLC system for GAs, high levels of incorporation were eluted at time pre-

dicted for kaurene oxidative products, including GA<sub>12</sub>-aldehyde and kaurenoic acid (Koshioka et al., 1983). It appears, therefore, that although the in vitro system can oxidise kaurene, its ability to catalyse subsequent steps to GA<sub>12</sub>-aldehyde is absent and how far oxidation of kaurene to the latter is uncertain. The polarity of these metabolites (kaurenol, kaurenal, kaurenoic acid, 7β-hydroxykaurenoic acid and GA<sub>12</sub>-aldehyde) suggest that they should be located at the origin of TLC system H: standard [<sup>3</sup>H]-kaurenoic acid is found at this position.

Within the sterol pathway, those compounds beyond squalene, predominantly lanosterol and ergosterol (1.6), should remain at the origin. Using authentic markers of both, this was shown to be the case. A TLC system capable of separating lanosterol, ergosterol, kaurenoic acid and the prenyl alcohols (also considered to be at the origin), was developed (Table 5.6). When extracts of a 5 day, SG22, light-grown, AM culture cell-free incubation were applied to this system, no [<sup>14</sup>C] was found in these sterols, but was exclusively located in the prenyl alcohols and kaurenoic acid.

Unlabelled standards of geraniol, farnesol, nerolidol and geranyl geraniol were all shown to remain at the origin of TLC system H: the prenyl alcohols of PPPP and CPP were not available, but these, along with the prenyl alcohols of IPP, DMAPP and possible unknown prenyl pyrophosphate alcohol derivatives, were considered to be located in the 'origin' fraction.

The second approach to identification of the 'origin' components, was an attempt to separate them by consecutive TLC systems with differing polarities.



Table 5.6

$R_f$  Values of Prenyl Alcohols, Lanosterol  
and Ergosterol on Kieselgel G using  
 $\text{CHCl}_3$  as a Developing Solvent.

Metabolite	$R_f$
Geranyl Geraniol (GG-ol)	0.33
Farnesol (F-ol)	0.28
Geraniol (G-ol)	0.26
Nerolidol (N-ol)	0.31
Lanosterol	0.36
Ergosterol	0.23

The 'origin' was applied to TLC system I from which the radiolabelled bands were applied to TLC system J (2.2.21). The bands were identified with available standards and visualised with the spark chamber (2.2.17). This separation technique was used with extracts of cell-free systems from SG22 (5 day, AM, light-grown cultures; 2.2.14). In addition the incubations were carried out with and without F and NADPH. The reason for this was that while F should decrease incorporation into the alcohol derivatives (5.3.2), NADPH should increase kaurene oxidation, for which it is essential (1.5.2): it is also essential for squalene synthetase (1.1.3).

The effect of altering incorporation in these intermediates may aid their identification, and if authentic standards were not available, it may at least be possible to identify a prenyl alcohol as distinct from an oxidative product of kaurene.

The spark chamber photographs of TLC system are shown in Plate 5.1 (TLC system H), Plate 5.2 (TLC system I) and Plate 5.3 (TLC system J). The incorporation levels of each band and Rf values are shown in Table 5.7; those metabolites identified by cochromatography with authentic samples are named.

A slightly higher total incorporation in those incubations containing NADPH compared to those without, was found and in both cases the addition of F reduced the total incorporation, although only by consistently small amounts. The significance of this difference can only be determined from the incorporations in the individual components. One should note the small effect of F on total incorporations, is only since phosphatase inhibition does not interfere with total levels but only individual incorporations.

That NADPH has no effect on incorporations into squalene

Plate 5.1

Spark Chamber Photograph of Cell-Free Systems of Mutant SG22 Grown on AM media in the Light Showing the Effects of NADPH and F on Incorporation Levels into the Terpenoids Separated on TLC System H (Table 2.2.2)

Plate 5.2

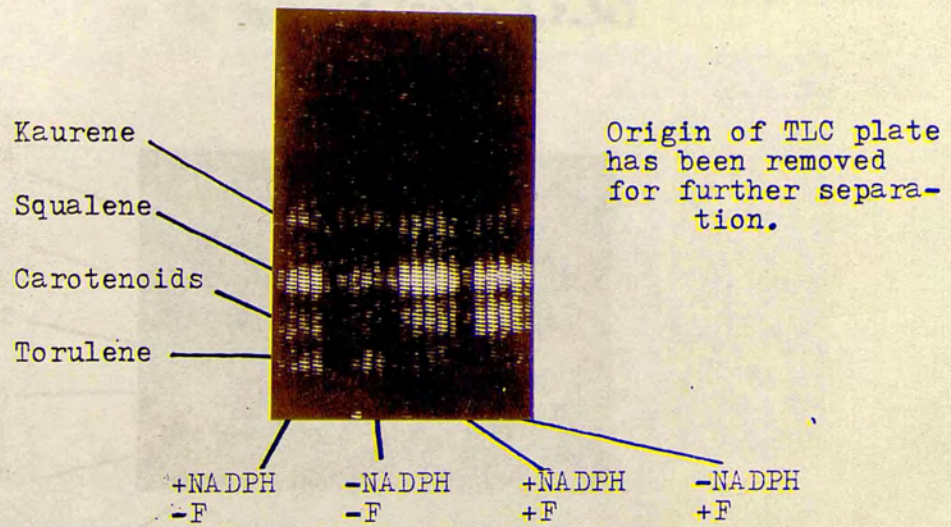




Plate 5.2 Spark Chamber Photograph of Cell-Free Systems of Mutant SG22 Grown on AM media in the Light Showing the Effects of NADPH and F on Incorporation Levels into the Terpenoids Separated on TLC System I (Table 2.2.3a)

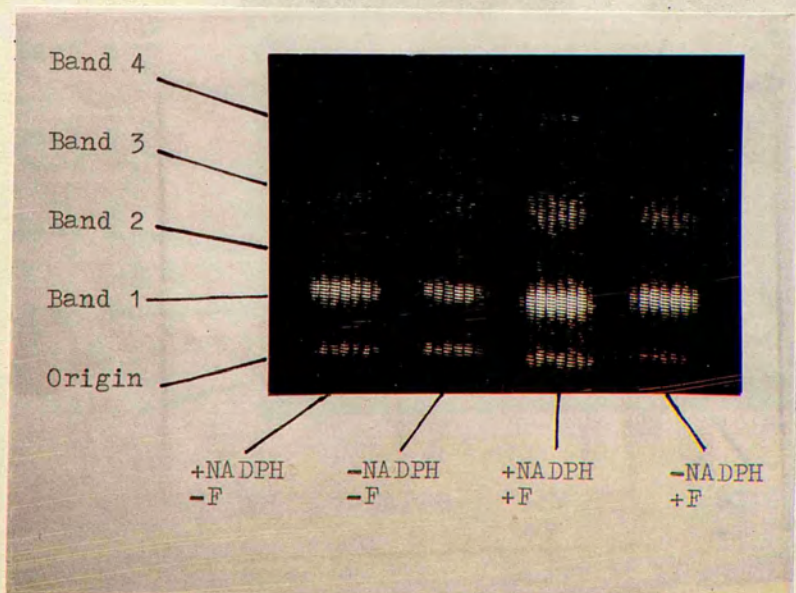


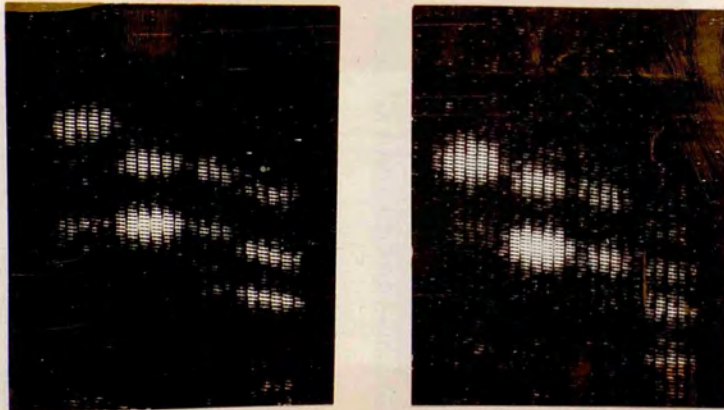


Table 5.7

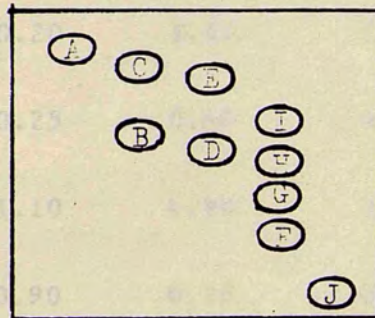
Plate 5.3

Spark Chamber Photograph of Cell-Free Systems of Mutant SG22 Grown on AM media in the Light Showing the Effects of NADPH and F on Incorporation Levels in the Terpenoids Separated on TLC System J (Table 2.2.3b)

Metabolite	+ or - NADPH +F	+ or - NADPH -F
Other Carotenoid		
Terulene		
Squalene		
Kaurene		
Total **		
*Bands of Origin:		
A; Geranic (R <sub>f</sub> 0.6)		
G; Kaurene Acid (R <sub>f</sub> 0.4)		
B; Geranyl Geraniol (R <sub>f</sub> 0.36)		
E; Farnesol (R <sub>f</sub> 0.50)		
D; Kaurenol? (R <sub>f</sub> 0.30)		
I; Unknown (R <sub>f</sub> 0.40)		
H; Unknown (R <sub>f</sub> 0.27)		
G; Prephytylene Alcohol (R <sub>f</sub> 0.4)		
F; Kaurenol? (R <sub>f</sub> 0.3)		
J; Unknown Origin		



Scheme of TLC System J indicating Designation of Bands seen in Spark Chamber Photograph. Identification is discussed in 5.3.4 and Table 2.2.3b



\*Carotenoids from *S. aureus* are not shown here but are present in the extract.

\*\* Total neutral lipids, before separation, after extraction.

\*\*\* see Table 2.2.3b

Table 5.7

Incorporation Levels of  $[2-^{14}\text{C}]$ -MVA  
into Terpenoids in Cell-Free Systems of  
Mutant SG22 Incubated in the Presence and  
Absence of NADPH (2 $\mu\text{mol}$ ) and F (10mM)

Metabolite	Incubation Conditions			
	NADPH: +	+	-	-
	F: +	-	+	-
	dpm X10 <sup>-3</sup>			
Other Carotenoids *	1.10	4.30	10.5	5.00
Torulene	7.50	2.50	7.0	2.80
Squalene	11.4	5.80	12.0	4.90
Kaurene	3.30	1.20	3.20	1.50
Total **	105.0	130.0	180.0	190.0
'Bands' from Origin				
A; Geraniol (R <sub>f</sub> 0.6)	2.50	4.60	2.50	3.90
C; Kaurenoic Acid (R <sub>f</sub> 0.53)	10.5	2.00	11.2	2.80
B; Geranyl Geraniol (R <sub>f</sub> 0.36)	10.2	60.2	12.1	50.1
E; Farnesol (R <sub>f</sub> 0.50)	0.45	1.00	0.65	0.60
D; Kaurenol? (R <sub>f</sub> 0.30)	1.10	0.20	0.40	0.20
I; Unknown (R <sub>f</sub> 0.40)	0.20	1.20	0.35	1.50
H; Unknown (R <sub>f</sub> 0.27)	0.25	0.40	0.10	0.60
G; Prephytoene Alcohol? (R <sub>f</sub> 0.17)	1.10	4.90	0.95	4.45
F; Kaurenal? Origin***	0.90	0.30	0.90	0.32
J; Unknown Origin	-	-	0.20	0.20

\*Carotenoids from G.fujikroi (see 3.3.1), other than torulene and NX

\*\* Total neutral lipids, before separation, after extraction

\*\*\* see Table 2.2.3b

and kaurene, suggests that enough endogenous NADPH is present in the incubation to allow production of these metabolites and addition of excess has no stimulatory effect.

The major differences in incorporations was found in those incubations with and without F. The levels of the carotenoids, squalene and kaurene all increase by 2-3 fold with F. This has been shown in SG78 (5.3.1.ii) and explained by F reducing the breakdown of intermediate prenyl pyrophosphates by phosphatase action, thereby increasing incorporation into terpenoid pathway products.

The compounds in the 'origin', separated on TLC system J (Table 2.2.3b) gave 10 radioactive bands designated A-J. The major components of this fraction have been predicted to be prenyl alcohols and the oxidative products of kaurene.

The results indicate that F had the predicted effect on the prenyl alcohols. Authentic geraniol (A), geranyl geraniol (B) and farnesol (E) cochromatographed with these radiolabelled bands and F addition reduces incorporations, supporting their identification as alcohols. On the other hand, [ $^3\text{H}$ ]-kaurenoic acid cochromatographed with (C) and incorporations increase with F, as expected.

Bands (H), (G) and (I) show an analogous behaviour to the prenyl alcohols with F (in both NADPH +/- conditions) whilst bands (D) and (F) appear to be oxidation products of kaurene: band (J) produced incorporation levels too low to discern any differences.

Since (G) showed the TLC properties expected of the prenyl alcohol of PPPP, it had an  $R_f$  similar to that reported for prephytoene alcohol on the same system (Kushwaha et al., 1977), it was assigned as prephytoene alcohol.

Since (D) and (F) behaved as kaurene oxidative products (not found in any other unidentified bands), it was assumed that these bands were kaurenol and kaurenal. By R<sub>f</sub> values (Dennis and West, 1967 and Bearder et al., 1974) kaurenol being more polar than kaurenal will have a higher R<sub>f</sub> than the latter on the reverse phase TLC system J. Therefore it is proposed that (D) is kaurenol and (F) kaurenal.

Bands (H), (I) and (J) could not be conclusively identified apart from (H) and (I) being tentatively assigned as alcohol derivatives of the prenyl pyrophosphates, possibly of CPP and IPP, as these are the only 2 prenyl alcohols unidentified.

Conclusive identification is only possible by mass spectrometry.

Apart from the prenyl alcohols and sterols, other polar compounds such as oxidative products of kaurene e.g 7  $\beta$ -hydroxykaurenoic acid, kaurenoic acid, 6  $\beta$ , 7  $\beta$ -dihydroxykaurenoic acid, GA<sub>12</sub>-aldehyde and the kaurenolides, may be produced in vitro, since only the GAs beyond GA<sub>12</sub>-aldehyde have been shown not to be produced. Also, the prenyl alcohols have not been fully assigned and squalene 2,3-oxide may be formed and located in the 'origin'.

Nevertheless, most of the unknown metabolites can be reasonably assigned to a particular class of compound. What is more, the numbers fit the amounts of possible intermediates, including all the prenyl pyrophosphates and the 3 oxidative products of kaurene.

The presence of only 3 consecutive, oxidative metabolites of kaurene correlates with the idea that these compounds are formed by a single enzyme (1.5.2) and further suggests that kaurenoic acid oxidase is inactive in this cell-free system.



The behaviour of F towards these metabolites seems to fit a predicted pattern, though it should be considered that this does not mean the pattern will be followed by all the metabolites: unknown phenomena may be operative, including the action of F, and incorporation levels may not change as expected.

NADPH did not alter incorporation levels in any way, and it can be assumed that the in vitro system preparation contains enough endogenous NADPH to allow fully functional kaurene oxidase and squalene synthetase.

#### 5.3.5 Subcellular Fractionation of In Vitro Activities

Mutant SG22 was grown in the light on AM medium for 5 days, and the crude cell-free preparations i.e S<sub>10</sub> fractionated into S<sub>105</sub> and P<sub>105</sub> (2.2.14).

Using a combination of 3 consecutive TLC systems; H (Table 2.2.2) followed by I (Table 2.2.3a) and then J (Table 2.2.3b) as in 5.3.4, the incorporation [2-<sup>14</sup>C]-MVA into kaurenol, kaurenal, kaurenoic acid and kaurene, the GA metabolites, squalene, phytoene, the other carotenoids and the prenyl alcohols, were determined (Table 5.8).

About 80% of the total activity from S<sub>10</sub> was recovered in the S<sub>105</sub> fraction, indicating that a majority of the active cell-free system enzymes are soluble.

The prenyl alcohols were completely synthesized in S<sub>105</sub>, suggesting that the enzymes from MVA kinase to prenyl transferase(s) are soluble, as has been found in other systems (1.1.1); the phosphatase activity must also be soluble.

The levels of carotenogenic activity for post-phytoene enzymes was found to be very low in the S<sub>105</sub> fraction,

Table 5.8 Effects of Fractionation by Differential Centrifugation on Terpenoid Activities in Cell-Free Systems of Mutant SG22 Grown in the Light and on AM media.

Metabolite	S <sub>105</sub> Activity*	S <sub>105</sub> + P <sub>105</sub> Activity*
Kaurenol	50	40
Kaurenal	50	40
Kaurenoic Acid	50	40
Kaurene	35	15
Squalene	28	100
Phytoene	54	100
Other Carotenoids**	9	96
Prenyl Alcohols	100	100
Total	81	100

S<sub>10</sub> = Cytosolic and Microsomal Preparation

S<sub>105</sub> = Cytosolic Preparation

P<sub>105</sub> = Microsomal Preparation

\* % Activity with respect to that in S<sub>10</sub> (= 100%)

\*\* Carotenoids from G.fujikuroi (see 3.3.1) other than phytoene and NX

suggesting these enzymes are membrane bound, as suggested from previous studies (1.3).

Phytoene, squalene and kaurene activities were present in  $S_{105}$  but at reduced levels compared to those in the  $S_{10}$  fraction. Apparently the synthetases involved in production of these compounds are partly soluble, and the method of cell-free preparation 'releases' a certain amount of each enzyme into the cytosol. This has not been the case in other studies. Kaurene synthetase has been found to be fully soluble and cytosolic in G.fujikuroi (Evans and Hanson, 1972) and a number of higher plant systems (1.5.1). Squalene synthetase has been shown to be microsomal in a number of systems (Popjak and Agnew, 1979) though in P.blakesleeanus (Bramley, 1973) and A.giganteus (El-Jack, personal communication) it is soluble. It has been shown that while phytoene synthetase is also membrane bound in P.blakesleeanus (Gregonis and Rilling, 1974), it can be almost fully released from the membrane by high molarity salts, suggesting it is a loosely bound, peripheral, membrane protein (Bramley and Taylor, 1985).

These 3 synthetase enzymes represent branch points in the terpenoid pathway. Therefore, they may be important sites of terpenoid regulation (1.8). One important means of regulation is compartmentation (reviewed by Douce et al., 1984) by which different enzymes are located in different regions of the cell. In this case the apolar nature of the respective products means that they partition into the membrane. However, the polar substrates (the prenyl pyrophosphates) predict a close location to the cytosol, such that one may propose that these enzymes are peripheral. The results from

G.fujikuroi seem to confirm this, suggesting that the enzymes are detachable and loosely bound. It is possible that the varying results found in other systems for the exact location of these 3 enzymes are a result of their peripheral nature, coupled to the degree of severity to which they are subjected during preparation. The strength of binding to the membrane may also vary between systems.

One possible explanation for the reduced activity of kaurene synthetase in the  $S_{105}$  fraction especially in view of the fact it has hitherto been found to be soluble (1.5.1), is that there is a non-catalytic protein involved in kaurene metabolism. This has been suggested in homogenates of P.sativum seeds (Moore et al., 1972) and G.fujikuroi mycelia (Hanson et al., 1980) in which a soluble carrier protein, analogous to that seen in sterol biosynthesis (1.7.4) has been found to enhance kaurene synthesis. Although the evidence is inconclusive and has been questioned by some workers (reviewed by Hedden, 1983), perhaps kaurene synthetase is fully soluble but the preparatory technique for  $S_{105}$  removes or inactivates the efficient functioning of such a carrier protein.

The results seen for kaurene oxidase products i.e kaurenol, kaurenal and kaurenoic acid, show identical incorporation levels to each other in  $S_{105}$  and are all 50% of the activity found in  $S_{10}$ . That the activities are of equal magnitude suggests that the enzymes for these steps are present in similar amounts and detachable from the membrane to equal levels. The data also support the possibility that the steps are carried out by a multi-subunit complex, each subunit catalysing one step in the overall reaction (West, 1980

and reviewed by Hedden, 1983).

The reduced level of kaurene oxidase activity in  $S_{105}$  may reflect the low activity of kaurene synthetase in this fraction, thus reducing the incorporation into oxidase products and not reflecting the degree of oxidase solubility. Therefore kaurene oxidase may be totally soluble. However, the results do suggest that at least a percentage of kaurene oxidase is soluble. Although the previous studies have shown it to be microsomal and to be a cytochrome P-450 linked oxygenase (reviewed by Hedden, 1983). There is only one P-450 linked enzyme, camphor-5-exo-hydroxylase from Pseudomonas putida which has been reported to be in a soluble form (reviewed by Ralph et al., 1985). Possibly, the Gibberella system will afford a eukaryotic model to study a P-450 linked enzyme.

Confirmation of the solubility of kaurene oxidase is required, however, especially as contamination of the  $S_{105}$  fraction by microsomal fragments is possible (see 6.2.7).

The extraction technique may have detached kaurene oxidase from the membrane in a functional form. A peripheral, loosely bound location of this enzyme, as found for kaurene, phytoene and squalene synthetases, could be inferred from the hydrophilic nature of its substrates and products, indicating the necessity for the enzyme to be associated with the cytosol. Kaurene will need to be located in a more hydrophobic environment, such as the membrane.

Attempts to reconstitute enzymic activity of the  $S_{10}$  fraction by recombining  $S_{105}$  and  $P_{105}$  fractions, restored full carotenogenesis and squalene synthesis, but not kaurene synthetase or apparently kaurene oxidase activities. Consequently, for carotenoids and squalene, the centrifugal fractionation had

separated microsomal and cytosolic enzymes which can be recombined to give a fully functional pathway. However, for kaurene synthetase, not only is activity not fully restored, it is reduced still further on addition of P<sub>105</sub> to S<sub>105</sub>. This may be an artifact produced by a raised activity in other pathways thus reducing substrate levels of kaurene synthetase.

The small incorporations into kaurene on reconstitution of the S<sub>10</sub> fraction, may account for the apparently low activity of kaurene oxidase in the reconstituted preparation.

### 5.3.6 Conclusions and Summary

A cell-free system from G.fujikuroi has been developed for the terpenoid pathway. In the carotenoid pathway incorporation of [2-<sup>14</sup>C]-MVA can be achieved as far as torulene, but not into NX. This is the first time a carotenogenic in vitro system has been developed for this fungus.

Cell-free systems in the GA pathway have been developed in G.fujikuroi from [2-<sup>14</sup>C]-MVA as far as GA<sub>12</sub>-aldehyde (West, 1973), although the later steps, considered to be soluble oxidases, have been less well characterised in vitro. This is somewhat surprising considering the soluble nature of these enzymes. From the cell-free system, [<sup>14</sup>C] incorporation was identified as far as kaurenoic acid. Whether later steps are active cannot be determined, generally through lack of authentic standards, although the absence of incorporation into un-designated metabolites which could be kaurene oxidative products, suggests that this is the full extent of GA in vitro activity. This also supports the idea that there is a specific kaurene oxidase for kaurenoic acid production, and another oxidation enzyme involved in the following steps which is not active in this system. Such evidence comes from the

inhibitor studies using ancymidol, which specifically inhibits kaurene oxidase but not kaurenoic acid oxidation (Coolbaugh and Hamilton, 1976 and Coolbaugh et al., 1978). These workers also showed ancymidol to be specific for P-450, such that kaurene oxidation is not carried out by a cytochrome P-450 linked enzyme.

The in vitro system is also capable of incorporation of [ $^{14}\text{C}$ ] into squalene, but not the sterols. It has been possible to characterise a number of the properties of the terpenoid pathway. These include the effects of age of culture, indicating the specific times that each individual pathway is active i.e at 3 days for the sterols, 5 days for carotenogenesis and 8-11 days for the GAs. This reflects the in vivo data and time of production in relation to their possible functions. It has been further demonstrated that there is a relationship between each individual pathway within the total terpenoid pathway i.e an increased activity in one pathway sees a concurrent reduction in other pathway activities.

It is possible to use the cell-free system to indicate the intracellular location of the pathway enzymes and these results suggest phytoene, squalene and kaurene synthetases to be loosely bound peripheral membrane proteins. The earlier enzymes are soluble, while carotenogenic dehydrogenases, cyclases and isomerase are membrane bound along with the post-squalene enzymes. It is possible that kaurene oxidase is soluble, or at least loosely membrane bound, and that kaurene synthetase is fully soluble.

The inter-relationships of age, mutation, media and light as they effect terpenoid biosynthesis in Gibberella, are discussed in Chapter VII and illustrated in Fig 7.1.

CHAPTER VI

RESULTS

*Assay and Some*  
*Properties* of Phytoene Synthetase

"Truth is never pure,  
and rarely simple"

Oscar Wilde



## 6.1 Introduction and Aims

The main aim of this part of the project was an attempt to purify the enzyme, phytoene synthetase. This enzyme converts PPPP or GGPP via PPPP to phytoene; whether this involves a single enzyme from PPPP or GGPP via PPPP as an intermediate, or whether it involves a protein complex with 2 individual catalytic sites is uncertain, but could be clarified by purification.

The intracellular location of phytoene synthetase is also uncertain (1.8). Generally it is considered to be microsomal (see 1.8), though in some strains it can be easily dissociated as a soluble enzyme, such as in P.blakesleeanus (Bramley and Taylor, 1985) and A.giganteus (El-Jack, personal communication). In purification of phytoene synthetase from G.fujikuroi it will be necessary to confirm the intracellular location of this enzyme for this species, and if it is microsomal, attempt to obtain it in a soluble form.

An integral part of any enzyme purification is to develop an assay system for the enzyme under investigation. This assay should allow a direct measurement of the enzyme activity and not be prone to any metabolic artifacts. Thus in considering phytoene synthetase, an assay must be developed which supplies the substrate and allows measurement of the direct product, phytoene. Since it is uncertain as to the true identity of the precursor of this enzyme, both PPPP and GGPP should be supplied in an assay. Furthermore, to allow a sensitive assay of phytoene, [ $^{14}\text{C}$ ]-labelled substrate should be used such that the assay involves detection of a radiolabelled product.

The requirements in an assay are encompassed in a coupled

assay. In this, an initial, cell-free incubation is prepared such that  $[2-^{14}\text{C}]$ -MVA is incorporated into GGPP and PPPP, which are then coupled to phytoene synthetase (2.2.24).

Once the assay system has been developed, a purification protocol will be followed. This would incorporate an initial, centrifugal fractionation and identification of a microsomal or cytosolic activity. If soluble, a crude preparation can be applied to column chromatography, in particular fast protein liquid chromatography (FPLC), and subject to gel filtration, ion exchange chromatography and chromatofocusing. These techniques separate proteins according to molecular weights, ionic charge and isoelectric points, respectively and the most suitable combination to effect a purification can be determined.

If the protein is found to be microsomal, it will be necessary to solubilize the enzyme before chromatography.

One method of solubilizing a microsomal phytoene synthetase may be to use high salt concentrations or, if this proves inefficient, detergents, both of which have been used for carotenogenic enzymes in *P. blakesleeanus* (Bramley and Taylor, 1985), while detergents alone have been used for carotenogenic enzymes in daffodil (Beyer et al., 1985) and *Capsicum* (Camara and Dogbo, 1986).

If the enzyme is membrane bound, the membrane itself may be integral for phytoene synthetase activity. In solubilization, detergents may replace such components, for example they may mimic essential lipids. But such detergents may be removed for purification by column chromatography and so it may prove necessary to re-introduce certain membrane lipids or components to a purified enzyme fraction to allow activity

to be assayed.

That this is the case has been suggested for phytoene synthetase in N.crassa (Mitzka-Schnabel, 1985) where membrane bound phytoene synthetase is solubilized by detergents, but the enzyme activity is lost once the detergents are removed by column chromatography.

The purpose of purifying phytoene synthetase from G.fujikuroi is to establish whether 2 isoenzyme forms exist, as was suggested from in vivo analysis of the strains SG43 (3.3.4) and SG78 (3.3.5). Through purification it may be possible to identify isoenzymic forms which may be present at different levels in these mutants.

The 2 possible enzyme forms may actually be identical but possess different induction mechanisms at a genetic level. This may prove an interesting area of study, aided through purification of phytoene synthetase. Once purified, antibodies may be developed which may then be used for rapid purification of the enzyme and to compare, by cross-reactivity, homology with phytoene synthetases from other e.g. fungal sources. Antibodies may also be used in isolating phytoene synthetase gene (from G.fujikuroi or perhaps other fungi) from which one may identify the possible photoinduced and chemically induced sequences (discussed in Chapter VII).

## 6.2 Results and Discussion

### 6.2.1 Stability of Enzyme Activity

The stability of phytoene synthetase activity was determined over time to ascertain whether the enzyme remains active on storage.

The total levels of incorporation of  $[2-^{14}\text{C}]$ -MVA into the terpenoids were measured over 28 days, from  $S_{10}$  preparations of SG78, light-grown on AM media for 5 days. During storage at  $-70^{\circ}\text{C}$ , the incorporation levels of the preparation show a steady fall with time (Fig 6.1).

As the  $S_{10}$  system contains a number of terpenoid enzymes and phytoene incorporation may represent only 10% of the total incorporation (5.3.2), it can only be assumed that the stability of phytoene synthetase is reflected by the stability of the total terpenoid in vitro system. This is not necessarily the case, however, as different enzymes may possess different rates of inactivation. The steady rate of decline in activity does imply a fall in activity of all the enzymes (or one in particular at a relatively fast rate) at a similar rate.

Therefore, after 28 days storage, phytoene synthetase activity still represents about 10% of total activity. It was concluded that  $S_{10}$  preparations of G.fujikuroi can retain a measureable (i.e.  $>10 \times 10^3$  dpm), in vitro activity up to 1 month storage at  $-70^{\circ}\text{C}$ .

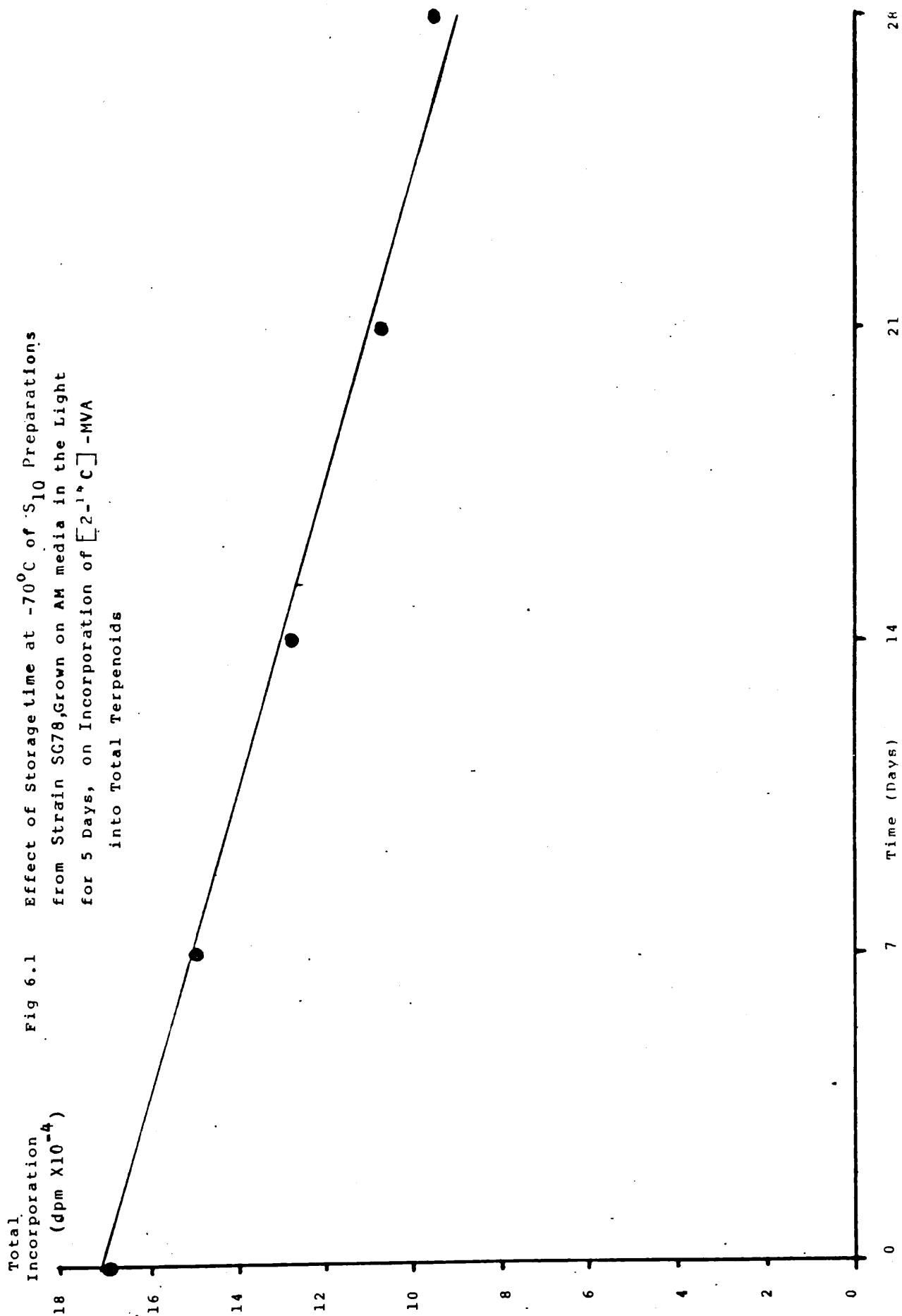
Storage of the  $S_{10}$  preparation at  $-70^{\circ}\text{C}$ , in 30% glycerol, had no beneficial effect on retention of activity, while storage at  $4^{\circ}\text{C}$  accelerated activity degradation such that it is virtually lost after 7 days.

#### 6.2.2 Effect of Tris-HCl Buffer Concentrations on the Solubilization of Phytoene Synthetase

From preliminary investigations on intracellular, fraction terpenoid activities (5.3.5), phytoene synthetase appears to be a loosely bound, peripheral membrane protein which may be

Total  
Incorporation  
(dpm  $\times 10^{-4}$ )

Fig 6.1 Effect of Storage time at  $-70^{\circ}\text{C}$  of  $\text{S}_{10}$  Preparations  
from Strain SG78, Grown on AM media in the Light  
for 5 Days, on Incorporation of  $[2-^{14}\text{C}]\text{-MVA}$   
into Total Terpenoids



dissociated from the membrane. Work with P.blakesleeanus (Bramley and Taylor, 1985) has indicated that the level of phytoene synthetase that can be solubilized from the membrane is dependent on the salt concentration present in the preparation of an S<sub>105</sub> fraction: a high concentration favours dissociation. The disadvantage of using a high salt concentration is that the preparation must be de-salted before applying to an ion-exchange column such that the sample is in a low salt concentration buffer.

To determine the most suitable buffer concentration to 'release' sufficient phytoene synthetase activity from the microsomes while avoiding, if possible, any additional steps before column chromatography, phytoene synthetase activity was assayed in the S<sub>105</sub> (which had been prepared in a range of Tris-HCl buffer, pH 8.0 concentrations; 50mM-400mM).

The results (Table 6.1) indicate that phytoene activity is highest in the S<sub>10</sub> fraction prepared in 400mM buffer, and decreases concurrently with a decrease in the Tris-buffer concentration. Apparently a higher salt concentration favours phytoene synthetase activity, perhaps by conferring stability to the enzyme structure. Alternatively, the fall in phytoene activity may be an artifact resulting from raised activity in the rest of the terpenoid pathway; this is indicated by kaurene and 'origin' (see 5.3.4) ; representing the alcohol derivatives of the prenyl pyrophosphates and the oxidative products of kaurene, including kaurenol, kaurenal and kaurenoic acid) activities which tend to increase with falling Tris-buffer concentrations, implying that high salt levels inhibit these activities. Perhaps both phenomena are operative in these changes in enzymic activities.

Table 6.1 Effect of Concentrations of Tris-HCl Buffer, pH 8.0 on Terpenoid Pathway Activities from Cell-Free Systems of Mutant Strain SG78

Supernatant Fraction and Buffer Conc.	'Origin*' (dpm/mg protein)	Squalene (dpm/mg protein)	Kaurene (dpm/mg protein)	Phytoene	
				(dpm/mg protein)	%S <sub>10</sub> in S <sub>105</sub>
400mM S <sub>10</sub>	1200	365	10	2075	100
400mM S <sub>105</sub>	400	25	-	540	26
200mM S <sub>10</sub>	1815	325	15	1765	100
200mM S <sub>105</sub>	2420	300	140	253	20
100mM S <sub>10</sub>	2820	825	1185	1175	100
100mM S <sub>105</sub>	2526	537	6526	196	17
50mM S <sub>10</sub>	1596	503	1465	763	100
50mM S <sub>105</sub>	1300	190	4690	110	14

'Origin\*'; represents the alcohol derivatives of the prenyl pyrophosphates and oxidative products of kaurene, including kaurenol, kaurenal and kaurenoic acid (see 5.3.4)

Phytoene synthesis in the different  $S_{105}$  fractions indicates a lower enzyme activity in each case, than that found in the  $S_{10}$  fraction. This has already been found (5.3.5) and reflects the loosely bound properties of this peripheral, membrane protein. However in these earlier studies, over 50% of the  $S_{10}$  phytoene synthetase activity was recovered in the  $S_{105}$  fraction, prepared in 400mM buffer, whereas in this case only 26% activity is retained: this anomaly is also apparent for the other terpenoid metabolites, kaurene and squalene, whose specific activities in  $S_{10}$  and  $S_{105}$  fractions in 400mM buffer are different to those found earlier (5.3.5). Although SG22 was used in the earlier study, the results support the proposal (5.3.5) that loosely bound, membrane, peripheral enzymes kaurene synthetase, squalene synthetase and phytoene synthetase are detachable from the microsome to varying degrees depending on the growth conditions and age of culture. That all 3 enzymes may be solubilized to varying extents, may in turn lead to further artifacts by redistribution of activities according to the levels of each enzyme that are present. Generally though, the solubility of phytoene synthetase in 400mM Tris-HCl buffer is variable and not reproducible.

In the lower salt concentration buffers, phytoene synthetase activity recovery in  $S_{105}$  fractions, is less than that seen in 400mM buffer and falls with lowering salt concentration.

High salt buffer concentrations remove greater levels of phytoene synthetase from the membranes, as seen in P.blakesleeanus (Bramley and Taylor, 1985), indicating the need for a hydrophilic environment to disrupt the peripheral membrane structure and remove the proteins in active configurations.



Again, these buffer concentration differences may reflect an artifact due to increased activity in the  $S_{105}$  of kaurene synthetase and the 'origin', which shows a general increase compared to that found in  $S_{10}$  in decreasing buffer concentrations (though it decreases slightly at 50mM) which may remove possible substrate from phytoene synthetase.

The increase in kaurene synthesis and incorporation in the 'origin' fraction, may reflect a high inhibitory action by salts and may also be due to the effects of the buffer on kaurene synthetase carrier protein (5.3.5).

In summary, 400mM Tris-HCl pH 8.0 buffer containing 5mM DTT, appears to be the most suitable for obtaining phytoene synthetase activity in the  $S_{105}$  fraction. However, the percentage activity found in  $S_{105}$  compared to  $S_{10}$  tends to vary with culture preparations, dependent on age, growth conditions, possibly the mutant and other terpenoid pathway enzyme variables. Nevertheless, this is an essential purification step for phytoene synthetase to isolate the protein from the microsomes.

### 6.2.3 Fractionation of Phytoene Synthetase Activity in Mutant Strains SG43 and SG78

The in vivo carotenoid levels in SG78 and SG43 suggests that there are 2 forms of phytoene synthetase; a photoinduced form and a chemically induced type. The latter is found in light- and dark-grown SG78 (3.3.5), with the photoinduced form in light-grown SG78 (3.3.5) and solely in light-grown SG43 (3.3.4). Dark-grown SG43 contains no phytoene synthetase (3.3.4).

Though the different forms may be identical with different mechanisms of induction, it is possible that they are

present as isoenzymes. Furthermore, in either case the 2 types may be located in different intracellular regions e.g. in an extreme case, one may be microsomal the other cytosolic. This may explain the partial recovery of the enzyme in the  $S_{105}$  fraction of SG78, the remainder being microsomal (6.2.2).

If the isoenzymes are in different intracellular locations, the 2 forms may be separated by fractionation.

The mutant strains, SG43 and SG78, were light- and dark-grown on AM media for 5 days and cell-free extracts were assayed for  $[2-^{14}\text{C}]$ -MVA incorporation levels into phytoene, in both  $S_{10}$  and  $S_{105}$  fractions. Levels of incorporation into kaurene and squalene were also determined.

From the results (Table 6.2), 100% of phytoene synthetase activity in  $S_{10}$ , is recovered in the  $S_{105}$  fraction of light-grown SG78. This is contrary to other results where 25% activity (6.2.2) and 50% activity (5.3.5) were recovered (though this was for SG22 in the latter case). Therefore, it appears that phytoene synthetase solubility is extremely variable in different mycelial preparations (though not from the same mycelial batch); this in turn may mean the results from this experiment are meaningless when comparing enzyme solubility from different mycelia. However, although results are variable, and so cannot confirm the intracellular location of phytoene synthetase isoenzymes. The fact that 100%  $S_{10}$  activity is found in  $S_{105}$  of light-grown SG78, suggests that the possible enzyme forms are not located in distinctly separate regions. If the 2 types exist, it is probable that they are both peripheral proteins, or both genuinely soluble.

Another result of some interest is that dark-grown SG43

Table 6.2

The Effect of Fractionation on Phytoene Synthetase and Squalene Synthetase Activity in Light- and Dark-Grown SG78 and SG43

<u>G.fujikuroi</u> Strain	Growth Conditions	%S <sub>10</sub> Activity in S <sub>105</sub>	
		Squalene	Phytoene
SG78	Light	30	100
	Dark	50	80
SG43	Light	35	50
	Dark	50	-

S<sub>10</sub> activity: SG78, light, squalene; 85000 dpm  
 " " phytoene; 14500 dpm  
 " dark, squalene; 78000 dpm  
 " " phytoene; 9000 dpm  
 SG43, light, squalene; 56000 dpm  
 " " phytoene; 7600 dpm  
 " dark, squalene; 65000 dpm  
 " " phytoene; -

shows no phytoene production, suggesting the total absence of phytoene synthetase. This confirms the in vivo results (3.3.4) and the fact that the enzyme is totally photo-inducible in this strain.

Though kaurene levels are too low to be measurable (due to high kaurene oxidase activity or low kaurene synthetase in young cultures), the recovery of squalene activity in S<sub>105</sub> is also seen to be variable in the different strains. This difference is inexplicable with respect to the mutant differences and is more probably related to the variability of the enzyme solubility from different mycelial batches, as with phytoene synthetase.

It seems the variability of recovery of phytoene synthetase and squalene synthetase in the soluble cellular fraction may be related to their peripheral location and the degree of membrane binding of each enzyme. It may also be related to the levels of associated cell-wall polysaccharides and glycoproteins which could be high in the high C medium (AM) and may also vary in different batches.

It should also be noted that on separation of soluble enzymes from microsomal fractions a number of terpenoid pathways e.g sterols, carotenoids and GAs, are disrupted and the overall activities and incorporation levels will be redistributed. This means that in vitro data is susceptible to a number of artifacts making it difficult to determine the degree of enzyme dissociation from the membrane in this manner.

#### 6.2.4 Identification of [<sup>14</sup>C]-Prenyl Pyrophosphates in Cell-Free Incubations

In developing a coupled assay, it was necessary to produce

[<sup>14</sup>C]-PPPP and -GGPP (6.1). Consequently, it is important to be able to identify the levels of these intermediates produced in cell-free systems, to ascertain the most suitable coupling system.

A system had been developed to identify the prenyl pyrophosphate intermediates (5.3.4) which could be used to indirectly identify GGPP and PPPP, but it must be confirmed that such alcohol derivatives exist as their parent pyrophosphates within the incubation: the alcohol derivatives or monophosphates of the intermediates will not be used by the enzymes of the terpenoid pathway.

Cell-free incubations of mutant SG78, light-grown for 5 days on AM medium, were extracted and subject to one of 3 TLC systems; K,L and M (Table 2.2.4) capable of separating prenyl pyrophosphates; and 3 TLC systems J,N and O (Table 2.2.5) to separate the alcohol derivatives of the prenyl pyrophosphates (2.2.22.i and ii).

The levels of [<sup>14</sup>C] in the alcohol derivatives of the prenyl pyrophosphates are given in Table 6.3, identified by co-chromatography with authentic standards and/or R<sub>f</sub> values from the literature (Table 2.2.4).

The [<sup>14</sup>C]-incorporation in the prenyl pyrophosphates themselves are shown in Table 6.4, having been identified by comparative literature R<sub>f</sub> values and comparing incorporation levels with the known alcohol derivatives of the prenyl pyrophosphates (Table 6.3).

The incorporation levels of alcohol derivatives in the different TLC systems (Table 6.3), show good complementation with generally similar levels in each, or combined, intermediates. Only system J shows separation of all 4 identified

Table 6.3 Incorporation of [ $^{14}\text{C}$ ] into the Alcohol Derivatives of the Prenyl Pyrophosphates from the Cell-Free Incubations of SG78 as Separated on Different TLC Systems

Alcohol Derivatives	TLC Systems*		
	J	N (dpm)	O
Unknown	18600	14700	15500
Prephytoene Alcohol	21800	22600	18000
Geranyl Geraniol	28600	} 31330	24700
Farnesol	6900		} 11600
Geraniol	3400		

\* See Table 2.2.5

(i.e prephytoene alcohol, geranyl geraniol, geraniol and farnesol). System N only separates prephytoene alcohol from geranyl geraniol, with geraniol and farnesol located in one band, but the total incorporation level is what is expected for a combination of these 3 intermediates compared to system J. The same is found with system O, although only farnesol and geraniol are located together while prephytoene alcohol and geranyl geraniol separate, again with similar incorporation levels to those found in J and N.

In each system, J, N and O, there is an unknown band containing similar levels of [ $^{14}\text{C}$ ] located in a less polar position on TLC (J, origin; N, origin and O, Rf 0.88). This may represent the CPP derivative copalol or the alcohol derivative of IPP.

The prenyl alcohol derivative incorporation levels (Table 6.3) are similar to those of the parent prenyl pyrophosphates (Table 6.4). This is as to be expected, since the incubations are identical and indicates that the acid hydrolysis of the prenyl pyrophosphates to form the alcohols (2.2.22.ii) does not degrade the metabolites. It also means the alcohol derivative systems can be used to aid identification of the prenyl pyrophosphates in TLC systems K, L and M. However, of these systems only K shows a good separation of all the intermediates. In this system the [ $^{14}\text{C}$ ] levels in the prenyl pyrophosphates show very good comparison with the levels in the respective alcohol derivatives (Table 6.3), especially J where GPP, FPP, GGPP and PPPP [ $^{14}\text{C}$ ]-levels are almost identical in the 2 systems: the unknown band shows increased levels in system K compared to system J implying that acid hydrolysis degrades these components in some way. Generally,

Table 6.4 Incorporation of [ $^{14}\text{C}$ ] into the Prenyl Pyrophosphates from the Cell-Free Systems of SG78 as Separated on Different TLC Systems

Prenyl Pyrophosphates	TLC Systems*		
	K	L (dpm)	M
Unknown	39500	} 62100	} 31400
PPPP	20200		
GGPP	24600	22600	26000
FPP	6200	} 11100	} 7400
GPP	3800		

\* See Table 2.2.4



the prenyl intermediates formed in vitro are mainly pyrophosphates.

Systems M and L are only suitable for the separation of GGPP from the other intermediates, since FPP and GPP, together with PPPP and the unknown band, develop as 2 bands identified by literature Rf values (Table 2.2.4) and [ $^{14}\text{C}$ ]-incorporation levels compared to system K and the alcohol derivative systems.

In conclusion, system J is the most suitable to separate the alcohol derivatives, whilst K is the best for the prenyl pyrophosphates themselves. The results imply that the alcohol derivatives from acid hydrolysis derive mainly from the pyrophosphates, and not the monophosphates which are apparently absent. Therefore, the prenyl alcohols reflect the pyrophosphates in a cell-free incubation. In any case, it is possible to determine the [ $^{14}\text{C}$ ]-GGPP and -PPPP levels in in vitro systems.

#### 6.2.5 The In Vitro Formation of [ $^{14}\text{C}$ ]-GGPP and [ $^{14}\text{C}$ ]-PPPP in Different Fungal Strains

The G.fujikuroi mutants SG4, SG75, and SG76 are GA producers (4.3.5) but do not synthesize phytoene (Avalos et al., 1985), and therefore may accumulate GGPP or PPPP.

Dark-grown A.giganteus does not produce phytoene (El-Jack, personal communication) and so may form GGPP or PPPP as end products of the terpenoid pathway.

The N.crassa strains, 911 and 4014 are affected at the al-2 locus which is considered to be a particulate enzyme involved in phytoene synthesis from PPPP (Harding and Turner, 1981) and so may accumulate the latter.

All these strains were grown on specific media (2.2.1) and

cell-free systems prepared (2.2.14). The in vitro systems (2.2.15) were analyzed for the incorporation levels of  $[2-^{14}\text{C}]$ -MVA into the prenyl pyrophosphates, in particular GGPP and PPPP, with respect to time of incubation using TLC system J (2.2.5) on the alcohol derivatives (2.2.22.ii), (Table 6.5).

Both N.crassa strains accumulate high levels of  $[^{14}\text{C}]$ -prephytoene alcohol, thus confirming the proposed site for the mutational block i.e al-2, phytoene synthetase from PPPP (Kushwaha et al., 1978 and Harding and Turner, 1981). The levels of  $[^{14}\text{C}]$ -geraniol, -geranyl geraniol and -farnesol are all low (< 9000 dpm) with -farnesol being absent showing that the in vitro systems are very efficient in PPPP production. With strain 4014 the  $[^{14}\text{C}]$ -prephytoene alcohol levels increase to a maximum (1.5h) while  $[^{14}\text{C}]$ -geranyl geraniol and -geraniol levels do not show a regular pattern with time, except for an apparent fall concurrent with the increase in prephytoene alcohol, suggesting as expected an assimilation of the intermediates into later products.

The pattern in strain 911 is similar to that in 4014, except that maximum  $[^{14}\text{C}]$ -prephytoene alcohol levels are reached earlier (1h).

Dark-grown A.giganteus incubations produce reasonably high  $[^{14}\text{C}]$ -prephytoene alcohol levels (ca. 30000 dpm), indicating that phytoene synthetase, using PPPP as a substrate, is fully photoinducible in this fungus (light-grown cultures produce phytoene, in vivo and in vitro; El-Jack, personal communication). Maximal  $[^{14}\text{C}]$ -prephytoene alcohol levels are reached early in the incubation (within 0.5h), while  $[^{14}\text{C}]$ geranyl geraniol levels remain low until later (2h).

Table 6.5 Incorporation of [ $^{14}\text{C}$ ] into Alcohol Derivatives of the Prenyl Pyrophosphates in Cell-Free Systems of Different Fungal Strains over Time of the Incubation

Strain and Alcohol Derivative	Time of Incubation (Min)				
	0.5	1	1.5	2	3
<u>N. crassa</u> 4014					
Prephytoene Alcohol	23800	36500	56400	56000	60500
Geranyl-geraniol	3700	7500	4400	3100	-
Farnesol	-	-	-	-	-
Geraniol	6400	3800	-	-	-
<u>N. crassa</u> 911					
Prephytoene Alcohol	55000	68700	55300	48400	54100
Geranyl-geraniol	9000	4200	-	3200	5300
Farnesol	-	-	-	-	-
Geraniol	7500	2300	-	-	-
<u>A. giganteus</u> (Dark-Grown)					
Prephytoene Alcohol	29500	30100	30100	35000	18400
Geranyl-geraniol	3100	5900	3900	24000	15600
Farnesol	3900	2900	3300	6500	9000
Geraniol	20800	15400	15700	18700	23800

Table 6.5

Incorporation of [ $^{14}\text{C}$ ] into Alcohol  
Derivatives of the Prenyl Pyrophosphates in  
Cell-Free Systems of Different Strains,  
over Incubation Time

Strain and Alcohol Derivative	Time of Incubation (Min)				
	0.5	1	1.5	2	3
<u>G.fujikuroi</u> SG4					
Prephytoene Alcohol	13200	20400	39400	35400	32200
Geranyl - geraniol	30800	28400	38100	28800	26200
Farnesol	7000	7300	8200	3700	4200
Geraniol	23800	25600	16100	14400	14200
<u>G.fujikuroi</u> SG75					
Prephytoene Alcohol	9800	28200	26000	25600	39000
Geranyl - geraniol	10500	17800	20000	22400	15700
Farnesol	7800	9200	8600	10400	14600
Geraniol	38400	28000	31600	25000	15600
<u>G.fujikuroi</u> SG76					
Prephytoene Alcohol	10000	25200	27600	25000	31000
Geranyl - geraniol	9000	15000	19600	21000	22700
Farnesol	7000	8700	9200	10000	11300
Geraniol	35300	29200	32000	29600	25300

[<sup>14</sup>C]-farnesol is produced but at relatively low levels (<9000 dpm) which change little over the incubation period, except late when they rise slightly (2h). [<sup>14</sup>C]-geraniol levels remain quite high (ca. 20000 dpm) throughout the incubation.

The G.fujikuroi mutants SG4, SG75 and SG76 all produce [<sup>14</sup>C]-prephytoene alcohol, maximal levels (1h for SG75 and SG76, 1.5h for SG4) being greatest in SG4 (ca. 40000 dpm), [<sup>14</sup>C]-geranyl geraniol levels are also quite high compared to the other strains tested (20000-40000 dpm), although they do not show a consistent pattern of change with time of incubation; perhaps a slight increase with time. Again, [<sup>14</sup>C]-geranyl geraniol levels are greatest in SG4 (ca 40000 dpm) after the same time period (1.5h) for maximal pre-phytoene alcohol.

[<sup>14</sup>C]-geraniol levels are all of similar magnitude (20000-40000 dpm), whilst [<sup>14</sup>C]-farnesol levels are quite low, as in the other strains (<15000); again there is no apparent pattern to the changes in these intermediates over time.

In all the strains, incubation time has some affect on the levels of [<sup>14</sup>C]-prephytoene alcohol which tend to increase with time. Generally, [<sup>14</sup>C]-farnesol is the lowest in each strain and tends not to accumulate at all, suggesting a high activity of squalene synthetase and possibly of prenyl transferase: it also reinforces the need for care when analyzing in vitro results, in that low incorporation levels do not necessarily mean low enzymic activities.

Another pattern in the [<sup>14</sup>C]-intermediate levels of each strain is the inconsistent levels of each intermediate, except prephytoene alcohol, with respect to time of

incubation. This also shows the problems in analyzing in vitro intermediate levels, since within a pathway there may be a number of regulatory and metabolic effects.

Since all the strains tested accumulate [ $^{14}\text{C}$ ]-prephytoene alcohol, they all lack phytoene synthetase (i.e. prephytoene pyrophosphate to phytoene). This in turn implies that this activity is in fact separate, and independent of, PPPP synthetase and that the formation of phytoene from GGPP involves 2 distinct, separate enzymes. However, since one cannot be sure that this is the case, nor of the efficiency with which the enzyme can use PPPP, it was considered that the best strain to use in a coupled assay should be one that produces a high [ $^{14}\text{C}$ ]-PPPP and -GGPP level.

N.crassa strains and A.giganteus cannot be used for a coupled system since their [ $^{14}\text{C}$ ]-GGPP levels are too low. Of the G.fujikuroi mutants, SG4 after 1.5h of incubation produces the highest levels of [ $^{14}\text{C}$ ]-PPPP and -GGPP. Since it is possible that the coupled system may allow all the prenyl pyrophosphates to be available for eventual use by phytoene synthetase (e.g. [ $^{14}\text{C}$ ]-GPP and -FPP), then SG4 produces the greatest total, prenyl pyrophosphate levels which may be used by phytoene synthetase (> 100000 dpm).

That a G.fujikuroi mutant appears to be the most suitable for coupling may also be more advantageous in that the system for coupling of purified phytoene synthetase from G.fujikuroi will consequently be homogenous and less prone to artifacts: a heterogenous couple between this system and cell-free extracts of N.crassa or A.giganteus may not produce a compatible couple.

### 6.2.6 Coupled Assay

Having determined that mutant SG4 of G.fujikuroi was the most suitable for use in a coupled assay of phytoene synthetase extracts (6.2.4), attempts were made to couple the system to produce [ $^{14}\text{C}$ ]-phytoene from -GGPP and -PPPP in cell extracts containing phytoene synthetase.

Incubations of SG4 (1.5h) were coupled (2.2.24.i) to S<sub>105</sub> extracts of 5 day-old, light-grown on AM, SG78, phytoene and other labelled terpenoid intermediates were measured over time from coupling (Table 6.6).

Incubations of SG4 at zero couple time (1.5h) produce low levels of squalene and kaurene, while phytoene is absent. The alcohol derivative levels indicate that [ $^{14}\text{C}$ ]-PPPP and -GGPP production is lower than that previously determined (6.2.4), and so total levels may vary from different batch cultures and preparations. Nevertheless, the total [ $^{14}\text{C}$ ]-prenyl pyrophosphate levels should be sufficient to qualitatively determine the presence, or not of phytoene synthetase.

On coupling with SG78 extracts, [ $^{14}\text{C}$ ]-phytoene levels increase to over 15000 dpm within 10min. This is the maximum level reached; all times beyond have slightly lower levels (12000 dpm). [ $^{14}\text{C}$ ]-Squalene levels also increase to a maximum after 10min of coupling, suggesting that in SG78 squalene synthetase is present in the soluble fraction. [ $^{14}\text{C}$ ]-Kaurene levels rise only slightly on coupling, as does the 'origin' (representing the alcohol derivatives of the prenyl pyrophosphates and the oxidative products of kaurene, including kaurenol, kaurenal and kaurenoic acid; see 5.3.4 ), suggesting a low activity of kaurene synthetase and (or high activity of) kaurene oxidase in this fraction;

Table 6.6 The Effect of Coupling a 1.5h, SG4 Cell-Free Incubation with SG78, S<sub>105</sub> Cell-Free Extracts on [<sup>14</sup>C]-prenyl pyrophosphate Alcohol Derivatives over Time of Coupling

Metabolites	Time of Couple (Min)				
	0*	10	20	30	60
	(dpm)				
Squalene	1700	11500	10000	11000	13000
Kaurene	100	200	300	250	250
Coloured Carotenoids	-	-	-	-	-
Phytoene	180	15900	12200	12000	12300
Prephytoene Alcohol	11900	2900	4800	15000	46000
Geranyl-geraniol	14000	43000	43700	43200	43900
Farnesol	7200	8200	6900	5300	1300
Geraniol	13000	7100	6100	2000	200

\* At zero time, incorporation levels represent those of SG4 incubations (1.5h)



this may be due to the young age of culture with respect to GA biosynthesis.

Only [ $^{14}\text{C}$ ]-prephytoene alcohol and -geraniol show concurrent falls in incorporation on coupling, while [ $^{14}\text{C}$ ]-geranyl geraniol shows a marked increase. That there is an increase in [ $^{14}\text{C}$ ]-phytoene, -squalene and -geranyl geraniol levels, greater than the fall in [ $^{14}\text{C}$ ]-prephytoene alcohol and -geraniol suggests that substrate must be available from earlier intermediates in the pathway, which are assimilated through the whole pathway i.e. [ $^{14}\text{C}$ ]-IPP and -MVAPP; [ $2\text{-}^{14}\text{C}$ ]-MVA cannot be used since the unlabelled MVA added to the incubation (2.2.24.i) was shown to completely prevent any labelled incorporation by MVA kinase.

Therefore, on coupling, all the terpenoid pathway enzymes to phytoene synthetase, from SG78 extracts, utilize the available [ $^{14}\text{C}$ ]-precursors and effect a redistribution of the intermediate levels, thus complicating analysis of the changes in their levels.

The [ $^{14}\text{C}$ ]-farnesol and -geraniol amounts, fall on continued incubation (beyond 10min), [ $^{14}\text{C}$ ]-geranyl geraniol remains the same while [ $^{14}\text{C}$ ]-prephytoene alcohol rises again suggesting a 'channeling' of the prenyl pyrophosphate intermediates into PPPP by the soluble enzymes of SG78. However, phytoene synthetase activity appears to have fallen off completely after 10min, either due to rapid denaturation of the enzyme or because maximal activity is reached within 10min and the enzyme is at equilibrium (with phytoene turnover), and/or under regulation.

Generally, the couple is complete for phytoene synthesis within 10min, beyond which there is no further incorporation

accumulating [ $^{14}\text{C}$ ]-PPPP. On the whole, the terpenoid intermediate levels fluctuate in a complicated manner on coupling, due to the many possible enzymic activities within the whole system. Nevertheless, it appears to be a system capable of producing labelled precursor for phytoene synthetase which can use the substrate quickly and efficiently: an additional coupled incubation indicated that 10min is the optimum coupling period since a 5min incubation produces half the level of [ $^{14}\text{C}$ ]-phytoene found after 10min.

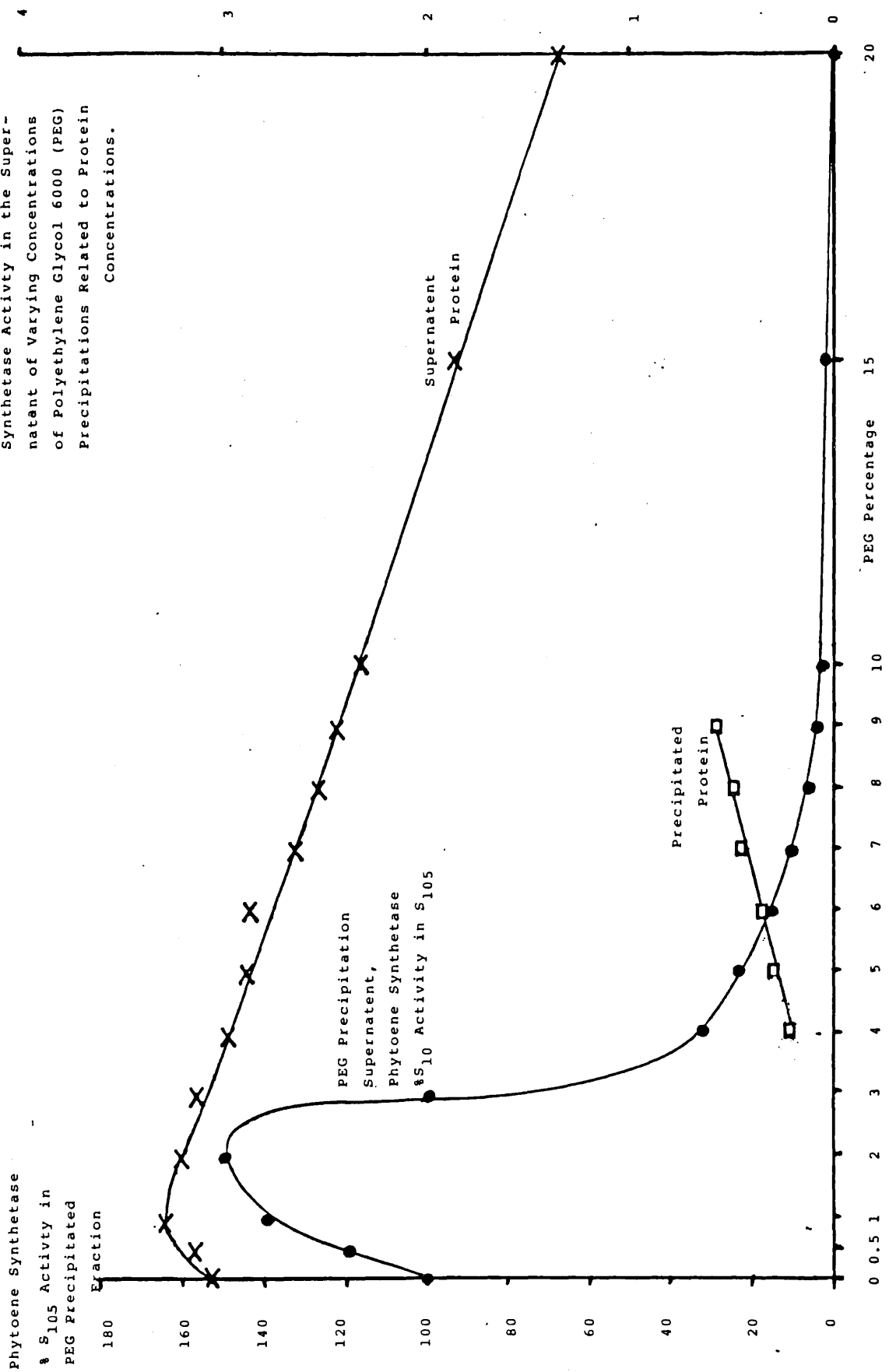
#### 6.2.7 Precipitation of Phytoene Synthetase by Polyethylene Glycol 6000

Cytosolic extracts of SG78, light-grown on AM for 5 days, were treated with varying concentrations of polyethylene glycol 6000 (PEG; 2.2.22). The supernatant and protein precipitates at various PEG concentrations were assayed for phytoene synthetase activity, using a coupled assay with preincubated SG4 extracts (2.2.24), (Fig 6.2).

At low PEG levels ( $\leq 2\%$ ), phytoene synthetase activity is greater than that in non-precipitated  $S_{105}$  fractions. This apparent 'activation' of phytoene synthetase activity by PEG may reflect the removal (by PEG) of proteins or factors inhibitory to the enzyme activity. Alternatively, or additionally, it may result from the removal of proteins which use phytoene synthetase substrates such as kaurene synthetase, thus allowing an increase in phytoene incorporation levels. This latency of phytoene synthetase activity has also been seen in *P.blakesleeanus* (Bramley and Taylor, 1985) using detergents to solubilize the enzyme where it was suggested a synergistic action making the active site on the enzyme more accessible to its substrate was the reason for the

Protein  
(mg/ml)

Fig 6.2 The Percentage of S<sub>10</sub> Phytoene Synthetase Activity in the Supernatant of Varying Concentrations of Polyethylene Glycol 6000 (PEG) Precipitations Related to Protein Concentrations.



phenomenon. Whatever the case, it cannot be due to increased phytoene synthetase levels (although overall protein levels also imply this), and may only serve a purpose in the purification if, in increasing the activity, it enables a more sensitive location of phytoene synthetase from subsequent purification steps.

At higher PEG concentrations (>2%), the activity begins to precipitate rapidly, such that by 5% PEG only 20% of the activity remains in the supernatant, and at 10% PEG it is almost completely precipitated.

The protein levels in the S<sub>105</sub> PEG supernatant also show an unusual increase at low PEG concentrations (0-2%). This may possibly be due to PEG removing certain large proteins or cell-debris components from S<sub>105</sub> thus revealing proteins which may have been complexed or 'hidden' such that they could not be detected in a protein assay. This speculative theory may also explain the latency in phytoene synthetase activity.

Beyond the initial, apparent rise, in the supernatant protein levels, the amount falls steadily with increasing PEG concentrations, while the precipitated protein levels rise at a concurrent rate. However, the rate of protein removal is much less than that of phytoene synthetase activity precipitation, indicating a major purification of the enzyme at low PEG levels (<10%) and furthermore, that it may be a large protein (Ingham, 1984).

However, a major problem with the PEG precipitation of phytoene synthetase activity, was that at those percentages where the enzyme appears to have been precipitated (4-9%), the activity could not be found in the precipitate.

The main problem was that the precipitate could not be redissolved in the buffer, even by leaving overnight at 4°C. An explanation for this phenomenon may be suggested by the observation that S<sub>105</sub> actually consists of 3 layers (2.2.14 and 6.2.7) and that the lower, turbid layer may contain membrane components, such as glycoproteins, polysaccharides and lipids, which do not fully precipitate (in the P<sub>105</sub>) and are associated with phytoene synthetase (6.2.7). Consequently, PEG precipitation is actually sedimenting these large complexes, which are associated with phytoene synthetase, and form a complex precipitate which cannot be redissolved due to the 'binding' or 'sticky' nature of these components.

Consequently the apparent precipitation of phytoene synthetase activity by PEG, may be an artifact resulting from the precipitation of large complexes and associated components. In any case, it may prove necessary to treat the S<sub>105</sub> fraction (or PEG precipitate) with detergents in the same way as membrane bound proteins are treated (1.8, 6.1 and discussed in Chapter VII) to obtain phytoene synthetase in a soluble form.

#### 6.2.8 Location of Phytoene Synthetase Activity Within the S<sub>105</sub> Fraction

The 3 layers seen in an S<sub>105</sub> preparation (2.2.14) of 5 day-old, light-grown on AM medium, SG78, were assayed by a coupled assay for phytoene synthetase activity (2.2.24) and specific activities were determined in each layer (Table 6.7).

While there is no activity in the top 'lipid' layer, and a certain amount of the middle 'clear' layer, the lower 'turbid' layer contains a majority of the phytoene synthetase activity (ca 87%): this distribution is also found for

Table 6.7

The Levels of [ $^{14}\text{C}$ ]-Phytoene and [ $^{14}\text{C}$ ]-Squalene  
Produced by the Different Layers of S<sub>105</sub>  
Prepared from SG78

Fraction	[ $^{14}\text{C}$ ]-Phytoene (dpm)	[ $^{14}\text{C}$ ]-Squalene (dpm)	Protein (mg/ml)
S <sub>105</sub> ; Top "Layer"	-	-	1.5
Middle "Clear"	1500	11000	8
Lower "Turbid"	11300	53000	15
S <sub>10</sub>	13100	72000	18

squalene synthetase (ca 83%), while kaurene was not detected in any fraction layer.

It may be that while the S<sub>105</sub> fraction contains mainly soluble, cytosolic proteins and the precipitate (P<sub>105</sub>), mainly microsomal proteins, the lower 'turbid' layer represents the loosely bound, peripheral membrane proteins and components. The activity found in the middle layer may represent contamination from the lower layer of enzymes that have not been fully sedimented. This explanation implies that it may be possible to solubilize phytoene synthetase from the turbid layer and retain activity. The upper 'clear' layer is probably lipid.

An analogous situation has been found in N.crassa (Spurgeon et al., 1979), where a turbid layer in a S<sub>100</sub> preparation was shown to be essential for the conversion of [<sup>14</sup>C]-GGPP to -phytoene while the remaining 'clear' layer contains the prenyl transferases and IPP isomerase.

This supports the idea that phytoene synthetase (and squalene synthetase) is a peripheral, membrane bound enzyme and such enzymes are located in the lower 'turbid' layer of an S<sub>105</sub> preparation from G.fujikuroi. It may prove necessary to treat this fraction in the same way as microsomal enzymes are treated to obtain soluble carotenogenic systems e.g by the use of detergents; in P.blakesleeanus (Bramley and Taylor, 1985); N.crassa (Mitzka-Schnabel, 1985); Capsicum (Camara, 1985) and daffodil (Beyer et al., 1985). However, initial attempts to solubilize phytoene synthetase in N.crassa (Mitzka-Schnabel, 1985) suggest that the enzyme activity requires associated, undefined lipids which are removed on solubilization and it may therefore prove

necessary to re-introduce such components when assaying for phytoene synthetase in purified fractions.

#### 6.2.9 Summary and Conclusions

A system capable of identifying prenyl pyrophosphates was developed and established the production of PPPP in a number of fungal strains that do not produce phytoene. This implies there are 2 distinct enzymes, PPPP synthetase and phytoene synthetase, since inactivation of the latter does not affect the former.

Of the strains investigated, SG4 produced the greatest levels of [ $^{14}\text{C}$ ]-GGPP and -PPPP. Using cell-free extracts these precursors could be incorporated into [ $^{14}\text{C}$ ]-phytoene in a coupled system with SG78 extracts.

Using the coupled assay for phytoene synthetase, the enzyme appears to be located in a turbid layer found in the  $S_{105}$  fraction. The enzyme is apparently peripheral and detachable from the membrane (along with squalene synthetase) but remains associated with other peripheral membrane components and fragments. It may be removed from the membrane by high buffer concentrations, but the level of detachment varies in different preparations (from 25%-100%). This variability is not found in *P.blakesleeanus* (Bramley and Taylor, 1985) or in *A.giganteus* (El-Jack, personal communication) and is possibly linked to the absence of a turbid layer in the  $S_{105}$  preparations, which is found in *G.fujikuroi* (2.2.14): that it is also found in *N.crassa* (Spurgeon et al., 1979) indicates that it may be a trait of this taxonomic group of fungi (the Prenomycetes), of which *P.blakesleeanus* and *A.giganteus* are not members. This layer may represent high levels of



membrane components such as glycoproteins, polysaccharides and lipids, which may interfere with solubilization of peripheral membrane proteins.

Therefore, although phytoene synthetase is peripherally located in G.fujikuroi, it remains associated to some membrane fractions on detachment. These may possibly be removed by detergents for further purification, but in doing this such components may have to be re-introduced to assay the enzymes, as they may be essential for catalytic activity.

CHAPTER VII

CONCLUSIONS

"Life is the art of drawing  
sufficient conclusions from  
insufficient premises"

S. Butler

## 7.1 Summary

The mutants of G.fujikuroi that were available for characterization (1.10.1) have been analyzed within the terpenoid, biosynthetic pathway by both in vivo and in vitro techniques.

Generally, the mutations were found to be structural producing inactivation of certain enzymes and accumulation of precursors. These included 2 mutations in phytoene dehydrogenase, SG43 and SG78; a mutant in the enzyme(s) involved in NX biosynthesis from torulene, SG68, and 3 mutants affected in phytoene synthetase, accumulating PPPP, SG4, SG75 and SG76. Of all the mutants, only one was a regulatory mutant (though other structural mutants were derived from this) in which there were elevated levels of terpenoids; SG22.

From the studies of the mutants, a number of general observations and conclusions can be made, with respect to the terpenoid pathway in G.fujikuroi:

(i) The identification of nine carotenoids from G.fujikuroi (Chapter III), indicates the sequence of the carotenoid pathway in this fungus. This implies that  $\gamma$ -carotene formation is via  $\beta$ -zeacarotene, and not lycopene. This is in contrast to other fungi, such as N.crassa (reviewed by Rau, 1976) and R.rubrum (Davies, 1970) and R.globiformis (Schmidt and Liaaen-Jensen, 1973) (see 1.3.1).

(ii) Carotenogenesis was shown to be photoinduced. Photo-regulation of carotenoid biosynthesis is a phenomenon in several organisms and a limited number of carotenogenic enzymes have been shown, unambiguously, to be photoinduced (see 1.2.3.iii). This is the first report of the phenomenon in G.fujikuroi, and only phytoene synthetase is fully photo-

induced (see 3.3.4 and 6.2.3).

(iii) There are some indications from the in vivo carotenoid levels of the mutants SG43 (3.3.4) and SG78 (3.3.5), that 2 types of regulation of phytoene synthetase exist: photo-regulation and chemical regulation. This may be through the existence of isoenzymes under different regulation, or a single enzyme affected by 2 regulatory mechanisms.

(iv) The HPLC technique that was developed (2.2.13), enabled separation and identification of 5 major GAs: GA<sub>3</sub>, GA<sub>4</sub>, GA<sub>7</sub>, GA<sub>13</sub> and GA<sub>14</sub>. These were also found to be the major GAs in this fungus by Hanson (personal communication).

(v) GA biosynthesis at early, logarithmic stages of growth, is photoinduced. This is especially marked in the regulatory mutants of G.fujikuroi (i.e SG22, SG78, SG75 and SG76, see 4.3.5, Table 4.4). However, GA biosynthesis in dark-grown cultures is induced later, i.e during stationary phase, such that in older cultures, light- and dark-grown differences in GA levels are negligible. Photoinduction of GA production has been noted in a number of higher plants (reviewed by Moore, 1979 and 1.4.3.iii) and to a limited degree in G.fujikuroi (see 1.4.3.iii), but results in these studies suggest that it is rarely noticed in the fungus since it is only apparent early in growth.

That dark-grown cultures produce GAs at the onset of stationary phase, suggests an additional induction mechanism may operate: this may be chemical since it is affected by the media constituents.

(vi) It appears that while the early steps in terpenoid biosynthesis, up to and including the prenyl transferases, are soluble, the enzymes, kaurene synthetase, squalene synthetase

and phytoene synthetase are peripheral, membrane proteins which may be detached from the microsomes to varying degrees; level of solubility also varies in mycelial preparations. The intracellular locality of these enzymes may vary between species and preparations from the same organism (see 1.8). This may reflect the peripheral nature of the enzymes such that different organisms or experimental conditions, solubilize the enzymes to varying degrees.

Enzymes beyond phytoene and squalene appear to be integral, as is found in other organisms (1.8). However, kaurene oxidase appears to be soluble in these preparations, unlike that found elsewhere (5.3.5). Furthermore, kaurene oxidase is considered to be a cytochrome P-450 linked enzyme (see 1.5.2) which means this is one of the few such enzymes to be obtained in a soluble form (see 5.3.5).

(vii) Considering the terpenoid pathway as a whole there appears to be an overall regulation and regulation of inter-related pathways.

All the terpenoid pathways examined i.e GA, carotenoid and sterol syntheses have elevated levels of production in the regulatory mutant SG22, (and mutant derivatives thereof) so the pathway must have a common, early regulatory point.

There also appears to be a light effect on the total pathway, elevating carotenoid levels and GA levels, although only early in growth. The sterol levels are identical late in growth but it is possible there is sterol photoinduction, early in growth, as in the GAs, such that there is a common photoregulatory point in the pathway.

Limited studies on media effects on the terpenoid pathway, suggest an overall regulation. Media with a high C:N ratio

induce GA biosynthesis while sterol and carotenoid levels are slightly lower (except phytoene which is higher) than the levels found in a low C:N ratio medium. Therefore the media can regulate each pathway within terpenoid biosynthesis.

The individual activities also maintain specific regulatory mechanisms. For example, there is always a light- and dark-grown carotenoid difference, at any stage of growth, and it is proposed that enzymes of the carotenogenic pathway itself are also photoinducible (in addition to early, common pathway enzymes) e.g. phytoene synthetase.

Also, as has been mentioned, the GAs are induced by a specific regulatory mechanism which is related to age of culture, and allows light- and dark-grown differences to be negated. This does not happen to the carotenoids and so must be specific to GA biosynthesis; it may also occur in sterol biosynthesis, though this has not been examined in the present studies.

The stage of growth has an effect on activities of each individual pathway, again indicating an overall, pathway regulation. It is seen that early growth produces a high activity in sterol biosynthesis, while GA and carotenoid synthetic activity is low. By mid-log. growth carotenoid biosynthetic activity is optimal while sterol synthesis is minimal. Finally, late in growth GA biosynthesis is stimulated, while the other pathways show low activities. These observations may result from pathway activities, or from rates of turnover of products, either of which imply an overall regulation.

The close links between these biosynthetic pathways is also seen when the pathways are blocked by mutation, as shown

Fig 7.1 Summary of the Regulatory Effects of Certain Factors, on Terpenoid Biosynthesis

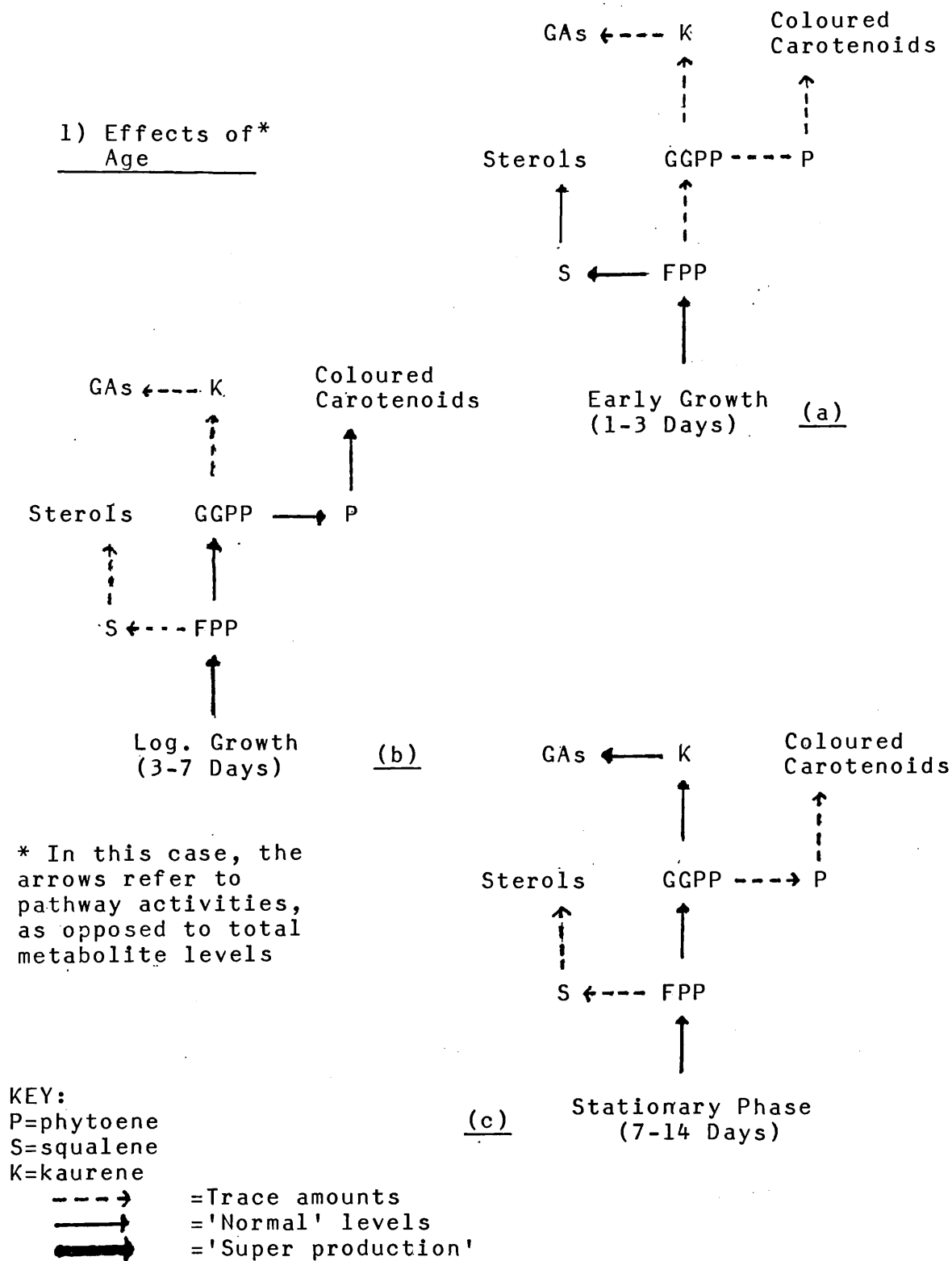


Fig 1.7 Contd.

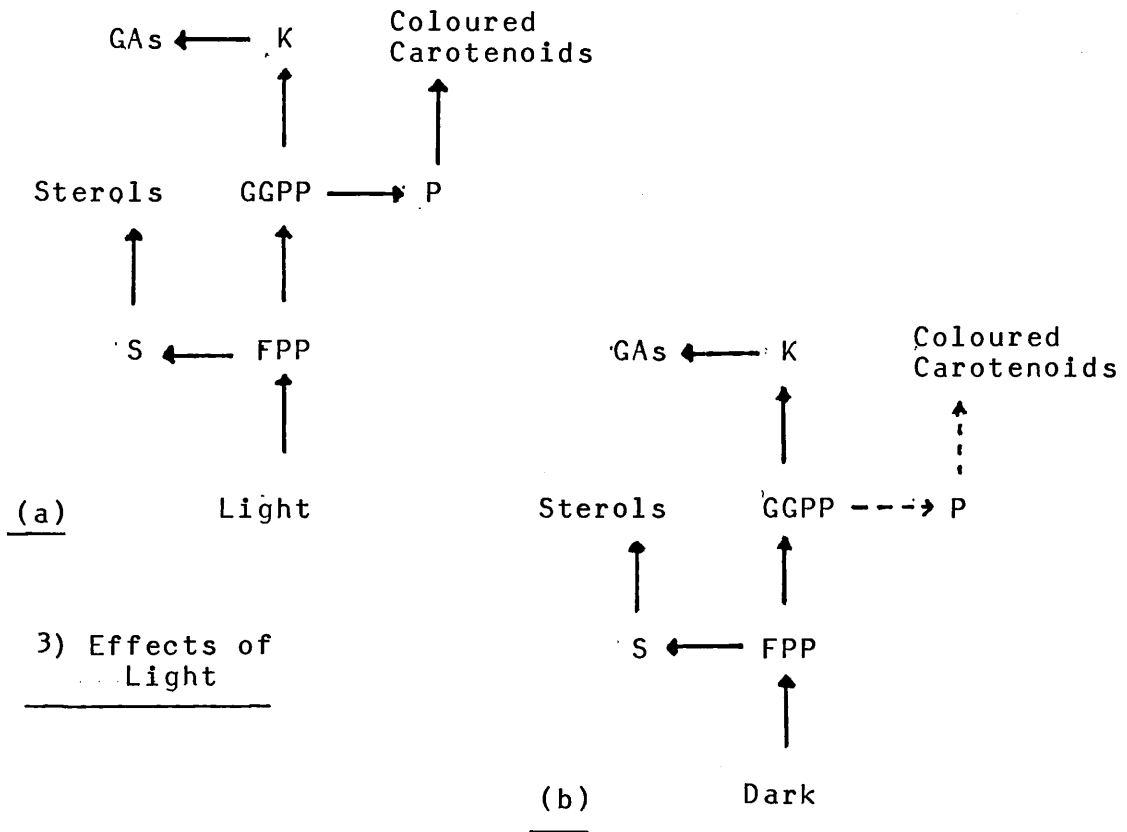
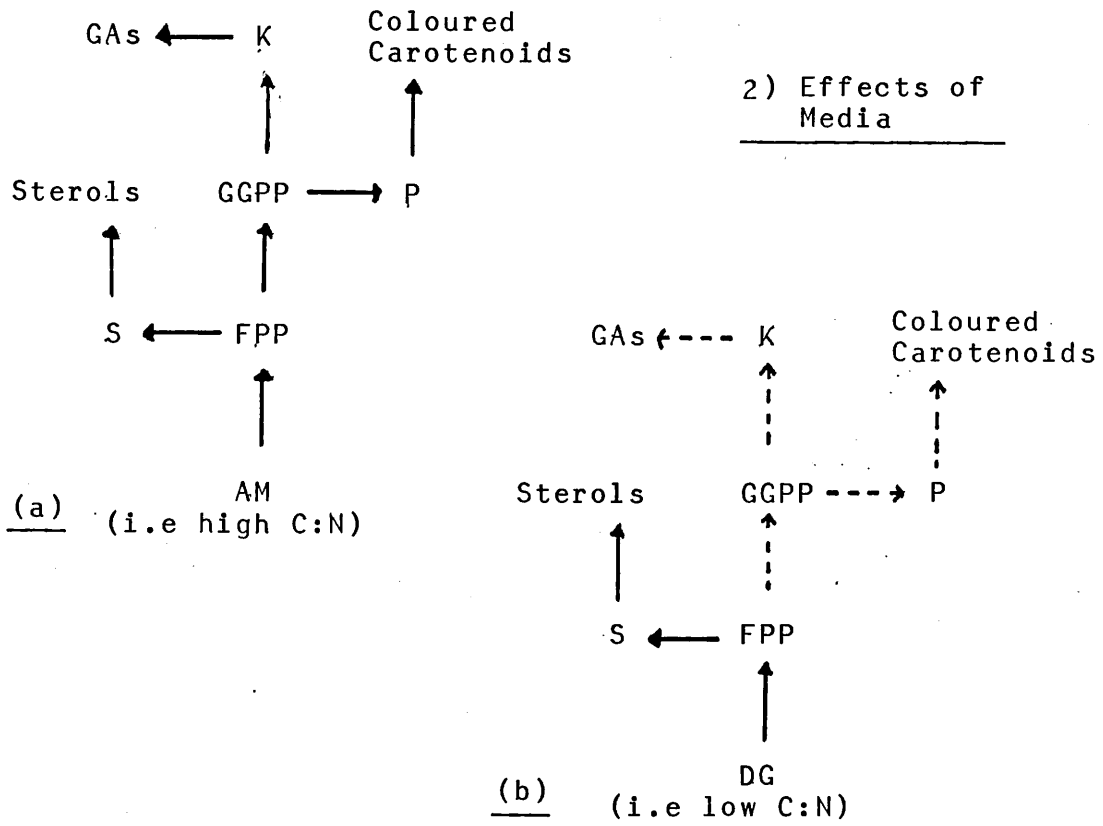
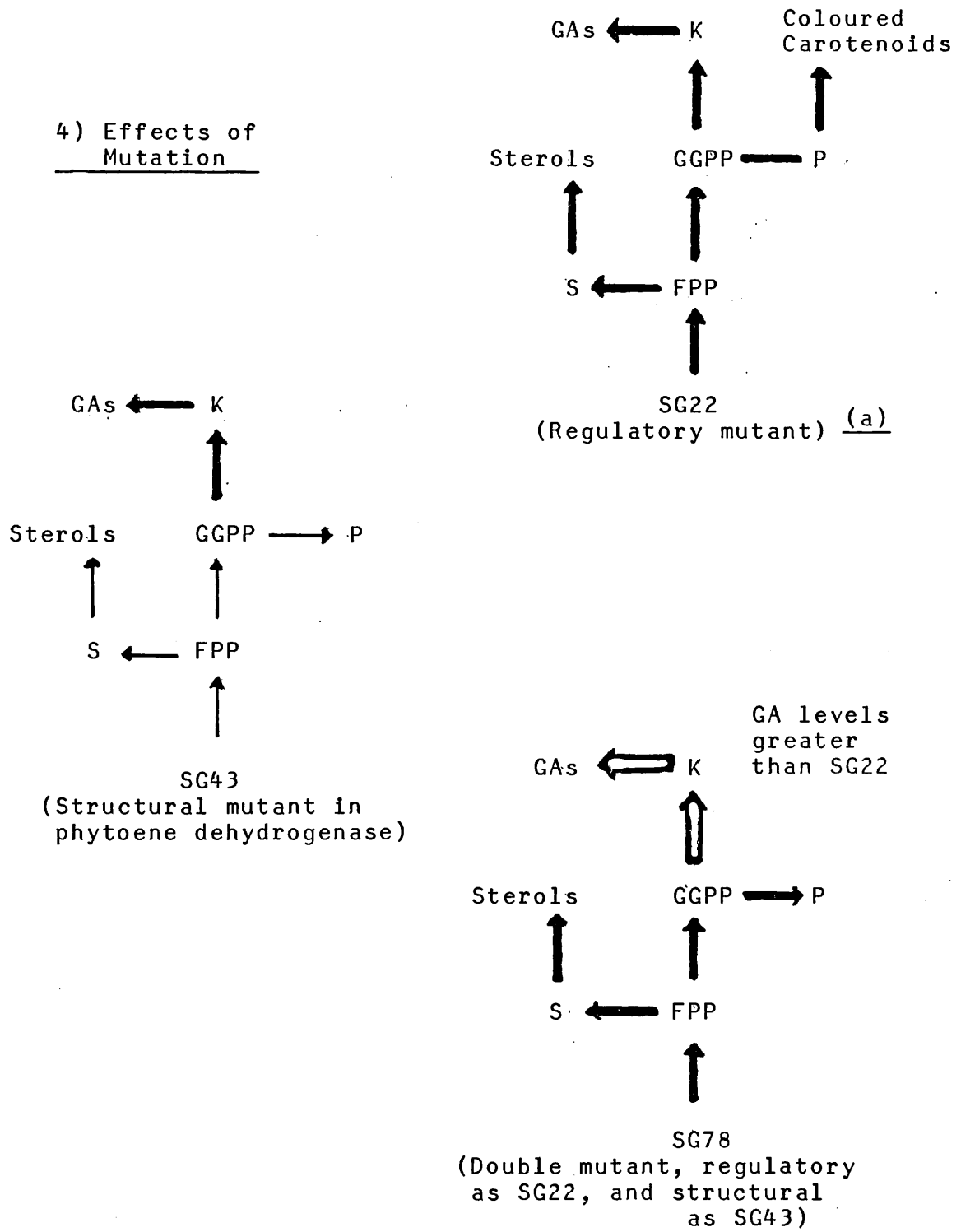




Fig 7.1 Contd.

4) Effects of Mutation



by increased levels in another unblocked pathway, by increasing precursor availability or channeling, to those other pathways.

The regulatory effects on the terpenoid pathway of all the aforementioned factors, are summarized in Fig 7.1.

## 7.2 Conclusions and Implications

In addition to the more general points, a number of other observations were made of a more specific nature; discussed in detail in each individual section. These studies have revealed several interesting areas, the implications of which indicate that they may prove potentially promising with respect to future research:

(i) The purification of phytoene synthetase. This enzyme appears to be located in the lower 'turbid' layer of an  $S_{105}$  preparation of G.fujikuroi (6.2.7) in a peripheral, membrane protein fraction. Consequently, it may prove necessary to solubilize the enzyme to enable further purification. This has been achieved for carotenogenic enzymes from; P.blakesleeanus with Tweens 40 and 60 and Zwittergents 3-08 and 3-10 (Bramley and Taylor, 1985); N.crassa with sodium cholate and CHAPS (Mitzka-Schnabel, 1985); Narcissus pseudo-narcissus with CHAPS (Beyer et al., 1985) and for lycopene cyclase from Capsicum with acetone powder and Tween 80 (Camara and Dogbo, 1986). Once in a soluble form, the protein may be applied to column chromatography for separation. However, in following these procedures one may remove components that are essential to the enzyme activity e.g. the membrane lipids; this was found in N.crassa (Mitzka-Schnabel, 1985) and Capsicum (Camara and Dogbo, 1986). These components

may have to be re-introduced to a protein fraction that has been purified, to enable the activity to be assayed.

Once the enzyme has been purified it will be possible to ascertain whether 2 isoenzymes (or more) exist, and these forms can be investigated with respect to regulation within the terpenoid pathway. Even if only one type exists, and the differences lie at the genetic level, a purified enzyme may be of use to elucidate genetic information on the protein.

The enzyme in a pure form can be used to develop antibodies, which in turn may be used to identify and isolate the respective mRNA. From this it may be possible to produce cDNA which may then be used to identify the genetic sequence, for phytoene synthetase, from a gene library for G.fujikuroi (preparation of which is in progress in the laboratory). From this it may be possible to identify associated regulatory sequences such as a photoregulatory sequence or a chemically induced sequence, if these possible phenomena operate at the gene level.

This method need not be limited to phytoene synthetase, and other possibly photoinduced enzymes may be purified, such as phytoene dehydrogenase, in order to identify possible genetic, photoregulatory sequences; purification of this membrane bound protein will also involve solubilization.

(ii) Carotenogenic gene isolation. The techniques involved in gene isolation (also necessary in 7.2(i)) may be facilitated by the use of auxotroph mutants which are also available in G.fujikuroi (Avalos et al., 1985);

Mutant	Phenotype	Possible Genotype
SG12	Leu <sup>-</sup>	Unknown, leucine auxotroph
SG26	Leu <sup>-</sup>	"

Some G.fujikuroi auxotrophs, contd.

Mutant	Phenotype	Possible Genotype
SG32	Leu <sup>-</sup>	Unknown, leucine auxotroph
SG17	Arg <sup>-</sup>	Enzyme before ornithine*, arginine auxotroph
SG34	Arg <sup>-</sup>	Ornithine transcarbamoylase* (OCTase) arginine auxotroph
SG30	Lys <sup>-</sup>	Unknown, lysine auxotroph
SG46	Ade <sup>-</sup>	Unknown, adenine auxotroph
SG47	Ade <sup>-</sup>	"

\*(Greenshields, personal communication)

These auxotrophs may be used in selecting transformants containing cDNA from phytoene synthetase (see 7.2(i)), or cDNA from a G.fujikuroi gene bank, especially SG34 since the OCTase gene has been isolated from A.nidulans (Berse et al., 1983) and has furthermore been introduced into a vector capable of transforming A.nidulans argB<sup>-</sup> (John and Peberdy, 1984).

To use this technique it will be necessary first to develop a transformation system in G.fujikuroi. This is currently in progress in the laboratory and involves producing protoplasts of the fungus, suitable transformation conditions and vectors and a protoplast regeneration method. These systems have been developed in A.nidulans and N.crassa (reviewed by Bennett and Lasure, 1985), and the method for G.fujikuroi may be a close adaption of these techniques.

An alternative method of selection for transformants would be to transform G.fujikuroi with vectors (carrying the cDNA) which carry an antibiotic resistance gene. Such genes have been isolated, such as the oligomycin resistance gene from A.nidulans which has been used to transform A.nidulans (Ward et al., 1986) and a benomyl resistance gene from a benomyl-resistant mutant of N.crassa which has been used to transform

N.crassa (Orbach et al., 1986).

If these antibiotic resistance genes do not transform into G.fujikuroi, it may be possible to isolate a gene specific for resistance in G.fujikuroi such as benomyl for which a naturally resistant mutant of G.fujikuroi has recently been isolated (Mackenzie, personal communication).

If a suitable vector carrying an antibiotic resistance gene, and an appropriate transformation system can be developed for G.fujikuroi, it may be possible to isolate certain G.fujikuroi genes, such as phytoene synthetase, without purifying the enzyme (see 7.2(i)). This may be possible by transforming an appropriate mutant (lacking the activity for the gene one wishes to isolate) with cDNA from a G.fujikuroi gene bank in a vector carrying antibiotic resistance. So for example, in the case of phytoene synthetase, one can transform SG4, SG75 or SG76 with G.fujikuroi cDNA (from a gene library) in an antibiotic resistant vector. The clones carrying the cDNA will grow in the presence of the particular antibiotic, while those, specifically carrying phytoene synthetase gene, will appear orange (as opposed to white) on transformation. This "double selection" method may also be used in the same way to isolate the phytoene dehydrogenase gene, from SG78 for example.

The drawback to this procedure is that a large number of transformants may have to be produced before isolating a clone carrying the gene of interest.

An alternative method to isolate photoregulated genes from G.fujikuroi, may be to use a selection technique in which only the transformants carrying the gene of interest may be selected immediately. Such a technique is feasible in N.crassa.

Furthermore, transformation systems, antibiotic resistance carrying vectors and extensive gene maps and gene banks are all available in N.crassa (reviewed by Bennett and Lasure, 1985). Once the gene has been isolated from N.crassa it can be used to isolate the analogous gene in G.fujikuroi (and other fungi such as Aspergillus spp. and P.blakesleeanus) by 'probing' the appropriate gene bank in which, if there is sufficient sequence homology with the corresponding gene, the gene of interest may thus be located.

The method used for N.crassa makes use of the extensive gene map for this fungi, in which it is found that a number of carotenogenic genes map closely to auxotrophic genes (in Catalogue of Strains, Fungal Genetics Stock Center, 1986). Such genes include;

Loci	Phenotype (Perkins et al., 1982)
arg-6	Arginine auxotroph, uses ornithine or citrulline, probably bifunctional for arginine-sensitive acetylglutamate kinase and N-acetylglutamyl-phosphate reductase.
al-2	Accumulates PPPP, defective in phytoene synthetase.
hom	Homoserine auxotroph, affected in aspartate B-semialdehyde dehydrogenase.
al-1	Accumulates phytoene, defective in phytoene dehydrogenase.
al-3	Accumulates FPP, considered to be blocked in GGPP synthetase.
inl	Inositol auxotroph, lacks D-myoinositol-1-phosphatase.

These particular loci map close together such that the following double mutants are available (from The Fungal Genetics Stock Center, University of Kansas);

arg-6 al-2; hom al-1; al-3 inl

Consequently, transforming these mutants from an N.crassa

gene library using an antibiotic resistance vector (see Orbach et al., 1986), one may select immediately for transformants carrying the relevant auxotroph and associated carotenogenic gene.

Once such a gene has been isolated it may be used for locating the same gene in other species.

(iii) Purification of kaurene oxidase. This enzyme is considered to be a cytochrome P-450 linked enzyme (see 1.5.2) and has been found possibly to be soluble, in these studies (5.3.5). Consequently it may afford an almost unique model system to study such oxidases, the majority of cytochrome P-450 linked enzymes are membrane bound and often integral (reviewed by Murray et al., 1985) and this limits the investigations of such activities. However, one P-450 linked enzyme has been found to remain soluble in Pseudomonas putida, camphor-5-exo-hydroylase (see Murray et al., 1985) and this has led to a wealth of information on the enzymology of P-450 linked enzymes and even to x-ray crystallographic studies (Poulos et al., 1985). Therefore, since kaurene oxidase is at least loosely bound to the membrane, it may afford another enzyme whose investigation may elucidate phenomena useful in understanding the activities of other P-450 proteins.

(iv) Isolation of G.fujikuroi mutants in GA biosynthesis. None of the mutants used in this study were within the GA pathway. Although such mutants do exist, such as G.fujikuroi Bl-41a, defective in kaurene oxidase (Bearder et al., 1974), they are not common.

Obviously, such mutants may be invaluable in investigations of the GA biosynthetic pathway and its regulation. However,

isolation of these mutants is difficult, since a good selection method is not available. From these studies, it has been shown that the terpenoid pathway appears to have overall regulation (discussed in 7.1(vii)) and there is a total interrelatedness of the individual pathways e.g GAs and carotenoids. It may therefore be possible to select GA mutants indirectly, by isolating mutants within the carotenoids which may also have affected GA levels. The mutants used in these studies were isolated in this manner and some were found to be affected in the GA pathway e.g SG22. However, these are only mutants in which GA levels are raised while no blocks in the GA pathway or mutants with different GAs, were isolated.

A quick assay for GAs is necessary, but their lack of unique functional, chemical groups makes analysis difficult; for example the HPLC method developed would take far too long for routine screening of mutants. Alternatively it may be possible to use their inherent biological activity as a means of measuring GAs i.e a bioassay. A problem comes here in that it may still prove necessary to extract media for GAs which is time consuming. One possibility is to grow new mutants on agar plates and then, having removed the strains, using the agar as bioassay, growth base. However, it is reported that N levels in normal agar plates for G.fujikuroi prevent the production of GAs in static cultures (Lawrence, personal communication). Perhaps this may be by-passed by producing a medium which is limited in N levels and so will enable GAs to be produced and found within the agar.

(v) GA biosynthesis regulation. This is another area of study in which further investigations may lead to some important



insights into this pathway.

Apart from the overall terpenoid pathway regulation, which affects the total GA levels, in SG22 for example, and photo-regulation which increases GA levels early in growth (see 7.1(vii) and (v) respectively), there appear to be other possible regulatory mechanisms in operation. For instance, what mechanism initiates GA biosynthesis at stationary phase growth. That this appears to involve C:N ratio levels, implies a metabolic, nutrient regulation. Furthermore, other investigations indicate that the types of GAs produced can be affected by C and N levels (Aitken, personal communication). Surprisingly, published studies in this area are limited, despite the commercial demand for GAs, and the obvious fermentation investigations that may have been pursued within their industrial production; perhaps the work has been carried out but not published! This area may be revealing towards GA biosynthesis regulation.

That the GAs are apparently extruded into the media, also implies a possible regulatory phenomenon. The GAs may be actively secreted from the fungus and perhaps control of this mechanism may affect overall biosynthesis. Indeed, it is not impossible that the secretion mechanism itself is regulated by the medium, nutrient levels, and it is this which activates the GA biosynthesis at onset of stationary phase growth.

Alternatively, pH may serve some regulatory function. The characteristic fall and rise of the media pH during growth, in G.fujikuroi culture media, may simply be a result of metabolism, but may also induce metabolic activities.

pH has been shown to regulate the levels of a number of enzymes, in particular phosphatases, phosphodiesterase and

certain permeases, from A.nidulans (Caddick et al., 1986); the same workers propose such regulation to be at the genetic level. The studies carried out on G.fujikuroi have shown the effects of in vitro phosphatase activities on the terpenoid pathway and incorporation levels thereof (5.3.1). Perhaps endogenous phosphatases serve a regulatory function towards terpenogenesis (i.e preventing prenyl pyrophosphate assimilation into the terpenoids, by degradation of these intermediates), which in turn may be regulated by pH.

Also the secretion mechanism may be regulated by the media pH, since this is more likely to be exposed to the media environment, and maybe the rise in pH at the end of log. growth is what actually initiates the translocase and so indirectly, GA biosynthesis.

It is further worthwhile noting at this point, the number of phenomena in living organisms which are regulated by pH and pH gradients, in particular one of the actions of plant hormones, such as auxins and the GAs. The mechanism of action of these compounds is considered to be partly via a pH change which activates certain hormonal responses (reviewed by Moore, 1979).

Obviously, it may prove very interesting to investigate the effects of pH on GA production and maybe on the overall terpenoid biosynthesis.

(vi) Development of a GA in vitro system. The system in these studies was shown to be able to incorporate  $[2-^{14}\text{C}]$ -MVA into the GA pathway as far as kaurenoic acid. Whether incorporation goes further than this could not be shown for certain, but the implications were that no GAs were produced and that this is in fact the in vitro, GA pathway product.

However, in vitro systems have been developed which show incorporation of precursors into the GAs from 4 higher plant systems and G.fujikuroi (reviewed by Hedden, 1983). To develop an in vitro system capable of activity from early in the pathway to end product formation, should be possible and may aid tremendously in studying terpenoid biosynthesis and its overall regulation: if this can be developed too, within the sterol pathway, i.e beyond squalene, as is possible in P.blakesleeanus, as far as ergosterol (Bramley, 1973), this will aid such studies to an even greater extent.

By comparison with other systems in which GAs can be produced in vitro, there seems to be no reason why the system used in this study should not produce GAs. That it can produce kaurenoic acid implies that no more cofactors are necessary, since NADPH is the only additional cofactor required for synthesis beyond kaurenoic acid, and since it is also essential for kaurenoic acid oxidase, which is active in the system, therefore there is probably endogenous NADPH present. Maybe the endogenous NADPH is only sufficient to allow kaurenoic acid production and additional cofactor should be added to obtain further activity.

Perhaps the system is incapable of further activity since the preparation method may have inactivated an enzyme, such as that for oxidation of kaurenoic acid.

One limitation in studying an in vitro GA system, would be that of assaying. While a system to separate GAs produced in vivo was developed, which could be adapted for radiolabelled metabolites, it is rather time consuming (i.e 1h per sample, for HPLC alone). Alternatively, one could develop a TLC system which, in conjunction with GA markers and the spark chamber, may accelerate the assay time. Such a TLC

system has been developed (Aitken, personal communication) which appears to be remarkably sensitive and capable of separating a number of GAs (i.e. Kieselgel G developed in di-isopropyl ether:H0Ac, 95:5, v:v; Rf values GA<sub>1/3</sub> 0.11; GA<sub>4/7</sub> 0.37; GA<sub>2/8</sub> 0.04; GA<sub>5</sub> 0.31; GA<sub>6</sub> 0.25; GA<sub>9</sub> 0.79).

(vii) Points of Regulation. An important question which these studies have gone some way to answer (and complicate!), is that of where are the regulatory points, within the terpenoid pathway?

Phytoene synthetase appears to be photoregulated and also chemically regulated. Apart from this it is unclear as to other regulatory points, although others must exist. For example, are the later carotenogenic enzymes photoregulated, such as phytoene dehydrogenase and the cyclase in A.giganteus (El-Jack, personal communication)? Do the enzyme(s) involved in NX production from torulene have a regulatory control mechanism?

As has been discussed (1.8), the 3 enzymes which effectively commit common precursors to specific terpenoid pathways, i.e. squalene synthetase, kaurene synthetase and phytoene synthetase, may be prime sites for regulation. Indeed, this seems to be the case for phytoene synthetase, but is it also that the other 2 enzymes are regulated in a similar manner? This may be clarified by examining the activities of purified enzymes; the purification of kaurene oxidase is currently in progress in the laboratory.

The studies also indicate that there is a common regulatory point, affected in SG22, which can regulate the whole terpenoid pathway. Since the regulation affects all the pathways, it is reasonable to assume that it is at a step prior

to, or involving, the prenyl transferase(s). Furthermore, that increased levels of activity in SG22 were also seen in vitro using [2-<sup>14</sup>C]-MVA, implies that the regulatory point must be after MVA i.e MVA kinase, MVAP kinase, MVAPP carboxylase, IPP isomerase or prenyl transferase(s).

It is also possible that even earlier enzymes are photoregulated. This has been found for HMG-CoA reductase in Rhodotorula minuta (Tada and Shiroishi, 1982). It is possible that the apparent lack of photoinduction of carotenogenesis in the in vitro systems (5.3.3), may be because it is these enzymes, prior to MVA kinase (such as HMG-CoA reductase), which are photoregulated; this does not explain why phytoene synthetase, shown to be photoregulated in vivo, is not photoinduced in vitro.

The regulation of GA biosynthesis has already been discussed (7.1(v)) and it is possible that some of these regulatory points serve to regulate overall terpenoid biosynthesis, such as media nutrient levels and pH.

(viii) Limitations of the system. Despite the fact that G.fujikuroi and its mutants offer a number of possibilities towards understanding the terpenoid biosynthetic pathway, the system (as in most model systems) also has certain limitations.

For example, while carotenogenesis is prevalent, the pathway does not display any properties that have already been investigated, such as in N.crassa where the carotenoids and regulation thereof, are quite similar. In particular, examining photoinduced enzymes, such as phytoene dehydrogenase which has been studied in more detail in other strains e.g. A.giganteus (El-Jack, personal communication) where it is

photoinduced; it is unknown whether the same enzyme is photoregulated in G.fujikuroi. Also the system appears to be poor in NX production, compared to say phytoene, and in view of the difficulties in purifying xanthophyll producing enzymes (reviewed by Bramley, 1985), it would seem to be a poor choice of organism to study such steps. Note that the high phytoene levels in G.fujikuroi, and the speculated different forms of regulation, make it an interesting model for this particular carotenogenic enzyme.

In vitro systems on the whole, while offering certain invaluable methods of analysis e.g the assaying of phytoene synthetase in a coupled system, also are prone to spurious results. Consequently, one is limited in the use of a cell-free system to examine individual steps in complete isolation, or total pathways as a whole, but not individual steps in a complete pathway.

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By any other name would smell as sweet"

W.Shakespeare

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