INHIBITION OF CAROTENOGENESIS IN CELL FREE SYSTEMS OF APHANOCAPSA AND PHYCOMYCES

A Thesis submitted by

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ABSTRACT

A number of herbicides have been identified as inhibitors of carotene biosynthesis. Because of the multiple effects associated with inhibition of carotenogenesis *in vivo*, it was considered opportune to investigate the effects of such compounds on carotene biosynthesis in cell free systems.

The carotenogenic cell free system from *Phycomyces blakesleeanus* was optimized with respect to concentrations of NAD^+, $NADP^+$, ATP, Mg^{2+} and Mn^{2+}. A new photosynthetically active, carotenogenic cell extract from *Aphanocapsa*, that incorporates radioactivity from [2-^{14}C] GGPP, was discovered and the cofactor requirements elucidated.

The preparation from *Aphanocapsa* was used to investigate the structure-bleaching activity relationship of

(i) a series of norflurazon and difunone derivatives with respect to phenyl ring substitution;

(ii) a series of alkyl phenoxy-benzamides with respect to alkyl chain and phenoxy ring substitution. Selected alkyl phenoxy benzamides were also tested with the *Phycomyces* system.

The *Phycomyces* extract was used to investigate the effects of a number of diphenyl compounds, e.g. diphenylamine, on *in vivo* carotenogenesis. Selected diphenyl compounds were tested against the *Aphanocapsa* system.

A hypothetical mechanism for the inhibitory action of alkyl phenoxybenzamides and diphenyl compounds is discussed and a hypothesis forwarded to explain the superaccumulation of radioactivity into phytoene under certain circumstances.

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ABBREVIATIONS

The abbreviations used in this text are those recommended by the Biochemical Journal (1983) with the following additions:

CPTA	2-(4-Chlorophenylthio) - triethylamine hydrochloride
DMAPP	Dimethylallyl pyrophosphate
DPA	Diphenylamine
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
GPP	Geranyl pyrophosphate
HMG	3-Hydroxy-3-methylglutaric acid
IPP	Isopentenyl pyrophosphate
MVA	Mevalonic acid
MVAP	5-Phosphomevalonic acid
MVAPP	5-Pyrophosphomevalonic acid
PPPP	Prephytoene pyrophosphate

CHAPTER 1

INTRODUCTION

1:1 CAROTENOID NOMENCLATURE

The semi-systematic names of carotenoids, as proposed by the Commission on Biochemical Nomenclature (1975), are listed here, together with the trivial names. Hereafter, carotenoids will be referred to only by their trivial names.

TRIVIAL NAME	SEMI-SYSTEMATIC NAME
Antheraxanthin	5,6-Epoxy - 5,6-dihydro - β,β-carotene - 3,3'-diol.
α- Carotene	β,ε - carotene.
β- Carotene	β,β - carotene.
γ- Carotene	β,ψ - carotene.
δ- Carotene	ε,ψ - carotene.
ε- Carotene	7,8,7',8' - tetrahydro - ψ,ψ - carotene
Lycopene	ψ,ψ - carotene
Lycopersene	7,8,11,12,15,7',8',11',12',15' - decahydro - ψ,ψ - carotene
Neurosporene	7,8 - dihydro - ψ,ψ - carotene
Phytoene	7,8,11,12,7',8',11',12' - octahydro - ψ,ψ - carotene
Phytofluene	7,8,11,12,7',8' - hexahydro - ψ, ψ - carotene
Violaxanthin	5,6,5',6' - diepoxy - 5,6,5',6' tetrahydro - β,β - carotene - 3,3' - diol
β - Zeacarotene	7',8' - dihydro - β,ψ - carotene
Zeaxanthin	β,β - carotene - 3,3' - diol

1:2 FUNCTION OF CAROTENOIDS

1:2:1 PHOTOPROTECTION BY CAROTENOIDS

1:2:1:1 Introduction. There have been many reports detailing the sensitivity of photosynthetic organisms to high light fluences, when synthesis of coloured carotenoids has been impaired through mutation or chemical inhibition (Goodwin, 1980 for review). In several species of pigmented micro-organisms it has been observed that cultures lacking carotenoids through mutation, growth condition or chemical inhibition are sensitive to light e.g. Micrococcus luteus (Mathews and Sistrom, 1960), Dacryopinax spathularia (Goldstrom and Lilly, 1965) and Neurospora crassa (Morris and Subden 1974). Carotenoids have been shown to protect whole bacteria (Corynebacterium poinsettiae) and isolated bacterial membranes (M. luteus) from an artificial photosensitiser in the presence of light (Kunisawa and Stanier, 1958; Prebble and Huda, 1973).

1:2:1:2 Photodestruction and Photoprotection. In the absence of coloured carotenoids, light and oxygen appear to interact in such a way as to be highly destructive (see 1:2:1:1). It was proposed that coloured carotenes protect photosynthetic organisms from chlorophyll — mediated photodynamic destruction (Griffiths et al., 1955). Furthermore, in view of the highly reactive nature of oxygen in the singlet energy state (Schenk, 1954), carotenoids were suggested to quench the singlet oxygen-generating triplet

state of chlorophyll (Fujimori and Livingston, 1957). Carotenoids were also found to quench singlet oxygen without being oxidised (Foote and Denny, 1968) possibly through the formation of carotenoids in the triplet energy state (Foote et al., 1970).

In non-photosynthetic organisms, carotenoids may protect against singlet oxygen formation from photosensitisation of a pigment other than chorophyll. The action spectrum for the photodestruction of carotenoidless cultures of Myxococcus xanthus is similar to the absorbtion spectrum of protoporphyrin X (Burchardt and Hendricks, 1969).

Triplet state chlorophyll formation is favoured by high light fluences and may be generated in the antenna complexes through intersystem crossing from excited (singlet state) molecules, or in the reaction centres through a reverse charge separation process (see Mathis and Schenk, 1982).

For carotenoids to be able to quench singlet oxygen the carotenoid triplet state energy must be below the 94.1 kJ/mol determined for singlet oxygen formation (Foote and Denny, 1968). The relationship between photoprotection and carotenoid conjugation above the nonaene level has been well documented (e.g. Mathews-Roth and Krinsky, 1970) and suggests that -

i) the carotenoid triplet state energy decreases with increased conjugation, and

ii) the triplet state energy of conjugated nonaene carotenoids (e.g. neurosporene) is close to that of singlet oxygen. It has been estimated that the triplet state energy of β-carotene is 73.4 kJ/mol (Mathis and Kleo, 1973).

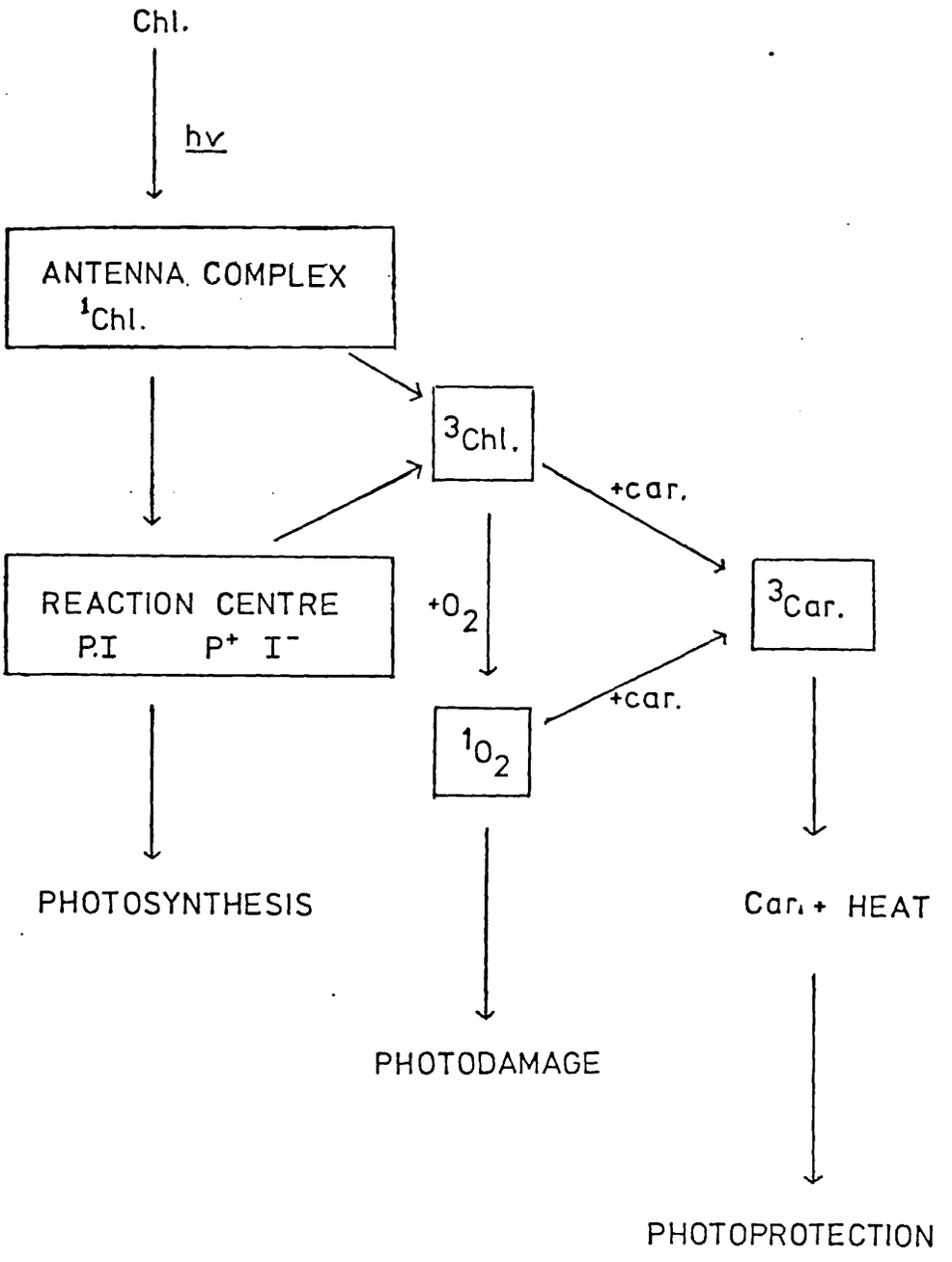
The processes of photodestruction and photoprotection are summarized in Fig. 1:2:1.

1:2:2 CAROTENOIDS IN PHOTOSYNTHESIS

1:2:2:1 Light Harvesting Function. The evidence from various photosynthetic action spectra suggests that light absorbed by carotenoids is used for photosynthesis in higher plants, algae and bacteria (Goodwin, 1980; for review). The efficiency at which carotenoid-absorbed light is used for photosynthesis varies enormously, from nearly as efficient as chlorophyll a in fucoxanthin-containing algae and diatoms (Tanada, 1951), through half that of chlorophyll a in higher plants and green algae (Emerson and Lewis, 1943), to minimal in red and blue-green algae (Emerson and Lewis, 1942) where phycobilin pigments fulfil this function.

The transfer of energy from illuminated carotenoids to chlorophyll (measured as chlorophyll fluorescence) has been shown to be absent from etiolated seedlings until 4h exposure to light (Goedheer, 1965; Margulies, 1965). Both observations implicate β-carotene rather than xanthophylls as the light harvesting carotenoid.

FIG. 1:2:1 PROCESSES OF PHOTODESTRUCTION AND PHOTOPROTECTION



It is believed that light-harvesting carotenes transmit energy to chlorophyll by a series of singlet - singlet energy state transfers. Carotenoids appear ideal for this type of transmission as it has been demonstrated that the carotenoid singlet-triplet crossover efficiency is very low (Bensasson et al., 1976), thus energy is not wasted in crossing over to the triplet state.

Detergent solubilization of photosynthetic membranes has allowed the isolation and characterization of several photosynthetic pigment-protein complexes. In addition to photoactive reaction centres, pigment-protein complexes have been isolated that have no photochemical activity but can sensitize chlorophyll fluorescence; these are believed to be light harvesting complexes.

Two light harvesting complexes have been extracted from purple photosynthetic bacteria; the B-800:850 complex containing three chlorophylls per carotenoid, and the B-870 complex containing two chlorophylls and two carotenoids (Codgell and Thornber, 1980). An analogous complex to B-870 has been isolated from a carotenoidless mutant of Rhodopseudomonas sphaeroides and spheroidene or neurosporene (not β-carotene) shown to be incorporated into a specific binding site (Davidson and Codgell, 1981) where carotenoid incorporation restores the chlorophyll sensitizing properties of the complex. The properties of light harvesting complexes extracted from higher plants, depend on the isolation method used (Boardman et al., 1978 for

review). However, a basic stoichiometry of 3 chlorophyll *a*: 2 chlorophyll *b*: 1 carotenoid has been observed (Mathis and Schenck, 1982) and it has been suggested, from low temperature studies, that each basic sub-unit contains 1 carotenoid (Mathis *et al.*, 1979).

1:2:2:2: Carotenoids Associated with Reaction Centres.

There have been many reports suggesting that certain carotenoids are closely linked with the photo-active reaction centres of both green plants and photosynthetic bacteria (e.g. Barrett and Anderson, 1980; Codgell, 1978). However, experiments with various carotenoidless or carotene deficient photosystem I preparations suggest that β-carotene is not essential for photochemical activity (Searle and Wessels, 1978), although activity is increased in the presence of β-carotene and there is great affinity in the reaction centre for β-carotene (Ogawa and Vernon, 1970). Similarly photosystem II preparations devoid of β-carotene were still found to be photoactive (Knaff *et al.*, 1977)

Spectrophotometric analyses of flash illuminated chloroplasts have identified two carotenoid – associated absorbance changes. The effect associated with increased absorbance at 515nm is believed to be due to the response of certain permanently polarized carotenoids to the field created by photo-induced electron transport across the photosynthetic membrane (Witt, 1971; see Goodwin, 1980 for review). The effect associated with increased absorbance around 990nm is thought to be due to the creation of carotenoid radical cations that are either a part of the electron

transport chain between P-680 (photosystem II) and the oxygen evolving complex, or are formed at the expense of oxidized components in this chain (Mathis and Schenk, 1982).

1:2:2:3 The Xanthophyll (Violaxanthin) Cycle. It has been shown that due to a continuously active epoxidase and a light stimulated de-epoxidase, zeaxanthin is epoxidized to antheraxanthin and violaxanthin in the dark, whilst in the light these 5,6-epoxides are converted back to zeaxanthin (Sapozhnikov, 1973). These two enzymes have been purified and the difference in pH optima suggests that the de-epoxidase is situated on the inner surface of the thylakoid membrane whilst the epoxidase is situated on the outer (Hager, 1975). The biological significance of this cycle is not clearly understood.

1:3 BIOSYNTHESIS OF CAROTENES

1:3:1 THE BIOSYNTHESIS OF SOLUBLE CAROTENOID PRECURSORS

1:3:1:1 Biosynthesis of Δ^3 -Isopentenyl Pyrophosphate.

Carotenoids are members of the class of biological compounds named terpenoids (Ruzicka, 1953) that are derived from the polymerization of 5-carbon isoprenyl units. The incorporation pattern of radioactive β-carotene biosynthesized from [^{14}C] acetate shows that it is synthesized from two blocks of four isoprenyl units joined end to end (Grob and Butler, 1956).

Early studies, using a variety of possible carotenogenic precursors, showed that acetate, HMG CoA and MVA were all converted into β-carotene (for review see Britton, 1976).

The discovery that a soluble enzyme system from yeast required ATP for the formation of squalene from MVA suggested that terpenoid biosynthesis required phosphorylated intermediates. Bloch and co-workers (1959) demonstrated, using yeast autolysates, that MVA was phosphorylated into MVAP and again into MVAPP, the latter being converted into IPP with the loss of C-1 (of MVA) as CO_2.

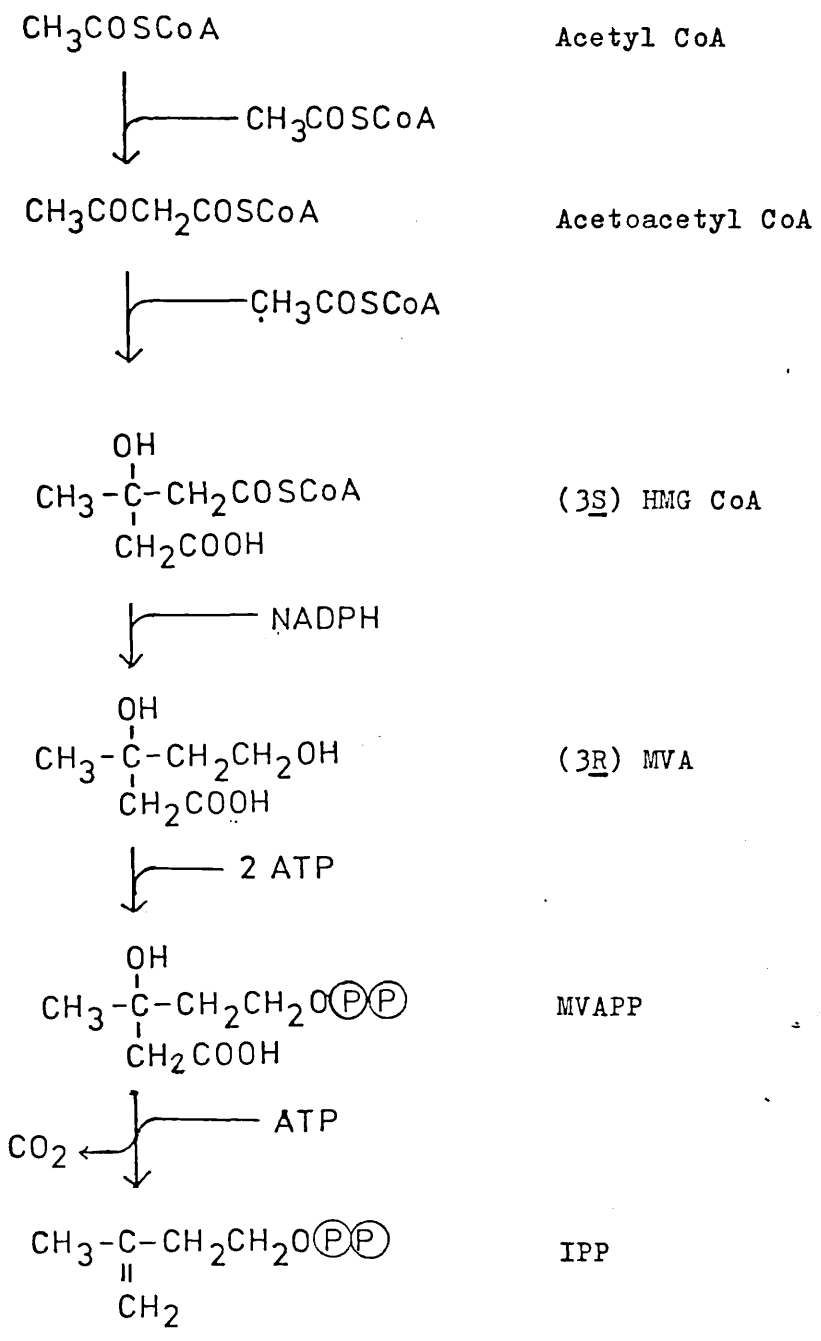
Radioactivity from the 5-carbon compound IPP in the absence of ATP was shown to be incorporated into squalene by yeast autolysates (Bloch *et al.*, 1959) and into lycopene by tomato fruit homogenates (Varma and Chichester, 1962). Thus it was shown that IPP was the 5-carbon isoprenyl 'building block' predicted from acetate labelling experiments, (Wuersch *et al.*, 1952).

The biosynthesis of IPP from acetate is outlined in fig. 1:3:1.

1:3:1:2 Formation of Polyisoprenyl Pyrophosphates

Polyisoprenyl pyrophosphates are generated by the prenyltransferases (prenylpyrophosphate synthetases, E.C. 2.5.1.1.) that catalyse the sequential condensation of a number of IPP molecules onto the allyl pyrophosphate end [$-C(CH_3)=\overset{CH}{\underset{\wedge}{}}CH_2OPP$] of a DMAPP 'anchor' molecule. The DMAPP anchor is itself an isomerization product of IPP and each condensation reaction results in the formation of a new

FIG. 1:3:1 BIOSYNTHESIS OF IPP FROM ACETATE

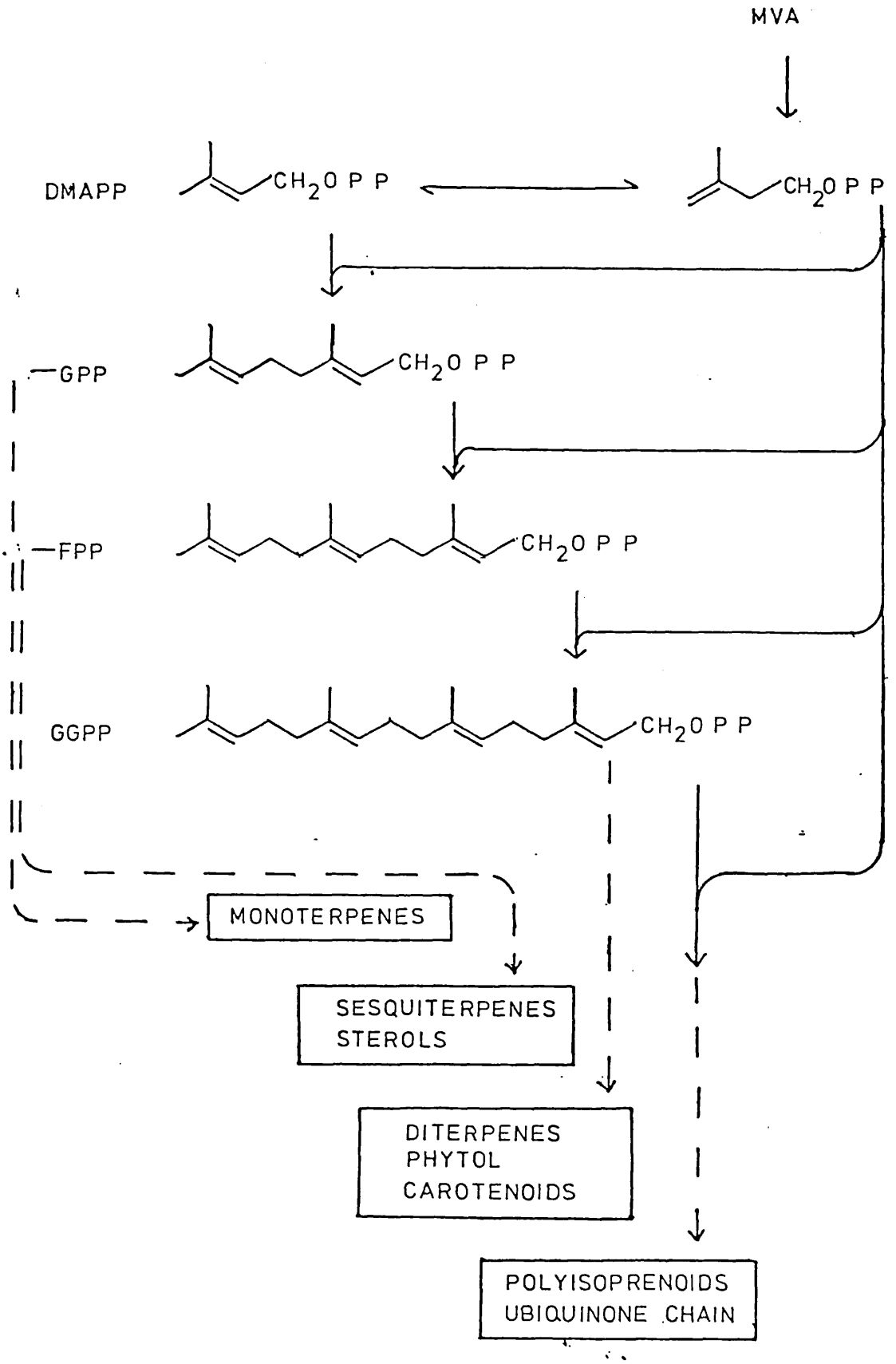


allyl pyrophosphate end group that allows the repetitive addition of IPP (Agranoff et al., 1959, 1960). These reactions are outlined in Fig. 1:3:2

Sterols are an important and widespread class of terpenoid, synthesized via the dimerization of FPP into squalene (Lynen et al., 1958). Thus prenyltransferases specific for FPP synthesis (FPP synthetases) are common. FPP-synthetases have been purified from chicken liver (Reed and Rilling, 1975) and yeast (Eberhardt and Rilling, 1975) and were both found to co-purify with dimethylallyl transferase activity, suggesting that in these organisms both GPP and FPP were synthesized by the same enzyme. However, with the FPP synthetase purified from pumpkin fruit, it was shown that GPP preferentially protected the geranyltransferase activity from heat denaturation whilst DMAP inhibited only the dimethylallyl transferase functions (Ogura et al., 1969). The same group showed that an IPP analogue (4-methyl-pent-4-enyl pyrophosphate) could act as co-substrate only with GPP (Ogura et al., 1974). The pumpkin FPP-synthetase has since been shown to consist of two sub-units (Reed and Rilling, 1975) and so it is possible that the above two activities are located on different proteins in the same complex.

The incorporation pattern produced by [^{14}C]acetate is similar in β-carotene and squalene and so it was suggested that carotenes were formed by an analogous dimerization of GGPP (Grob, 1959). The incorporation of radioactive FPP into B-carotene by a carotenogenic

FIG. 1:3:2 ISOPRENOID PYROPHOSPHATE SYNTHESIS



homogenate of P.blakesleeanus was negligible, except in the presence of MVA or IPP (Yokoyama et al., 1962). Prenyltransferases purified from chicken liver and yeast were found to produce GGPP only when incubated with high concentrations of IPP with FPP (Eberhardt and Rilling, 1975), suggesting that carotenogenic organisms may possess other prenyl transferases.

The incorporation rate of [^{14}C]IPP into polyisoprenyl pyrophosphates was compared between supernatant fractions of a Mycobacterium sp. before and after photo-induction of carotenogenesis (Johnson et al., 1974). Illumination doubled the incorporation rate of radioactivity from [^{14}C]IPP when FPP or GPP were used as co-substrates, but had no effect when either GGPP or DMAPP were used. It was concluded that photoinduction of carotenogenesis caused the synthesis of a GGPP synthetase specific for carotene biosynthesis.

The prenyltransferases from the carotenogenic bacterium Micrococcus luteus were found to have a greater affinity for FPP than for GPP or DMAPP, suggesting that GGPP is the preferred product (Kandutsch et al., 1964).

1:3:2 PHYTOENE BIOSYNTHESIS

1:3:2:1 Biosynthetic Pathway Since [^{14}C]acetate produced a similar incorporation pattern in both β-carotene and squalene, it was argued (Grob, 1959) that both were synthesized through dimerization of the appropriate polyprenyl pyrophosphate. Through analogy with terpene and

steroid synthesis, Lynen and Harding (1960) predicted that phytoene was synthesized from IPP through GPP(C_{10}),FPP(C_{15}) and GGPP(C_{20}) as intermediates (Fig. 1:3:2). It has been shown that radioactivity from [^{14}C]GGPP, synthesized by an Echinocystis preparation from [2-^{14}C]MVA (Oster and West, 1968), was incorporated into phytoene by several cell-free systems including that from P.blakesleeanus (Lee and Chichester, 1969).

By further analogy with squalene biosynthesis, GGPP should dimerise to form a cyclopropylcarbinyl intermediate, PPPP (PLPP) which is then reduced by NADPH into lycopersene, the analogous C_{40} equivalent of squalene. Altman et al., (1972) synthesized PPPP(PLPP) and showed that it could be produced from [^{3}H]GGPP by a cell-free extract from a Mycobacterium sp. grown in the light. Furthermore, the [^{3}H]PPPP thus synthesized could be converted by the same system into [^{3}H]phytoene. A similar experiment, using an extract from a phytoene accumulating mutant (albino 10) of P.blakesleeanus (Lee et al., 1972) revealed that incubation with [^{14}C]GGPP gave rise to a radioactive enzyme-bound intermediate, that could be isolated and re-incubated to yield [^{14}C]phytoene; presumably the bound intermediate was PPPP. Radioactive [^{14}C]PPPP was also seen to accumulate when purified yeast squalene synthetase or a system from an acetone powder of tomato fruit plastids were incubated with [^{14}C]GGPP in the absence of NADPH (Qureshi et al., 1972, 1973; Barnes et al., 1973): in the presence of NADPH, [^{14}C]lycopersene was

detected. It was found that both [^{14}C]PPPP and [^{14}C]lycopersene were converted into [^{14}C]phytoene or, in the presence of FAD^+ and $NADP^+$, into [^{14}C]lycopene by the tomato fruit plastid system. Radioactive lycopersene also accumulated when a particulate fraction of Neurospora crassa mycelIa was incubated with [1-^{14}C]GGPP in the presence of NADPH (Grob et al., 1961). The same laboratory also reported that lycopersene accumulated in cultures of N. crassa when grown in the presence of diphenylamine (Grob and Boschetti, 1962).

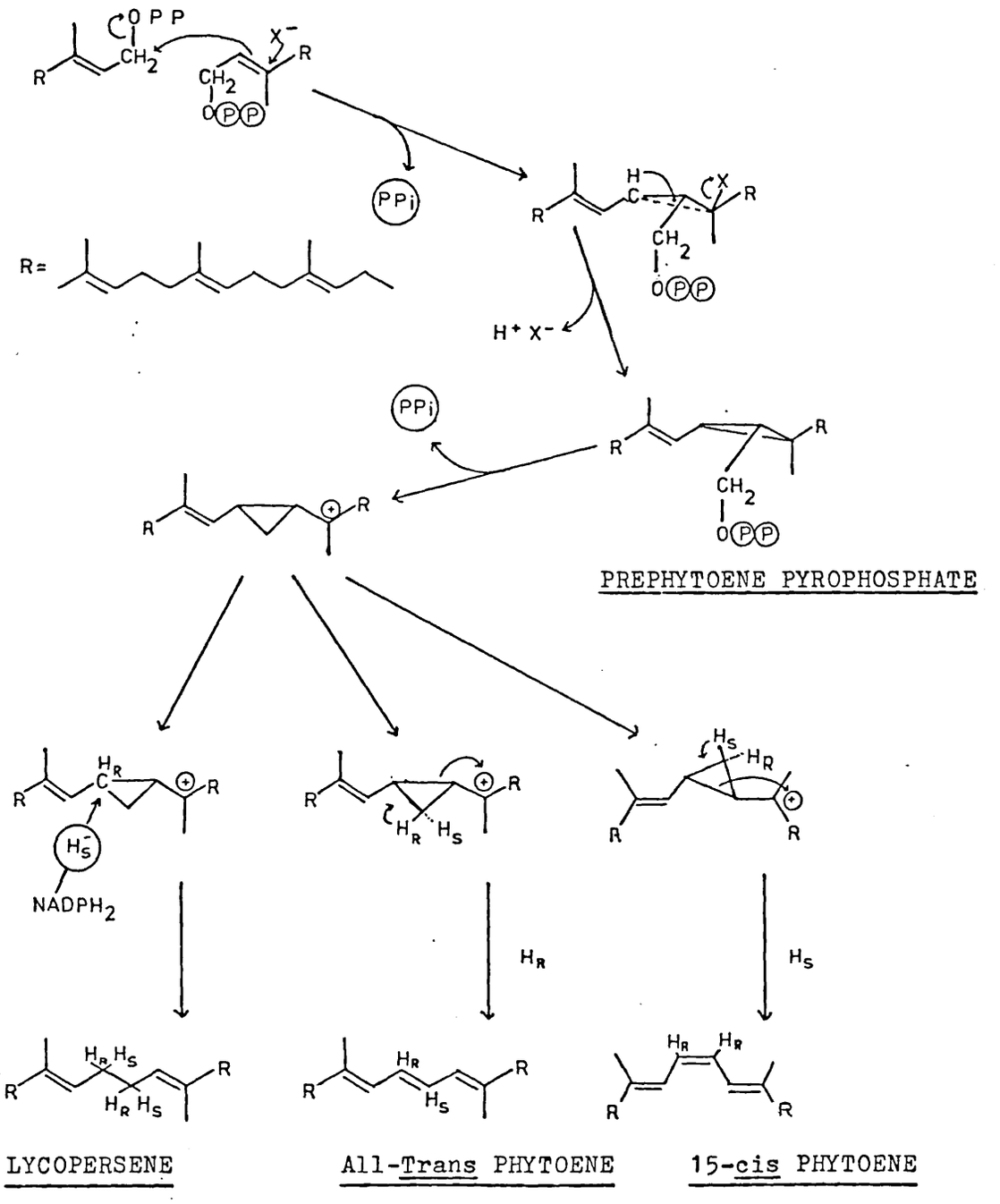
Whilst PPPP has been accepted as a bona fide intermediate in carotenogenesis, lycopersene has not. Lycopersene was first proposed as an obligatory intermediate by Grob et al., (1961) but by 1970 this suggestion was "largely discounted" (Britton, 1971) since there were reports of several enzyme systems incorporating radioactivity into phytoene and other carotenoids, but none of incorporation into lycopersene. Also, phytoene-accumulating mutants of Chlorella vulgaris (Claes, 1954), Chlorella pyrenoidosa (Kessler and Czygan, 1966), N. crassa (Haxo, 1956), Rps. sphaeroides (Griffiths and Stannier, 1956) and P.blakesleeanus (Meissner and Delbruck, 1968) had been described, but none accumulating lycopersene. Altman et al., (1971) proposed that the central conjugated triene of phytoene could be formed directly from the cyclopropylcarbinyl moiety of PPPP, implying that lycopersene need not be formed. However, the debate over whether or not lycopersene is involved in carotene biosynthesis was resumed following the work of Qureshi et

al., (1972, 1973) and Barnes et al., (1973, see above). Gregonis and Rilling (1974) using enzyme systems from a phytoene accumulating mutant of P.blakesleeanus and from a Mycobacterium sp. prepared radioactive phytoene in vitro from [4-^{14}C, 1-$^{3}H_2$]GGPP. Both the radioactive 15-cis phytoene (from the Phycomyces system) and the radioactive all-trans phytoene (mostly from the Mycobacterium sp. preparation) retained two of the four original tritium atoms around the central (15-15') carbon bond. These results, and other labelling studies, support the assertion that phytoene is formed directly from PPPP (Altman et al., 1971; (see Fig.1:3:3)).

It has been suggested that the formation of radioactive lycopersene from [^{14}C]GGPP is a result of lack of absolute substrate specificity by squalene synthetase (Davies and Taylor, 1976). The phytoene synthetase from tomato fruit plastids has now been extensively purified (Porter et al., 1980) and appears to be a multienzyme complex of 200,000 daltons, containing at least three separable peptides: no lycopersene synthesizing capability for this enzyme complex has been reported.

1:3:2:2 Stereochemical Aspects of Phytoene Synthesis. By incubating a liver enzyme system with either [2-^{14}C,(4R)-4-$^{3}H_1$]MVA or [2-^{14}C, (4S)-4-$^{3}H_1$]MVA, Popjak and Cornforth (1966) were able to show that the pro-4S hydrogen atom of MVA was stereospecifically eliminated by the IPP:DMAPP isomerase and the DMAPP and GPP transferases. When the enzyme system was incubated with [2-^{14}C,(4S)-4-$^{3}H_1$]MVA, only

FIG. 1:3:3 FORMATION AND POSSIBLE CONVERSIONS OF PREPHYTOENE PYROPHOSPHATE



radioactivity from ^{14}C was found in FPP and squalene, yet when incubated with the (4R)-4-^{3}H substrate the ^{14}C:^{3}H ratio found in FPP and squalene was that of the substrate. Stereospecific elimination of the pro-4S hydrogen of MVA suggests the formation of all-trans polyprenyl pyrophosphates. Goodwin and Williams (1966) showed with similar substrates that phytoene biosynthesis (in P. blakesleeanus and tomato slices) also results in loss of the 4S-tritium label, thus implying that carotenes too are synthesized from all-trans polyprenyl pyrophosphates. The radioactive GGPP isolated from Echinocystis macrocarpa seed endosperm (Oster and West, 1968) was identified as being all-trans and was incorporated into carotenoids by a number of systems.

Two phytoene isomers are found to accumulate in nature. In algae, fungi, photosynthetic bacteria and higher plants, the 15-cis isomer predominates, whereas all-trans phytoene is found in many non-photosynthetic bacteria. The phytoene 15,15' carbon atoms are derived from C-5 of MVA, thus by using [2-^{14}C,(5R)-5-$^{3}H_1$]MVA and [2-^{14}C,5-$^{3}H_2$]MVA it was shown that both hydrogen atoms of the central 15,15' carbon bond in 15-cis phytoene are derived from the pro-5R hydrogen of MVA in tomato slices (Williams et al.,1967A) and bean chloroplasts (Buggy et al., 1969). Gregonis and Rilling (1974) used [4-^{14}C,(1S)-1-$^{3}H_1$]GGPP to show that radioactive 15-cis phytoene, isolated after incubation with a P. blakesleeanus extract, contained no tritium, whilst radioactive all-trans phytoene isolated after incubation with a Mycobacterium sp. preparation contained one tritum

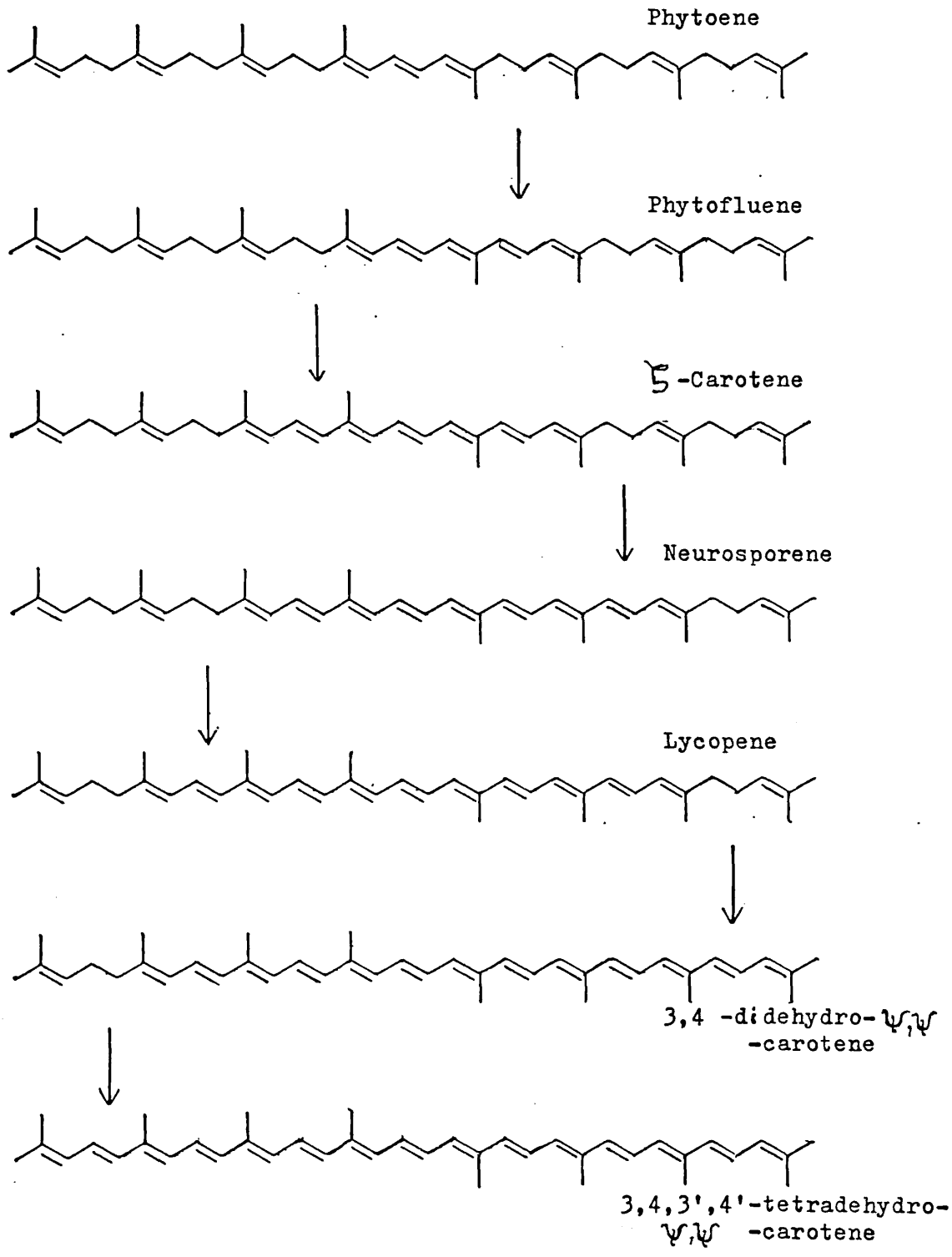
label around the central bond. These experiments lead to the conclusion that both phytoene isomers are derived directly from PPPP (as suggested by Altman et al., 1971) and that the hydrogen lost in the formation of PPPP was pro-5S (of MVA) as it is in PSPP synthesis (Popjak and Cornforth, 1966; see Fig. 1:3:3).

1:3:3 PHYTOENE DESATURATION

1:3:3:1 Biosynthetic Pathway. The structural determination of various desaturated intermediates (Davis et al., 1966) suggested that phytoene is desaturated by a series of didehydrogenations on alternate sides of an expanding polyene chromatophore (Fig. 1:3:4) to form those carotenoids proposed by Porter and Lincoln (1950) to be intermediates in the biosynthesis of lycopene. This sequence has since been enlarged to include the asymmetrical conjugated heptaene (7,8,11,12-tetrahydrolycopene) found in Rps. rubrum (Davies, 1973), 3,4-didehydro - ψ,ψ - carotene found in some fungi (Liaaen-Jensen, 1965) and 3,4,3'4'-tetradehydro - ψ, ψ- carotene found in Valencia oranges (Winterstein et al., 1960).

The desaturation steps from phytoene to lycopene have been demonstrated with various cell-free systems. Kushwaha et al. (1970) showed that enzyme systems from various genetic selections of tomato fruit incorporate radioactivity from [^{14}C] phytoene into phytofluene ζ- carotene, neurosporene and lycopene. Radioactivity from [^{14}C]phytoene was also incorporated into phytofluene and

FIG. 1:3:4 CAROTENE DESATURATION PATHWAY



lycopene by an enzyme system from spinach (Subbarayan et al., 1970). Using an enzyme system from a red tomato fruit plastid acetone powder, Qureshi et al., (1974a) demonstrated the incorporation of radioactivity from [^{14}C] phytofluene into ζ-carotene and from [^{14}C] ζ-carotene into neurosporene, lycopene and cyclic carotenes. The last step of the normal desaturation pathway, neurosporene into lycopene, has been demonstrated using [^{14}C] neurosporene with cell free preparations from Halobacterium cutirubrum (Kushwaha et al., 1976) and from P. blakesleeanus (Bramley et al., 1977). It has been shown that radioactivity is incorporated into more desaturated carotenoids when each of [^{14}C]phytoene, [^{14}C]phytofluene, [^{14}C] ζ-carotene and [^{14}C]neurosporene was incubated with the enzyme system from H.cutirubrum (Kushwaha et al., 1976).

1:3:3:2 Geometric Isomers In higher plants, fungi and most bacteria, PPPP is transformed into 15-cis phytoene. As the more desaturated carotenoids are all-trans, isomerization of the original 15-cis structure during the early stages of desaturation must take place.

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

Furthermore, the conversion of 15-*cis* [^{14}C]phytoene into all-*trans* phytoene and [^{14}C]phytofluene has been demonstrated using a cell-free preparation from *Flavobacterium* R1560 (Brown *et al.*, 1975).

The carotenogenic pathway in tomato fruits was studied by Porter and co-workers using a variety of radioactive carotenoid substrates incubated with enzyme preparations from different genetic selections of tomato fruit (see Porter and Spurgeon, 1979, for review). From these experiments it was concluded that all-*trans* carotenoids were synthesized from 15-*cis* phytoene via 15-*cis* phytofluene that was isomerised into all-*trans* phytofluene. Furthermore the poly-*cis* carotenoids proneurosporene and prolycopene, predominant in tangerine tomato fruit, were concluded to be synthesized from 15-*cis* phytoene via all-*trans* ζ-carotene isomerized to *cis*-ζ-carotene; the possibility of direct conversion of *cis*-phytofluene into *cis*-ζ-carotene was not eliminated. The carotenogenic pathway in tomato fruit, as proposed by Porter and co-workers, is summarised in Fig. 1:3:5*a*. However, since the structures of *cis* carotenes in tangerine tomato fruit were determined to be 9',15-di-*cis* phytofluene, 9,9'-di-*cis* ζ-carotene, 9,7',9'-tri-*cis* neurosporene and 7,9,7',9'-tetra-*cis* lycopene (Englert *et al.*, 1979; Clough and Pattenden, 1979), the validity of this scheme is unclear. Synthesis of prolycopene is thought to be due to a single mutation (Porter *et al.*, 1980) and it has been suggested that this causes the *trans*-$C_{9,10}$ bonds to isomerize following normal desaturation of $C_{11,12}$ whilst the *cis*-$C_{7,8}$ bonds are thought

FIG. 1:3:5A SCHEME FOR THE BIOSYNTHESIS OF CIS-CAROTENES AFTER PORTER AND CO-WORKERS (Porter and Spurgeon, 1979)

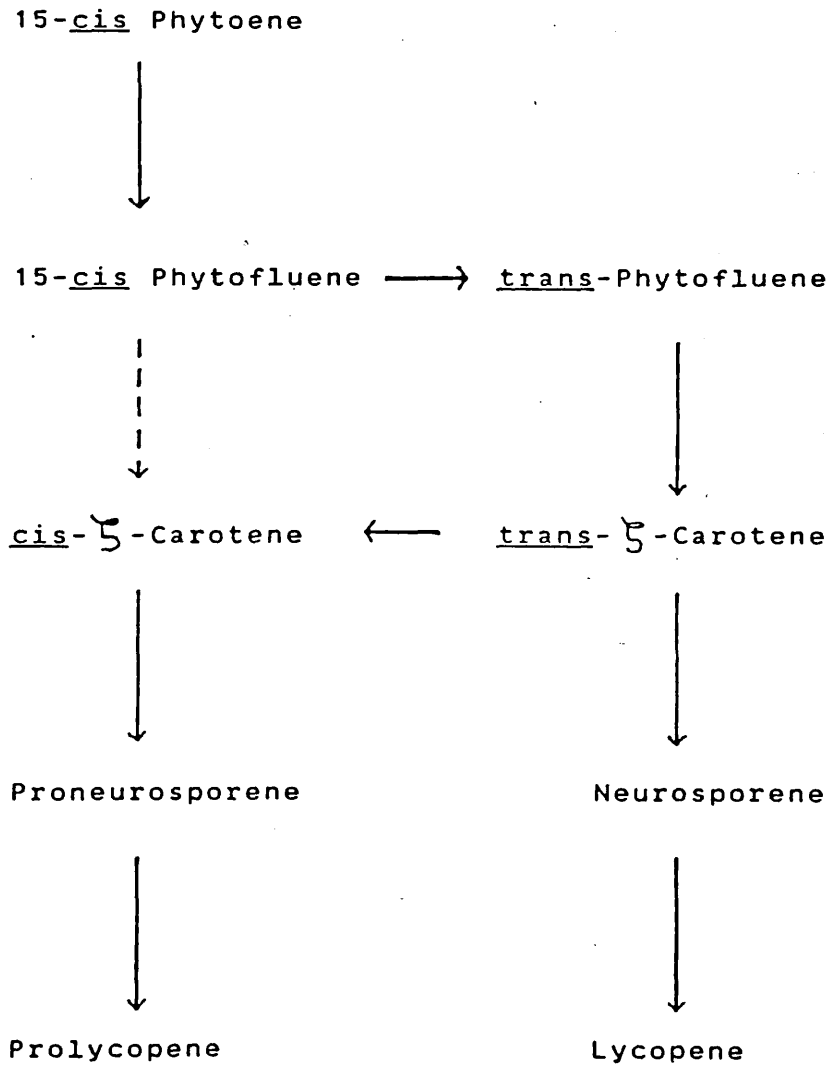
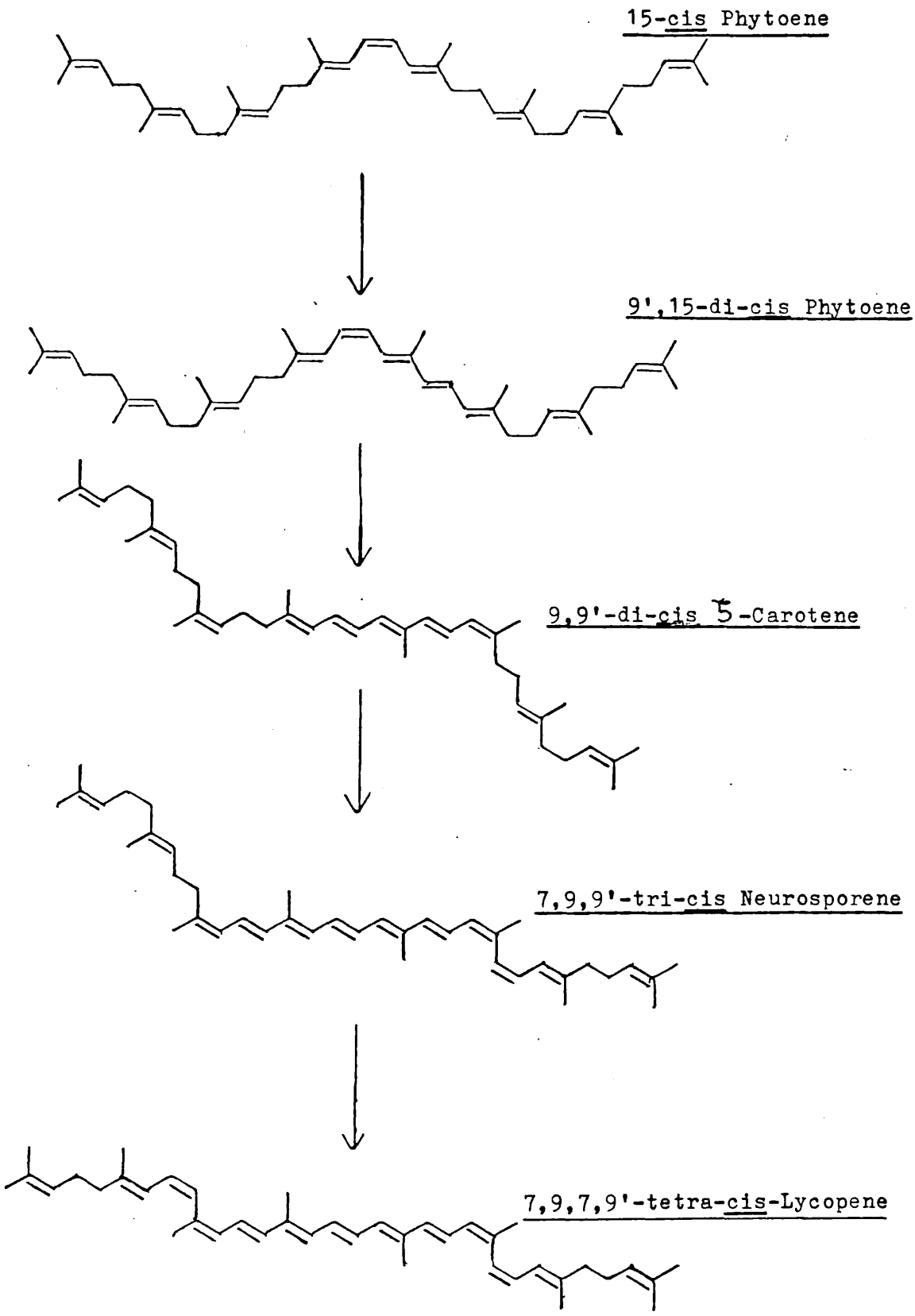


FIG. 1:3:5B SCHEME FOR BIOSYNTHESIS OF Cis-CAROTENES
AFTER ENGLERT ET AL. (1979) AND
CLOUGH AND PATTENDON (1979)



to be forced by the cis-$C_{9,10}$ bond such that a straight molecule is maintained (Fig. 1:3:5b; Goodwin, 1983). Presumably the cis bond is isomerized as suggested by Porter and co-workers. In Scenedesmus obliquus (PG1 Mutant) 15-cis=trans isomerization occurs at ζ- carotene (Powls and Britton, 1977).

1:3:3:3 Stereochemistry of Desaturation. Desaturation occurs around carbon atoms originating from positions 2 and 5 of MVA. When tomato slices were incubated with [2-^{14}C,(5R)-5-$^{3}H_1$]MVA and [2-^{14}C,5-$^{3}H_2$]MVA (Williams et al., 1967A) it was shown that desaturation caused the elimination of pro-5R hydrogen molecules. A similar experiment with [2-^{14}C,(2R)-2-$^{3}H_1$]MVA and [2-^{14}C,(2S)-2-$^{3}H_1$]MVA was non-conclusive. It was suggested that the prenyl transferases caused randomization of label between the pro 2S and pro 2R positions (Goad, 1970). This effect was overcome by synthesizing stereospecifically labelled GGPP using the Echinocystis macrocarpa system from 2S and 2R - tritiated [2-^{14}C]MVA substrates (West et al. 1969). The resultant [^{14}C, ^{3}H]GGPP when incubated with tomato slices showed that the pro-2S hydrogen atoms of mevalonic acid were eliminated during desaturation (Vose et al.).

The elimination of the pro-5R and pro-2S hydrogen molecules of mevalonic acid through desaturation has been also demonstrated with an enzyme system from a Flavobacterium species (McDermott et al., 1973).

1:3:4 BIOSYNTHESIS OF CYCLIC CAROTENOIDS

As a result of genetic studies with β-carotene and lycopene accumulating strains of tomato, it was concluded that B-carotene was (probably) a cyclisation product of lycopene (Porter and Lincoln, 1950). It was observed that lycopene was converted into β-carotene by carrot leaf chloroplasts (Decker and Uehleke, 1961) and into β-, γ-, and δ-carotenes by isolated tomato plastids or spinach chlorplasts (Wells et al., 1964). However, β-zeacarotene, a monocyclic conjugated nonaene, was found in DPA - inhibited cultures of P.blakesleeanus thus indicating that cyclisation may occur before complete desaturation of the carotenoid to a conjugated unadecaene (Williams et al., 1965). The same carotene has also been found in N.crassa (Davies, 1973).

Hill and Rogers (1969), using bean leaf chloroplasts and tomato plastids, demonstrated the incorporation of radio-activity into β-carotene from [^{14}C] lycopene. Radioactivity was also incorporated from [15,15' - $^{3}H_2$]lycopene into α-, β-, and γ- carotenes by spinach chloroplasts and soluble extracts from tomato fruit plastids (Kushwaha et al., 1969). Although these studies demonstrated that lycopene could be converted into β-carotene, they did not necessarily eliminate the possibility of cyclisation proceeding through neurosporene and β-zeacarotene either as an alternative or as the main pathway. Experiments with compounds that inhibit cyclase function (e.g. CPTA and nicotine) result in the accumulation of

lycopene in many systems (see 1:4:10), again this does not necessarily imply that lycopene is the substrate for cyclisation.

The existence of two possible pathways to γ-carotene, through either lycopene or β-zeacarotene, was demonstrated with an enzyme system from P.blakesleeanus (Bramley and Davies, 1976). Radio-activity from [2-^{14}C]MVA, usually incorporated into β-carotene by this system, was effectively diluted out by the addition of unlabelled γ-carotene and neurosporene, but not so effectively by either lycopene or β-zeacarotene. The relatively small amount of radio-activity trapped in lycopene and β-zeacarotene is consistent with the view that these carotenes are intermediates on different alternative biosynthetic routes. When this system was incubated with [^{14}C] neurosporene it was found, on re-isolation of unlabelled 'carrier' carotenes, that equal amounts of radio-activity had been trapped in lycopene and β-zeacarotene, suggesting that both pathways were of equal physiological importance (Bramley et al., 1977).

Genetic studies on heterokaryons have demonstrated that P.blakesleeanus contains a carotenogenic multi-enzyme complex consisting of four desaturases and two cyclases necessary for the conversion of phytoene into β-carotene (de la Guardia et al., 1971; cf. 1:3:6:2). As γ-carotene is just as likely to be synthesized from lycopene as from β-zeacarotene it has been suggested (Davies, 1973) that cyclase I is in a position in the complex such that it can

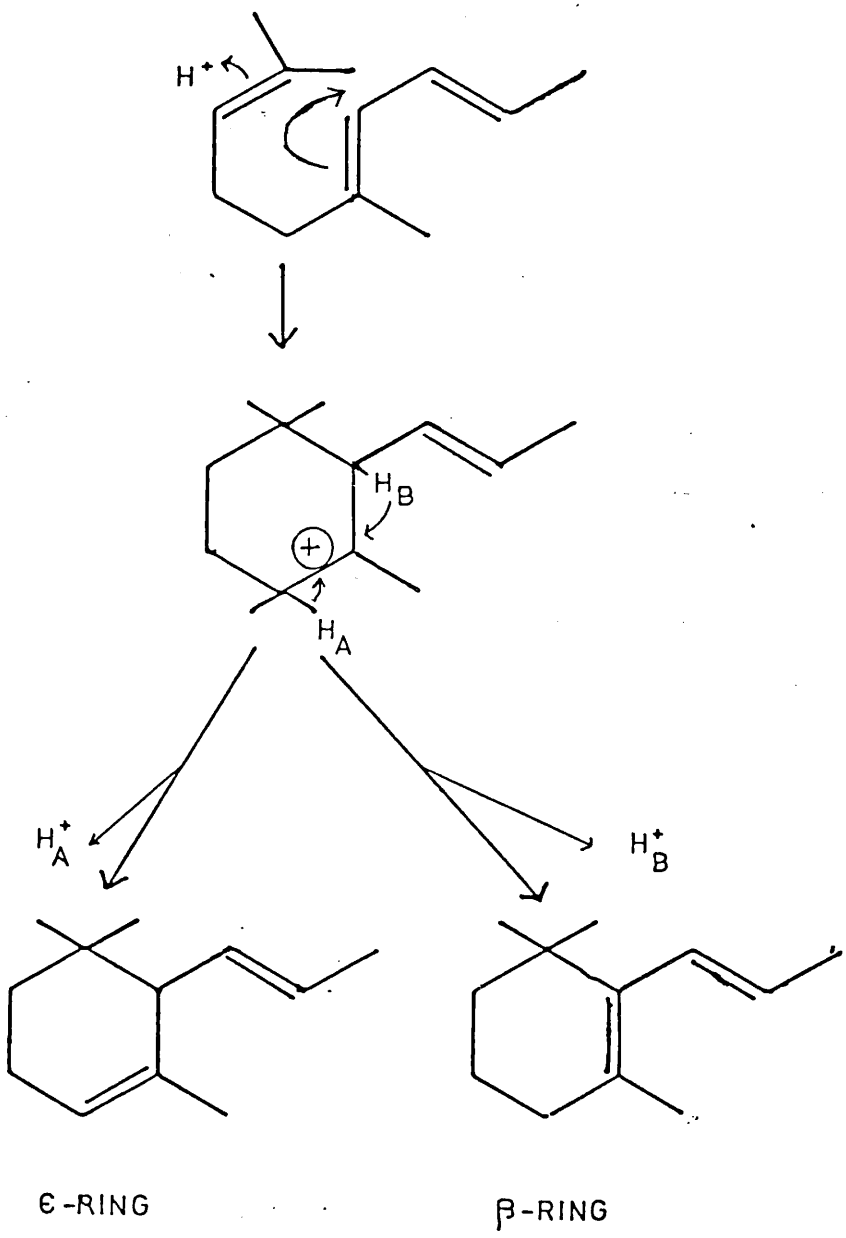
receive equally the product of desaturase III (neurosporene) or desaturase IV (lycopene). As both neurosporene and γ-carotene are substrates for the cyclases and 7,8,11,12 tetrahydro-β,ψ-carotene is detected in DPA inhibited cultures of P.blakesleeanus (Davies and Rees, 1973), it would appear that these cyclases have specificity for desaturated half carotene molecules or perhaps only unsaturated 7,8-carbon bonds.

Although it was suggested that the ϵ-ring was isomerized from the β-ring (Porter and Lincoln, 1950), inheritance studies with tomatoes suggested that both were formed independently from a common precursor (Tomes, 1967). From experiments with stereospecifically labelled [^{14}C,^{3}H]MVA, it was concluded that carotenoid cyclization involved the formation of an active cyclic intermediate (Fig.1:3:6) stabilized by loss of a proton from either C-4 to form an ϵ-ring, or from C-6 to form a β-ring (Williams et al., 1967 a, b).

The PG1 strain of Scenedesmus obliquus when grown heterotrophically in the dark accumulates ζ-carotene; Britton et al., (1977) harvested such cells, resuspended them in D_2O and extracted the bicyclic carotenoids (α-, β-carotenes, lutein and zeaxanthin) after a period of autotrophic growth. Mass spectrometric analysis demonstrated that each bicyclic carotenoid contained two atoms of deuterium at C-2 and C-2'. Deuterium incorporated into zeaxanthin (extracted from Flavobacterium R1519) was found to be located at the C-2, C-2' positions to give the 2S,2'S conformation as revealed by proton n.m.r. (Patel et al.,

FIG. 1:3:6 MECHANISM OF CAROTENE CYCLISATION

(After Williams, et al., 1967)



1977). This suggests that the proton attack, proposed by Williams et al., (1967b), is steriospecific for the re-re face in the C-1,2 double bond on the acyclic precursor.

1:3:5: COFACTOR REQUIREMENTS FOR CAROTENE BIOSYNTHESIS

Whilst many carotenogenic cell-free systems have been described, little attempt has been made to remove endogenous co-factors by either gel filtration or dialysis, to enable a true assessment of the cofactor requirements for a particular system. In addition, the majority of these systems are crude preparations and so cofactors that stimulate carotenogenesis may do so by indirect means.

1:3:5:1 Higher Plants. The carotenogenic enzymes of tomato fruit plastids have been extensively studied by Porter and co-workers and enzymes such as phytoene synthetase highly purified. The partially (350-fold) purified phytoene synthase preparation from tomato fruit plastids was found to catalyse the formation of phytoene from IPP; all intermediate activities copurified on gel filtration and the IPP:phytoene synthase complex molecular weight was estimated at 200,000 (Maudinas et al., 1977). The 200,000 dalton complex showed a strict requirement for Mn^{2+} (not replaceable by Mg^{2+}), phytoene synthesis was stimulated 6- to 7- fold by ATP (the ATP not participating in any reaction) and 1.5-3 fold by $NADP^{+}$. This complex has since been further purified to yield a 40,000 dalton IPP=PPPP synthetase (Islam et al., 1977) which in turn has been found to consist of a FPP=PPPP synthetase and an enzyme

that converts IPP into acid labile compounds below C_{20} (Porter et al., 1980): however, no additional information on the cofactor requirements of these component enzymes has been reported.

The same group have succeeded in partially purifying the enzymes for carotenoid desaturation and cyclisation. The partially purified desaturase preparation, prepared from ammonium sulphate fractionation of red tomato fruit acetone powder extracts, was found to require $NADP^+$ for the desaturation of phytoene to phytofluene and FAD^+ and Mn^{2+} for the conversion of phytofluene into lycopene (Kushwaha et al., 1970); glutathione and Mg^{2+} were found to be stimulatory but not essential. A similar preparation from lyophilized tangerine tomato fruit plastids required $NADP^+$ as well as FAD^+ and Mn^{2+} for incorporation of radioactivity from cis-[^{14}C]phytofluene into more desaturated carotenoids (Qureshi, et al., 1974a). It is possible that $NADP^+$ is associated with the cis-trans isomerization of phytofluene in tomato fruit. A partially purified cyclase preparation (from ammonium sulphate fractionation and DEAE-Cellulose chromatography of a tomato fruit plastid acetone powder extract) has been found to require FAD^+, Mn^{2+} and Mg^{2+} for maximal activity (Porter et al., 1980).

Studies on an ammonium sulphate precipitated system from spinach showed that radioactivity from [^{14}C]IPP was incorporated into carotenes only in light and was optimum in the presence of FAD^+, $NADP^+$ and a boiled extract

of spinach leaf supernatant (Subbarayan et al., 1970). Omission of the spinach leaf extract resulted in only a small amount of radioactivity being incorporated into carotenes, mostly into phytoene. Likewise omission of $NADP^+$ also reduced incorporation into carotenoids but most of the radioactivity was found to accumulate in lycopene rather than phytoene. Omission of FAD^+ did not result in reduced incorporation into carotenoids, but an accumulation of radioactivity in phytofluene at the expense of incorporation into lycopene. Previous studies with a soluble extract of spinach chloroplasts showed that radioactivity was incorporated from [15,15'-$^3H_2$]lycopene into B-carotene only in the presence of FAD^+; the reaction was stimulated by $NADP^+$ (Kushwaha et al., 1969).

1:3:5:2 Fungi. A dialysed cell free extract from P.blakesleeanus that incorporated radioactivity from [2-^{14}C]MVA into β-carotene was found to require $NADP^+$/NADPH in addition to ATP and Mg^{2+} (Yokoyama, et al., 1962); NAD^+ was found to inhibit this system. Later studies on a similar dialysed system from the R1 mutant of P.blakesleeanus showed that incorporation of radioactivity into lycopene from [^{14}C] GGPP was not dependent on NAD^+, $NADP^+$, NADPH or ATP (Lee and Chichester, 1969). However inclusion of these cofactors resulted in twice the amount of radioactivity being incorporated into lycopene without an increase in incorporation into total carotenoid. A dialysed carotenogenic preparation from the carB10 mutant (phytoene accumulating) of P.blakesleeanus showed no requirement for nicotinamide

nucleotides or FAD^+ for the *in vitro* synthesis of [^{14}C] phytoene from [2-^{14}C]MVA (Bramley, 1973).

1:3:5:3: Bacteria A cell free 270,000 x *g* supernatant prepared from *Halobacterium cutirubrum*, cells was found to incorporate radioactivity from both [2-^{14}C]MVA and [^{14}C]IPP into β-carotene and other carotenoids (Kushwaha *et al.*, 1976). The cell free preparation was found to require 4M NaCl and $NADP^+$ plus FAD^+ for the desaturation of phytoene, but no attempt was made to elucidate the cofactor requirement for each reaction step.

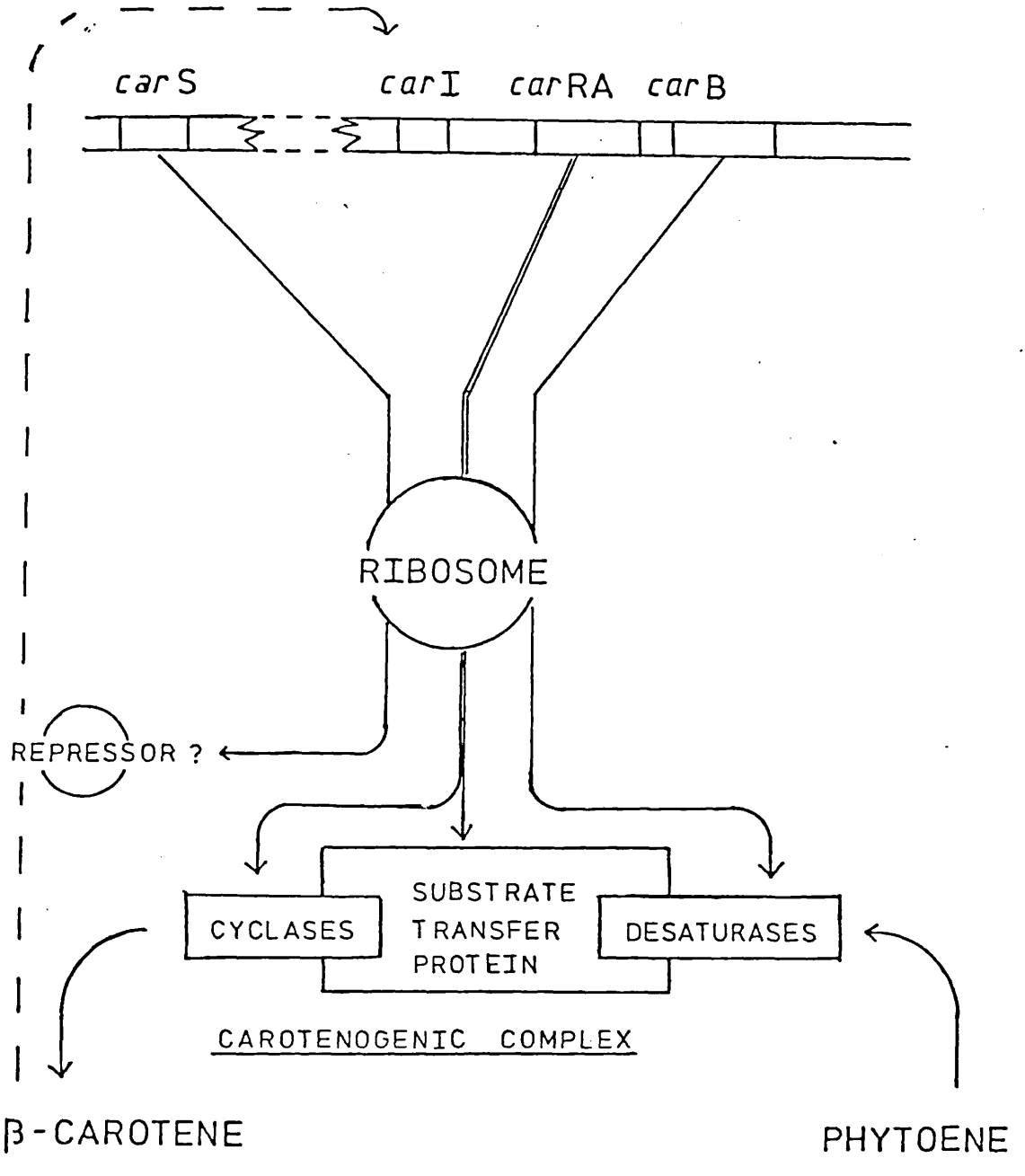
1:3:6: REGULATION OF CAROTENE BIOSYNTHESIS

1:3:6:1 Introduction. The nature and quantity of carotenoids found in living matter is dependent upon species, tissue, age and environment. Thus in any one species the carotenoid content will vary with the latter three considerations, so illustrating the complexity of carotenoid regulation. As a result of the large subject area that this topic encompasses and the relatively poor understanding of many interactions, only selected aspects of carotenoid regulation will be discussed here.

1:3:6:2 Genetics of Carotenoid Biosynthesis. Much work on the genetics of carotenoid biosynthesis has been performed using carotenogenic mutants of *P.blakesleeanus*. At least four genes have been identified that affect carotenogenesis in *P.blakesleeanus* (Fig. 1:3:7); genes *carB* and *carR* are structural genes that code for the desaturation and cyclising enzymes, respectively (Ootaki *et*

al., 1973), gene carS is thought to code for a diffusible product with operon-depressor-like qualities and is involved in β-carotene feedback inhibition (Murillo and Cerda-Olmedo, 1976), whilst gene carA is now believed to code for a protein that has substrate (i.e.. carotene intermediate) transfer activity (Murillo et al., 1981). Mutants with a defective carA gene contain very small amounts of carotene and so it was first thought that these mutants contained defective phytoene- synthesizing enzymes (Ootaki, et al., 1973). However, cultivation of these mutants in the presence of CPTA (a cyclase inhibitor) resulted in the accumulation of reasonable amounts of lycopene and γ-carotene; as this effect was cycloheximide-sensitive it was then suggested that the carA gene was involved in a negative control, sensitive to β-carotene, where carA mutants possessed an abnormally low threshold for this mechanism (Murillo, 1980). It had been previously shown that carA and carS functions were independent genes (Murillo and Cerda-Olmedo, 1976) and that white mutants failing to complement gene carA, frequently failed to complement carR as well (Ootaki et al., 1973). Later investigations showed that carA and carR represented different portions of the same gene, where carA was the larger portion situated towards the N-terminus of the peptide product (Cerda-Olmedo and Torres-Martinez, 1979). To explain the properties of C2*C9 (carA * carR mutants) heterokaryons, the product of gene carRA must be spliced after translation (Cerda-Olmedo and Torres-Martinez, 1979). Mapping experiments have now shown that gene carB (coding for the desaturase) is very near, but not a part of,

FIG. 1:3:7 GENES CONTROLLING CAROTENOGENESIS IN P.BLAKESLEEANUS



gene carRA; this gene cluster is thought to be 10 map units from the centromere of one of the chromosomes (Roncero and Cerda-Olmedo, 1982). However, results from gene-linking experiments suggest that gene carS is on the same chromosome as the above cluster, but not near it. Thus the organization of genes affecting carotenogenesis in P.blakesleeanus is reminiscent of the classical operon model, where carS is the repressor gene (Fig. 1:3:7).

Despite extensive searches for new mutants of P.blakesleeanus, no strain with a defective phytoene synthase or phytoene isomerase has been isolated to date. Strains S119 and S144 have been reported to be insensitive to retinol and CPTA; whilst the mutation in S144 mapped onto a new gene, carI, the mutation in S119 was found to be close to mutation carA5 in the A-section of gene carRA (Roncero and Cerda-Olmedo, 1982). As strain S106 (a carS mutant) is not stimulated by retinol, it was proposed that this carried an additional mutation (car102) that affects retinol stimulation (Murillo and Cerda-Olmedo, 1976). Retinol stimulation is thought to be linked to the A-section of gene carRA (Eslava et al., 1974). Light gives rise to many responses in P.blakesleeanus, of which increased carotenogenesis is one (section 1:3:6:4). Seven independent genes have been found to be involved in the light response, mad-A,B,C.D,E and G (Eslava et al., 1976), of which mutations in genes mad-A, mad-B and perhaps mad-D affect light induced carotenogenesis (Jayaram et al., 1980).

Mutants of the fungus Neurospora crassa defective in carotene biosynthesis were found to be of four types -

i) mutants that lacked all carotenoids;

ii) mutants that contained phytoene only;

iii) mutants that accumulated phytoene and partially desaturated carotenoids, and

iv) those defective in carboxylated carotenoids (e.g. neurosporaxanthin) synthesis (Haxo, 1956)

Complementation studies with white mutants reveal three genes al-1, al-2 and al-3 where al-1 and al-2 are closely linked (not all al-1 and al-2 mutants complement each other) and al-3 lies on a different chromosome (Subden and Threlkeld, 1970; Subden and Goldie, 1973). The al-1 mutant accumulated phytoene or, if leaky, a number of partially desaturated carotenoids, thus suggesting that gene al-1 coded for the desaturase (Subden and Goldie, 1973) whereas al-2 and al-3 mutants were carotenoidless (Kushwaha et al., 1978). Cell-free enzyme preparations from the al-3 mutant were able to incorporate radioactivity from [^{14}C]GGPP into phytoene, but unable to incorporate radioactivity from [^{14}C]IPP into GGPP, whereas the reverse is true of enzyme preparations from the al-2 mutant (Harding and Turner, 1981). As enzyme preparations from the al-3 mutant were able to incorporate radioactivity from [^{14}C]IPP into FPP it was postulated that gene al-3 coded for a prenyl transferase specific for the synthesis of GGPP whilst gene al-2 coded for phytoene synthase.

Yellow mutants of N.crassa synthesi e carotenoids, but not the carboxylated carotenoid, neurosporaxanthin. Complementation studies with those mutants show that the gene(s) coding for neurosporaxanthin synthesis (ylo) are unlinked to the al-1, al-2 cluster (cited from Cerda-Olmedo and Torres-Martinez, 1979).

The anther smut Ustilago violacea accumulates lycopene, yet mutants have been isolated that accumulate β-carotene or γ -carotene in addition to phytoene—accumulating and carotenoidless mutants (Garber et al., 1975). Phenotypes of different heterozygotes were explained by postulating three genes; the w-gene for desaturation, the o-gene for cyclisation-1 (γ -carotene forming) and y-gene for the second cyclisation to form β-carotene. In the wild type, o and y gene products were not active.

Seven independent genes affecting carotene biosynthesis have been identified in the photosynthetic bacterium Rhodopseudomonas capsulata (Marrs , 1982, for review). Mutants with defects in genes crtB or crtE are carotenoidless, whilst mutants with defective genes crtH and crtC were found to accumulate phytoene and neurosporene respectively. The remaining carotenoid genes, crtC, crtD, crtF and crtA are thought to code respectively for a hydratase, 3,4- dehydrogenase, O-methylase and oxygenase. All genes except for crtH, the desaturase, were found to be closely linked on a 40 megadalton chromosome segment that carries the genes specific for photosynthetic membrane and photopigment synthesis.

1:3:6:3 Chemical Control of Carotenogenesis. The increase in pigmentation observed in mating strains of P.blakesleeanus (Blakeslee, 1904) and other fungi was found to be induced by trisporic acid derived from β-carotene (Cagliotti et al., 1966; Bu'Lock et al., 1974; Cerda-Olmedo and Torres-Martinez, 1979). Trisporic acid stimulated pigment synthesis was reported to be cycloheximide sensitive (Thomas et al., 1967).

Retinol and β-ionone were found to stimulate β-carotene synthesis in the wild type strain of P.blakesleeanus, lycopene synthesis in carR mutants and phytoene synthesis in carB mutants (Eslava et al., 1974). At first it was thought that retinol (and β-ionone) was a mimic of trisporic acid; however, it has been shown that retinol stimulation is independent of both sexual stimulation and the carS gene (Murillo and Cerda-Olmedo, 1976). Intersexual heterokaryons expressing the car102 mutation (constitutive retinol stimulation) and possessing a defective carS gene accumulate up to 500 times the amount of β-carotene as does the wild type (Cerda-Olmedo and Torres-Martinez, 1979). Whilst carotenogenesis in carA mutants is stimulated by retinol, carotenogenesis in a carA,carR double mutant is not (cited from Roncero and Cerda-Olmedo, 1982). This might be due to a frameshift mutation rendering the whole of gene carRA nonsensical (Cerda-Olmedo and Torres-Martinez, 1979); a part of the A-section of this gene is thought to be involved with retinol stimulation (Eslava et al., 1974).

Whenever β-carotene synthesis is hindered, either through mutation (e.g. *carB*, *carR* mutants) or chemical inhibition (e.g. DPA, CPTA) the synthesis of carotenes prior to β-carotene is stimulated, suggesting end product (feedback) inhibition. The *carS* mutants of *P.blakesleeanus* appear to lack this type of inhibition as a *carB carS* double mutant does not accumulate any more phytoene than a single *carB* mutant (Murillo and Cerda-Olmedo, 1976). It has been observed that, *in vitro*, β-carotene specifically inhibits cyclase activity causing radioactivity from [2-^{14}C]MVA to accumulate in lycopene and γ-carotene, whilst in the same cell-free system, γ-carotene, lycopene, β-zeacarotene and neurosporene (but not β-carotene) were all found to inhibit phytoene desaturation (Bramley and Davies, 1976).

Certain onium compounds have been found to influence the carotenoid content in the flavedo of Marsh seedless grapefuit (see Yokoyama *et al.*, 1982, for review). Compounds with the general formula $(Et)_2NCH_2CH_2R$, where "R-" was either an aromatic or an aliphatic group, were found to induce the synthesis of all-*trans* carotenoids. Effectiveness as an inducer appeared to be a function of lipophilicity and where "R-" was an aromatic group, substitution of a lipophilic group into the "*para*- position improved potency. Compounds where "*R*" was bound through a hydrocarbon linkage induced more lycopene than β-carotene (but more β-carotene than control in numerous examples) whereas compounds such that "*R-*" was bound through an ester linkage induced more β-carotene. It was concluded that

compounds inducing more lycopene than β-carotene not only induced the synthesis of all-*trans* carotenoids but also inhibited cyclase activitiy. A second class of compounds, derivatives of dibenzylamine, were found to induce the biosynthesis of poly-*cis* carotenoids. Substituted *N*-benzyl-furfurylamines were found to be better inducers of poly-*cis* carotenoids than substituted dibenzylamines and substituted *N*-methyl, *N*-benzyl-furfurylamines even more effective.

1:3:6:4 Light Induced Carotene Synthesis. The phenomenon of photoinduced carotene biosynthesis has been noted in a number of non-photosynthetic micro-organisms. A number of studies indicate that the photoinduction process consists of a temperature-independent photochemical reaction and a temperature-dependent biochemical "dark" reaction in various species, including *Mycobacterium* sp. (Rilling, 1964), *N.crassa* (Rau *et al.*, 1968), *Fusarium aquaeductuum* (Rau, 1967 a, b) and *Rhodotorula minuta* (Tada and Shiroishi, 1982).

In bacteria the photochemical reaction is oxygen dependent (e.g. *Mycobacterium* sp. ; Rilling, 1964) whereas in fungi, the photochemical reaction proceeds under anaerobic conditions (e.g.*N.crassa*; Rau, 1969, and *R.minuta*; Tada and Shiroishi, 1982). Action spectra for the photochemical reaction all show that the blue-u.v. region is responsible for photoinduction and a number of photoreceptors have been proposed including porphyrins (Howes and Batra, 1970a), flavins-flavoproteins (Rau, 1967b) and β-carotene (De Fabo *et al.*, 1976). It has been observed

that once the photochemical reaction has taken place, the acquired ability to induce carotenogenesis is not lost on prolonged cold storage (Rilling, 1962). This "memory" was suggested to be a photochemical product that acted as an inducer (Rilling, 1964). Various chemicals have been reported that induce carotenogenesis in the dark in fungi and Mycobacterium marinum, but not through the same mechanism as light (Goodwin, 1980 for review).

Between the photochemical event and the appearance of increased carotenogenesis a lag phase has been noted during which protein synthesis is thought to occur (Batra, 1971). The duration of the lag period is dependent on species and temperature. It has been observed that photoinduction promotes the synthesis of a new protein in N.crassa (Subden and Turian, 1970) and that light stimulates protein biosynthesis in Verticillium agaricinum (Valadon et al, 1975). Although oxygen is necessary for carotenogenesis it is not vital for the lag phase process, as illuminated cells placed under nitrogen in darkness for a time synthesize carotenoids directly on exposure to oxygen even in the presence of cycloheximide (Lang and Rau, 1972). Light-induced carotenogenesis in N.crassa is inhibited by cycloheximide (Harding, 1974) and in Mycobacterium sp. by chloramphenicol and puromycin (Batra and Rilling, 1964; Howes and Batra, 1970a).

It has been demonstrated that in Mycobacterium sp. light induces the synthesis of prephytoene pyrophosphate synthetase at least (an activity absent from dark grown

cells) and raises the activity of GGPP synthetase several fold; light had no effect on the activity of IPP-isomerase (Gregonis and Rilling, 1974). In N.crassa, phytoene accumulates in the dark instead of β-carotene and neurosporaxanthin, thus suggesting that the post-phytoene synthetase enzymes are photo-induced (Harding et al., 1969). It has been reported that phytoene synthesis in the phytoene accumulating al-1 mutant is also stimulated by light (Lansbergen et al., 1976). Cell-free preparations from various albino mutants of N.crassa grown in light or darkness demonstrate that light induces increased phytoene synthetase and GGPP synthetase activities, but does not stimulate the synthesis of FPP (Harding and Turner, 1981).

The N.crassa wt-1 gene is believed to be a regulatory gene for photo-induced carotenogenesis since wt-1 mutants accumulate phytoene and have no light-stimulated GGPP or PPPP synthetase activity (Turner and Harding, 1981).

The biphasic light dose-response curve of P.blakesleeanus was reported to be composed of a low fluence response and a cycloheximide-sensitive high fluence response (Jayaram et al., 1979). It was found that high fluence light-induced carotenogenesis was affected by two or three of six 'mad' genes that regulate phototropic responses in this fungus (Jayaram et al., 1980).

Carotenoids, mostly xanthophylls, are present in the prothylakoids and prolamellar body of etioplasts (Wellburn and Hampp, 1979). On exposure to light the etioplast is transformed into the chloroplast with

concomitant increase in carotenogenesis . Light-stimulated carotenogenesis results in a change of the pattern of accumulated carotenoids such that the carotene =xanthophyll ratio increases. Chloroplast development inhibitors such as actidione inhibit light-induced carotene biosynthesis (Lichtenthaler, 1973).

Whilst blue light has been found to be more effective than red light to activate carotenogenesis in etiolated barley seedlings (Kleudgen and Lichtenthaler, 1974), carotenoid biosynthesis is thought to be controlled by phytochrome (Cohen and Goodwin, 1962). Mustard seedlings grown under constant far — red illumination have increased rates of carotene biosynthesis after 3h; this far-red stimulation has been found to be actidione sensitive (Virgin, 1967). Stimulation of carotenogenesis by far -red light has been reported in etiolated barley and horse-radish seedlings (Lichtenthaler and Becker, 1975). However, far red light has been reported to counteract the photoinductive effect of red light on carotene biosynthesis in etiolated maize seedlings (Cohen and Goodwin, 1962).

1:3:6:5 Sites of Carotenoid Biosynthesis. Carotenoids are found in the chloroplast grana of green tissues of higher plants. Despite their plastidal location, the carotenogenic enzymes are thought to be under nuclear control (Kirk and Tilney-Bassett, 1967). Although it is agreed that the chloroplast is the sole carotenogenic site, the identity of the fundamental metabolite used by the chloroplast for

carotene biosynthesis is a subject of much contoversy. Candidates for the carotenogenic precursor range from IPP (Kreuz and Kleinig, 1981) to CO_2 (Grumbach and Forn, 1980).

One of the key intermediates in carotenoid biosynthesis is thought to be acetyl-CoA, an important metabolite biosynthesized from pyruvate and formed as a result of glycolysis or β-oxidation of fatty acids. However, both glycolytic and fatty acid oxidative activities appear to be absent in chloroplasts (Stitt and ap Rees, 1979), implying that either acetyl CoA is synthesized through an unusual route, is transported into the chloroplast from outside (perhaps as another metabolite easily converted into acetyl CoA), or that chloroplasts do not biosynthesize carotenoids from acetyl CoA.

Spinach chloroplasts have been reported to incorporate $^{14}CO_2$ into fatty acids (Murphy and Leech, 1978) despite the assertions that chloroplasts cannot synthesize acetyl CoA from photosynthetically fixed CO_2 (Sherrat and Givan, 1973). Isolated spinach chloroplasts were also reported to be able to incorporate radioactivity from $^{14}CO_2$ into β-carotene (Bickel and Schultz, 1976), but the preparation had cytoplasmic contaminants. Radioactivity from $^{14}CO_2$, [^{14}C]pyruvate, [^{14}C]acetate and [^{14}C]MVA has been incorporated into β-carotene, mono- and di-galactosyldiglycerides, by an intact chloroplast preparation (Grumbach and Forn, 1980; Grumbach, 1980). It was concluded that spinach chloroplasts were autonomous for acetyl CoA, fatty acid and terpenoid biosynthesis where acetyl CoA was

synthesized from fixed CO_2 via phosphoglycerate and pyruvate (Grumbach, 1980).

In plants it now appears that acetyl CoA is synthesized exclusively in plastids (Ohlroge et al., 1979; Kuhn et al., 1981) where pyruvate (or acetate) is the likely substrate for the reaction. Pyruvate may be biosynthesized in the chloroplast directly from fixed CO_2 as postulated above; alternatively it may be synthesized in, or be derived from, a metabolite originating in the cytoplasm.

Although HMG-CoA reductase has been demonstrated in pea plastids (Brooker and Russell, 1975), there has been no clear demonstration of the source of HMG-CoA, thus leucine rather than acetyl CoA may be a substrate for carotenogenesis. The product of HMG-CoA reductase activity is MVA, and whilst several systems have been found to phosphorylate MVA [e.g. bean chloroplasts (Charlton et al., 1967); wheat etioplasts (Cooke, 1977)], this has been considered not to be unequivocal evidence for mevalonate phosphorylation within the chloroplast (Kreuz and Kleinig, 1981). Using a purified spinach chloroplast preparation, it was reported that whilst radioactity from [^{14}C]IPP was efficiently incorporated into terpenoids, radioactivity from [^{14}C]MVA, [^{14}C]MVAP, or [^{14}C]MVAPP (with appropriate cofactors) was not, except in the presence of cytoplasm (Kreuz and Kleinig, 1981). These authors demonstrated that IPP freely permeates the chloroplast and postulated that IPP synthesized in the cytoplasm was the fundamental metabolite used by the chloroplast for terpenoid biosynthesis. Similar

conclusions have been drawn for a system from purified daffodil petal chromoplasts that incorporates radioactivity from [^{14}C]IPP into β-carotene and other terpenoids, but not from [^{14}C]mevalonate derivatives (Beyer *et al.*, 1980; Kreuz and Kleinig, 1981). However, a similar system from chromoplasts of *Capsicum annuum* fruit has been reported to incorporate radioactivity from [2-^{14}C]MVA into carotenes and, to a lesser extent, xanthophylls (Camara and Brangeon, 1981). It has also been claimed that this system will reduce HMG-CoA to MVA (Camara and Brangeon, 1981).

The debate over the identity of the fundamental carotenogenic precursor in chloroplasts may be complicated by there being two pools of carotenogenic enzymes within the chloroplast. It was found that when [2-^{14}C]acetate and [2-$^{3}H_2$]mevalonate were simultaneously applied to six-day old radish seedlings, tritium label was incorporated into all carotenoids, whilst [^{14}C-label was incorporated into lycopene and β-carotene, but not into α-carotene or xanthophylls (Grumbach and Bach, 1979). The authors proposed that the chloroplast contained one pool of carotenogenic enzymes specific for β-carotene synthesis and another specific for α-carotene and xanthophyll syntheses.

Whilst radioactivity from [^{14}C]IPP has been reported to be incorporated into various terpenoids by isolated spinach chloroplasts (Kreuz and Kleinig, 1981), incorporation into carotenoids was not detected. Daffodil chromoplast stroma (possessing IPP:phytoene synthetase activity) co-incubated with a spinach chloroplast thylakoid

preparation, was found to incorporate radioactivity from [^{14}C]IPP into chlorophyll *a*$_{GG}$ rather than phytoene. This was interpreted as strong channelling of isoprenoid pyrophosphates by spinach chloroplasts into chlorophyll synthesis (Lutke-Brinkhaus *et al.*, 1982). However, daffodil chromoplast stroma co-incubated with a spinach chloroplast envelope preparation incorporated radioactivity from [^{14}C]IPP into phytoene, phytofluene, lycopene and tetradehydrolycopene. It was also found that the spinach chloroplast envelope incorporated radioactivity from [1-^{14}C]GGPP into phytoene, lycopene and tetradehydrolycopene and was thus concluded that the chloroplast envelope membrane contained not only the bound carotenoid desaturation enzymes, but a membrane bound phytoene synthetase (Lutke-Brinkhaus *et al.*, 1982). The failure to detect lycopene cyclase activity could not be explained.

Daffodil chromoplast homogenates efficiently incorporate radioactivity from [^{14}C]IPP into carotenoids and other terpenoids (Kreuz and Kleinig, 1981). Further investigations, similar to those above, have demonstrated that whilst the phytoene desaturation enzymes are tightly membrane bound, the phytoene synthetase is probably only loosely bound to the membrane (Kreuz *et al.*, 1982). Although phytoene synthetase activity is found in the soluble fraction, its activity has been shown to increase on addition of protein-free liposomes (Kreuz *et al.*, 1982).

The carotenogenic enzymes appear to be similarly organised in C.annuum fruit chromoplasts. Isolated chromoplasts from C.annuum incorporate radioactivity from [^{14}C]IPP into phytoene, β-carotene and other carotenoids as do recombined chromoplast stroma and membrane preparations and, to a lesser extent, chromoplast membrane preparations alone (Camara et al., 1982). However, preparations of C.annuum chromoplast stroma incorporate radioactivity from [^{14}C]IPP no further than phytoene; thus chromoplasts of C.annuum appear also to have loosely bound peripheral phytoene synthetase, but tightly membrane bound desaturating and cyclising enzymes (Camara et al., 1982).

Cell free preparations from the fungus, N.crassa have shown that incorporation of radioactivity into phytoene from either [2-^{14}C]MVA or [1-^{14}C]IPP needs both a soluble and a particulate fraction (Mitzka-Schnabel and Rau, 1981; Spurgeon et al., 1979), thereby suggesting that phytoene synthetase is membrane bound. The phytoene synthetase activity in cell free extracts of P.blakesleeanus is only partially precipitated at a centrifugal force of 105,000xg; whereas desaturase and cyclase activities are fully precipitated, this suggests that phytoene synthetase may be a loosely bound peripheral protein (Bramley, personal communication).

It has been observed that throughout growth the concentration of β-carotene in cell vacuoles of P.blakesleeanus (C115) remained constant, whereas the concentration in the lipid globules increased (Riley and

Bramley, 1982). The same authors demonstrated that the rate of incorporation of radioactivity from [2-^{14}C]MVA into terpenoids in both vacuoles and lipid globules was linear throughout the 5h test period. The lipid globules were suggested to be either the site of β-carotene biosynthesis or the primary accumulation site after synthesis prior to conversion into retinol or trisporic acid in other compartments (Riley and Bramley, 1982). Lipid globules have also been considered as the carotenogenic site in Mucor hiemalis (Herber, 1974) and Peridinium foliaceum (Withers and Haxo, 1978).

1:4 INHIBITORS OF CAROTENOGENESIS

1:4:1 SUBSTITUTED 2-PHENYLPYRIDAZINONES

It was shown that SAN 6706 (100μM) inhibits carotene biosynthesis in wheat seedlings causing the photo-destruction of 70S ribosomes and chlorophyll in bright light (Bartels and Hyde, 1970). Further experiments demonstrated that SAN 6706 inhibits carotene desaturation such that phytoene and phytofluene accumulate in place of coloured carotenoids; the biosynthesis of other terpenoids was not affected (Bartels and McCullough, 1972).

This herbicide was then shown to inhibit carotenoid desaturation in algae (Chlorella fusca; Kummel and Grimme, 1975) and in bacteria (Mycobacterium sp.; Kleinig, 1974).

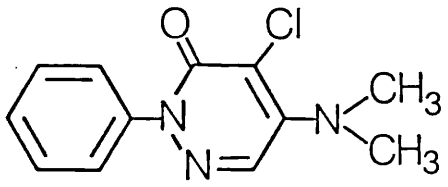
TABLE 1:4 TRIVIAL AND SYSTEMATIC NAMES OF INHIBITORS AND HERBICIDES

Acridine Orange	3,6 - Bis (dimethylamino)-acridine.
Amitrole	3-amino-1,2,4 - triazole.
BAS 44 521	4-chloro-5-methoxy-2-(3-trifluoromethyl-phenyl)- pyridazin-3(2H)-one.
CPTA	2-(4-chlorophenylthio)-triethylammonium chloride.
Dichlormate	3,4-dichlorobenzyl-N-methyl-carbamate.
Difunone	5-dimethylamino-methylene-2-oxo-4-phenyl-2,5-dihydrofurane-carbonitrile-(3).
EMD-IT 5914	See Difunone.
Fluridone	1-methyl-3-phenyl-5-(3-trifluoromethyl-phenyl)-4 (1H)-pyridine.
Haloxydine	3,5,-dichloro-2,6-difluoro-4-hydroxypyridine.
J-334	2-propyl-5-(2-chloro)-benzyl-6-methyl-pyrimidine.
J-739	2-methyl-5-benzyl-6-methyl-pyrimidine.
J-771	2-fluoro-3,5,6-tribromo-4-hydroxypyridine.
J-852	2-isopropylamino-5-isobutoxy-6-methylpyrimidine.
Metflurazon	4-chloro-5-dimethylamino-2-(3-trifluoromethyl-phenyl)-pyridazin-3(2H)-one.
Methylene Blue	3,7-Bis(dimethylamino)-phenazathiazine.
Neutral Red	3 -amino-7-dimethylamino-2-methylphenazine.
NK-049	3,3'-dimethyl-4-methoxybenzophenone.
Norflurazon	4-chloro-5-methylamino-2-(3-trifluoromethyl-phenyl)-pyridazin-3(2H)-one.
Pyriclor	2,3,5,-trichloro-4-hydroxypyridine.
SAN 6706	See Metflurazon.
SAN 9789	See Norflurazon.
Toluidene Blue	3-amino-7-dimethylamino-2-methylphenazathiazine.

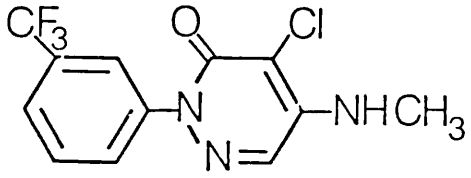
The green alga Ankistrodesmus braunii has been used to test the effect of other substituted 2-phenyl pyridazinones on carotene biosynthesis (Urbach et al., 1976). When grown for 5-7 days in the presence of a phenylpyridazinone (1μM), that contained the 2-(3-trifluoromethyl)-phenyl substitution together with either a 5-methoxy (BASF 44521) or a 5-methylated amine (SAN 6706, SAN 9789) substitution (Fig. 1:4:1), phytoene and phytofluene accumulated at the expense of the coloured carotenoids. Substituted phenyl pyridazinones that lacked either the 3-CF_3 or the above substitutions at position -5 had no effect on carotenogenesis. The herbicide SAN 9789 had also been reported to have a similar effect on wheat seedlings as SAN 6706 [E.Koren 1971, unpublished data cited in Ben-Aziz and Koren, 1974]. Further studies by Bartels and Watson (1978) on wheat seedlings showed that like SAN 6706, norflurazon (SAN 9789) caused the accumulation of phytoene and phytofluene at the expense of coloured carotenoid formation and caused the destruction of chlorophyll and ribosomes (70S) in light.

In the above experiments, wheat (and the green alga) was in the presence of herbicide from the beginning of cultivation. However, when SAN 6706 (100 μM) was added to five-and-a-half day old wheat seedlings (grown in the dark) 12h before being "greened" in light (2400 lx) , this herbicide had a differential effect on β-carotene and xanthophyll synthesis (Ridley and Ridley, 1979). These results showed that whilst β-carotene synthesis was inhibited almost instantaneously, the rate of xanthophyll synthesis

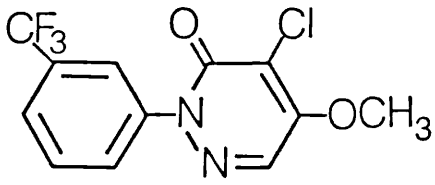
FIG. 1:4:1 THE PHENYLPYRIDAZINONE HERBICIDES



SAN 6706
(Metflurazon)



SAN 9789
(Norflurazon)



BAS 44 521

Please see Table 1:4 for systematic names.

was not affected until 8h after greening. The phytoene content of treated seedlings was high but constant for these 8h, after which the amount increased, whilst phytofluene appeared to accumulate at a steady rate throughout the greening period in SAN 6706 treated seedlings. No accumulation of phytoene or phytofluene was reported to be found in untreated seedlings. The evolution of photosynthetic oxygen was also monitored throughout greening in both control and SAN 6706 treated samples; no difference was found save for a 5h lag phase in treated seedlings.

Ridley and Ridley argued that since SAN 6706 has an I_{50} of 4μM against photosynthetic oxygen evolution (Hilton *et al.*, 1969) and 100 μM did not impair this evolution, SAN 6706 could not have entered the chloroplast. The inhibition of carotene and xanthophyll formation was effected from the cytoplasm as new enzymes were synthesized. Results from experiments using radioactive terpenoid precursors suggest the presence of two sets of carotenogenic enzymes, one specific for the formation of β-carotene, the other for the formation of xanthophylls, [Grumbach and Bach, 1979; Grumbach, 1979]. Thus Ridley and Ridley suggested that illumination induces the *de novo* synthesis of β-carotene specific synthetases in the cytoplasm that are affected by SAN 6706, whilst xanthophyll specific synthetases, already present in the chloroplast, are not affected by SAN 6706 until new xanthophyll synthetases are produced through enzyme turnover. The presence of phytoene and phytofluene in herbicide treated plants suggests that

SAN 6706 interferes with the proper functioning of the phytoene desaturating enzymes. This hypothesis is supported by experiments that show that inhibition of cytoplasmic protein synthesis destroys the bleaching activity of SAN 6706 (Grumbach and Drollinger, 1980).

Inhibition of carotenogenesis is not the only activity associated with these herbicides. Phenylpyridazinones have been shown to inhibit photosynthetic electron transport in isolated chloroplasts from barley (Hilton et al., 1969) and peas (Ridley, 1982), where the site of inhibition is probably close to that of DCMU (Tischer and Strotmann, 1977). Also, certain substituted phenylpyridazinones alter the fatty acid composition of lipids (St. John and Hilton, 1976). It has been shown that, whilst SAN 9789 is a poor inhibitor of photosynthetic electron transport (I_{50} of 33μM) and does not have any effect on fatty acid synthesis in the dark, SAN 6706 is a relatively potent electron transport inhibitor (I_{50} of 4μM; Hilton et al., 1969) and affects fatty acid synthesis in the dark (St. John and Hilton, 1976). It has also been reported that SAN 6706 prevents grana stacking in wheat grown in dim light (Bartels and Hyde, 1970).

The exact interaction between bleaching phenylpyridazinones and the mechanisms for carotenoid biosynthesis is still not known. It is, however, generally agreed that a carotenogenic cell free system from a higher plant would be a most useful model system in this respect. (Britton, 1979; Ridley, 1982).

1:4:2 DIFUNONE

Cultures of A. braunii, when grown in the presence of difunone (1μM) where found to accumulate phytoene and phytofluene in place of more unsaturated carotenoids (Urbach et al., 1976). Although structurally very different (Fig. 1.4.2), the action of difunone on A.braunii was similar to that of the bleaching phenylpyridazinones, SAN 6706, SAN 9789 and BASF 44521.

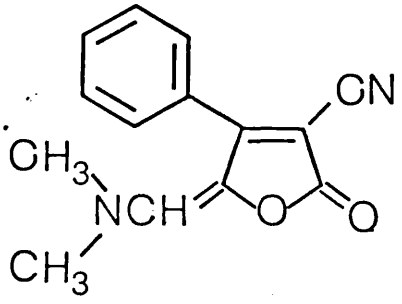
Although difunone was reported to be an inhibitor of porphobilinogenase (Hampp et al., 1975), studies with Scenedesmus showed that whilst 0.1μM difunone did not affect porphobilinogenase activity, the α-carotene content was only 30% of control. It was suggested that loss of chlorophyll was a consequence of loss of photoprotection rather than impaired porphobilinogenase activity (Kunert and Boger, 1978).

It has also been reported that difunone causes phytoene (and a little phytofluene) to accumulate in place of coloured carotenoids in barley seedlings grown in dim light (Ridley, 1982) and reduces the β-carotene content of cultures of P.blakesleeanus (Sandmann et al., 1979).

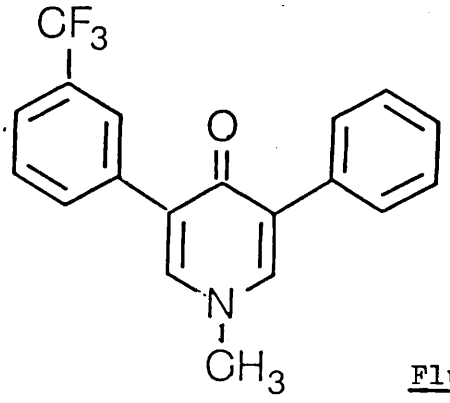
1:4:3 FLURIDONE

This herbicide was found to have a similar effect on wheat seedlings to SAN 9789 and SAN 6706 (Bartels and Watson, 1978). Wheat seedlings grown in the presence of fluridone (100μM) in the dark, lacked coloured carotenoids

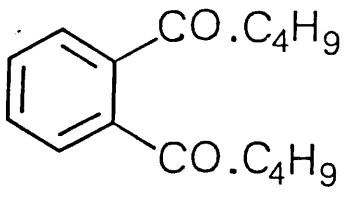
FIG. 1:4:2 STRUCTURES OF VARIOUS BLEACHING HERBICIDES - I



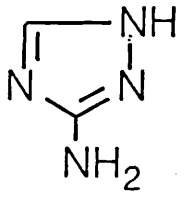
Difunone (EMD-IT 5914)



Fluridone



Di-n-Butyl Phthalate (DBP)



Amitrole

Please see Table 1:4 for systematic naming.

and accumulated phytoene and phytofluene. The chlorophyll and ribosomes (70S) were destroyed only in fluridone treated plants exposed to bright light.

Fluridone-treated barley seedlings accumulated traces of ζ-carotene in addition to phytoene and phytofluene with an I_{50} value of 0.8μM against light-induced β-carotene synthesis (Ridley, 1982).

Fluridone (Fig. 1:4:2) is structurally similar to the bleaching phenyl pyridazinones (Fig. 1:4:2) as both contain a 3-trifluoromethyl-phenyl group that is essential for bleaching activity (Urbach et al., 1976; Ridley, 1982). However, fluridone does not inhibit photosynthetic electron transport in isolated chloroplasts.

1:4:4 AMITROLE

Wheat seedlings, grown in the dark (six days), in the presence of 100μM amitrole (Fig. 1:4:2) have a reduced xanthophyll content (Burns et al, 1971) and phytoene, phytofluene and ζ-carotene are accumulated under these conditions. The chlorophyll in treated seedlings was found to be sensitive to bright light, although amitrole did not inhibit the synthesis of protochlorophyllide or chlorphyllide.

However, lycopene has been reported to accumulate in amitrole-treated maize (Guillot - Salomon et al., 1967), radish seedlings (Grumbach, 1981) and even wheat seedlings

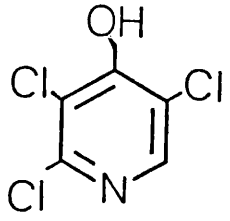
(Rüdiger et al., 1976). Rüdiger and Benz (1979) have since suggested that amitrole reduces the chlorophyll content through interference with phytol biosynthesis.

1:4:5 4-HYDROXYPYRIDINES

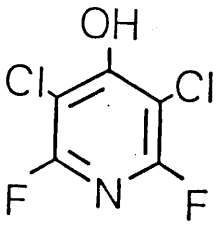
Pyriclor-treated wheat seedlings grown in the dark accumulate phytoene, phytofluene and ζ-carotene with concurrent loss of xanthophylls and normal carotenes; loss of chlorophyll in dim light is similar to that in amitrole-treated plants (Burns et al., 1971).

Two other 4-hydroxypyridines (haloxydine, J771; Fig. 1:4:3)) were found to inhibit carotene biosynthesis in oat seedlings (Ridley, 1982) where haloxydine was found to be the more potent, severely inhibiting both xanthophyll and α-, and β-carotene syntheses. Haloxydine treatment causes considerable phytoene accumulation whilst that with J771 is modest : phytofluene and ζ-carotene were reported to accumulate with both herbicides. Both of these 4-hydroxypyridines inhibit photosynthesic electron transport in isolated pea chloroplasts, where J771 is found to be potent (I_{50} of 0.15μM). However, the effect of J771 in vivo is of a weak carotenogenic inhibitor rather than a potent electron transport inhibitor and so it has been suggested that J771 does not affect carotenogenesis from within the chloroplast (Ridley, 1982).

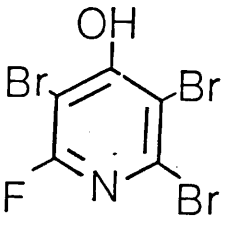
FIG. 1:4:3 STRUCTURES OF VARIOUS BLEACHING HERBICIDES - 2



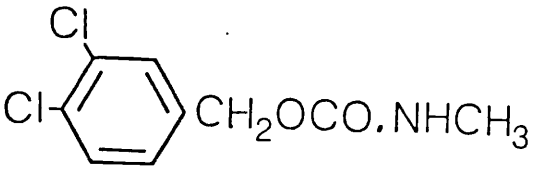
Pyriclor



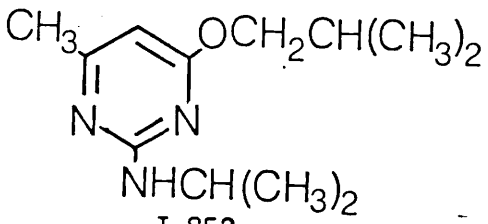
Haloxydine



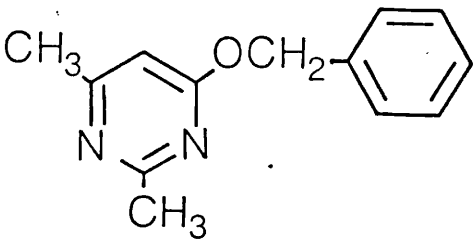
J-771



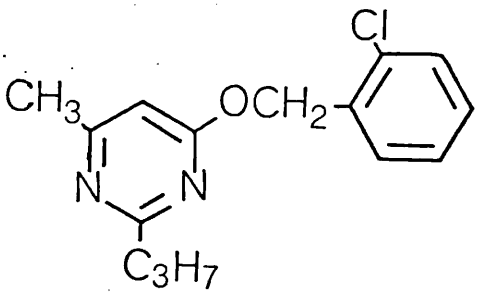
Dichlormate



J-852



J-739



J-334

Please see Table 1:4 for systematic names.

1:4:6 DICHLORMATE AND 6-METHYL PYRIMIDINES

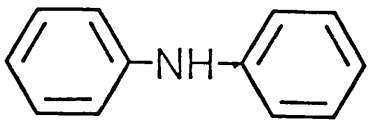
Wheat seedlings grown in the dark in the presence of 500μM dichlormate were found to have a reduced xanthophyll content, an extraordinarily high content of partially desaturated carotene intermediates and relatively little phytoene (Burns *et al.*, 1971). As treated seedlings grown in dim light contained substantial amounts of chlorophyll, it appeared that some β-carotene was being synthesized. Etiolated barley sprayed with 100μM dichlormate before being 'greened' was found to accumulate equal amounts of β- and ζ-carotenes where β-carotene was 78% of the control (Ridley, 1982).

Certain 6-methyl pyrimidines (J739, J852 and J334; Fig. 1:4:3) were found to have a similar effect on etiolated barley as dichlormate (Ridley, 1982). These 6-methyl pyrimidines caused ζ-carotene to accumulate rather than phytoene and phytofluene, although these too were accumulated in lesser amounts. Etiolated barley sprayed with a mixture of fluridone and J334 accumulated more phytoene and phytofluene than barley sprayed with either J334 or fluridone alone, less ζ-carotene than barley sprayed with J334 but more than that sprayed with fluridone (Ridley, 1982). This was interpreted as evidence that both fluridone and J334 act directly on the desaturation enzymes.

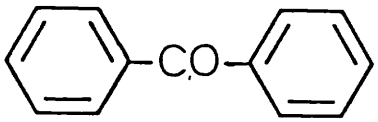
1:4:7 DIPHENYL INHIBITORS OF CAROTENOGENESIS

Over 30 years ago diphenylamine (DPA) was found to inhibit carotenogenesis in *P.blakesleeanus* (Garton *et al.*,

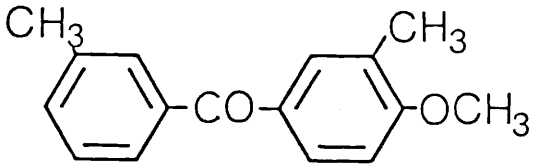
FIG. 1:4:4 DIPHENYL INHIBITORS OF CAROTENOGENESIS



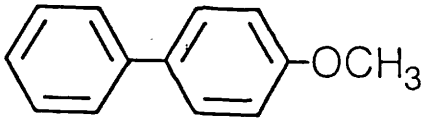
DIPHENYLAMINE



BENZOPHENONE



* NK-049



4-METHOXYBIPHENYL

* See Table 1:4 for systematic name

1951). Since that time, DPA has been reported to inhibit carotenogenesis in many other species of fungi, photosynthetic and non-photosynthetic bacteria (Goodwin, 1980 for review) and recently in an alga (Sandmann and Boger, 1983). Diphenylamine appears to inhibit phytoene desaturation such that phytoene and, to a lesser extent phytofluene and ζ-carotene, accumulate in place of β-carotene or other highly desaturated carotenoids. After comparing the effect that diphenylamine and various other compounds had on carotene biosynthesis in cultures of a Mycobacterium sp., Rilling (1965) postulated that DPA competed with unsaturated carotenoids for the active site on phytoene desaturase. Alternatively, it has been proposed that DPA inhibits the synthesis of mRNA (Shanmugam and Berger, 1969). However, a cell-free extract prepared from P.blakesleeanus (C115) mycelia grown in DPA(70μM) was found to incorporate radioactivity from [2-^{14}C]MVA into β-carotene if the extract was first passed through a Sephadex (G25) column, otherwise radioactivity only accumulated in phytoene (Clarke et al., 1983).

Phytoene formation is increased when the phytoene-accumulating C5 strain of P.blakesleeanus (carB mutant) is cultured in the presence of DPA (Lee et al., 1975). Furthermore, DPA increases the incorporation of radioactivity into phytoene by cell-free extracts of this Phycomyces strain (Clarke et al., 1983). Since carB mutants have defective phytoene desaturase enzymes, it has been suggested that DPA in Phycomyces at least, stimulates the

synthesis of phytoene as well as its desaturation thereby giving rise to a large accumulation of phytoene.

Diphenylamine is a strong inhibitor of carotene biosynthesis in cultures of the fungus Mucor hiemalis, but desaturation of one or both phenyl rings of DPA or methylation to N-methyldiphenylamine resulted in loss of inhibitor activity. Of the hydroxyl substituted diphenylamines, only 4-hydroxydiphenylamine was found to inhibit carotene biosynthesis in M.hiemalis (Herber et al., 1972).

Benzophenone was reported to inhibit carotenogenesis in cultures of a Mycobacterium sp. and thus it was postulated that, having a similar shape to DPA, it inhibited desaturation in an analogous manner (Rilling, 1975). This compound was also found to inhibit carotene biosynthesis in cultures of M.hiemalis, where cultures containing 400μM benzophenone failed to synthesize any coloured carotenoid, but accumulated phytoene (Herber et al., 1972). The same workers found that, whilst desaturation of one or both phenyl rings destroyed inhibitor activity, as in DPA, reduction of the central ketone of benzophenone to yield diphenylcarbinol and ensuing methylation to form 1,1-diphenylethanol, resulted in diphenyl products that were still potent inhibitors of carotene biosynthesis. However, hydroxyl substituted benzophenones were all found to have no effect on carotenogenesis. Whilst DPA, 2-hydroxybiphenyl and 9-fluorenone (see below) were found to have no effect on desaturation in a cell-free system from tomato fruits

(Bucholtz et al., 1977), a benzophenone derivative [3,3'-dimethyl-4-methoxybenzophenone; NK-049 (Fig. 1:4:4)] has been reported to be a useful herbicide against barnyard grass (Fujii et al., 1977). NK-049 apparently inhibits carotenoid desaturation, thereby removing the means for photoprotection (Fujii et al, 1977).

Carotene biosynthesis in cultures of M.hiemalis was found to be inhibited by certain substituted biphenyls (Herber et al., 1972). The most potent biphenyl compounds were the 4-methyl, 4-methoxy, 4-acetyl and 3,3'-dimethoxy derivatives. Biphenyl derivatives containing amino, halogen or nitro substituents were found to have no effect on carotene biosynthesis in M.hiemalis, whereas 2-hydroxybiphenyl was reported to inhibit carotene biosynthesis in Rps. spheroides and Rsp. rubrum (Maudinas et al., 1972); this compound was found to have no effect on carotene biosynthesis either in cultures of M.hiemalis (Herber et al., 1972) or a cell-free system from tomato fruit plastids (Bucholtz et al., 1977). It was observed that the biphenyl derivatives that inhibited carotenogenesis in M.hiemalis cultures were inactivated if one or both aromatic rings were saturated (Herber et al., 1972).

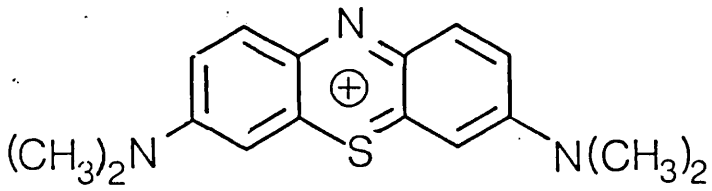
Inhibition of carotenogenesis in cultures of M.hiemalis by each of the above compounds resulted in the concomitant accumulation of phytoene, suggesting that these compounds inhibit phytoene desaturation.

1:4:8 DIPHENYL HETEROCYCLIC INHIBITORS

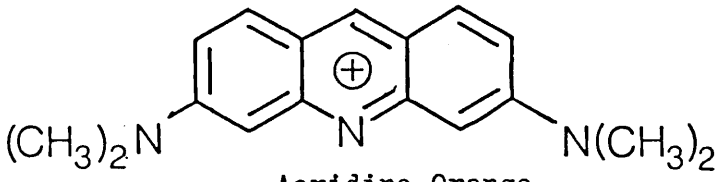
9-Fluorenone (Fig. 1:4:5) inhibits phytoene desaturation in cultures of both M.hiemalis (Herber et al., 1972) and in Verticillium agaricinum (Valadon et al., 1973). In each case phytoene accumulated in place of coloured carotenoids. Originally it was reported that 9-fluorenone did not inhibit phytoene desaturation in P.blakesleeanus (wild type) but caused a slight stimulation of phytoene synthesis (Medina and Micol, 1981). However, in liquid cultures of P.blakesleeanus (wild type, carS and carR mutants), 9-fluorenone proved to be a potent desaturation inhibitor such that much phytoene, particularly the all-trans isomer, accumulated (Valadon et al., 1981). Since DPA was found to stimulate the accumulation of very little all-trans-phytoene in P.blakesleeanus cultures (Clarke et al., 1983), 9-fluorenone and DPA may inhibit desaturation through different mechanisms. 9-Fluorenone had no effect on carotenogenesis in a cell-free system from tomato fruit plastids (Bucholtz et al., 1977).

Various dyes, namely acridine orange, methylene blue, neutral red and toluidine blue (Fig. 1:4:5), were found to be potent inhibitors of phytoene desaturation in cultures of a Mycobacterium sp. (Rilling, 1965). However, the dyes were found to have no effect on carotenogenesis in cultures of Mycobacterium under high oxygen tension, suggesting that the reduced (dihydro-) forms of these compounds were the active inhibitors.

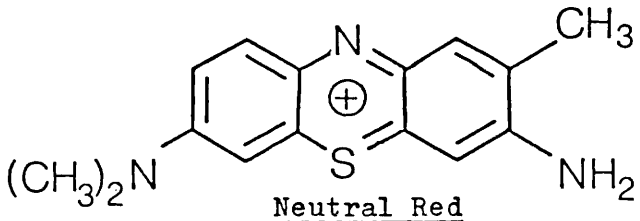
FIG. 1:4:5 HETEROCYCLIC DIPHENYL INHIBITORS OF CAROTENOGENESIS



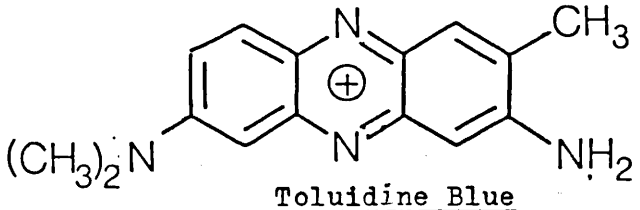
Methylene Blue



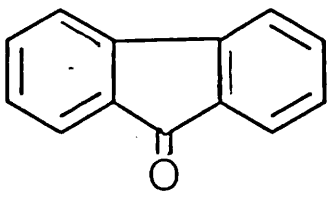
Acridine Orange



Neutral Red



Toluidine Blue



9-Fluorenone

Please see Table 1:4 for systematic names.

1:4:9 DI-*N*-BUTYLPHTHALATE

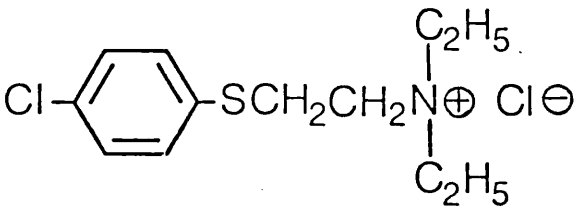
Di-*n*-butylphthalate (DBP) is extensively used as a plasticizer in certain paints and flexible PVC. Recently there have been several reports of DBP-containing products causing leaf damage to a variety of plants (Morner, 1979; Hannay, 1980; New Scientist, 1983). Radish seedlings and bluebell shoots cultivated in high levels of DBP vapour were found to accumulate phytoene in place of coloured carotenoids and it was postulated that leaf damage was a result of loss of photoprotection (Virgin *et al.*, 1981).

1:4:10 INHIBITORS OF CAROTENE CYCLISATION

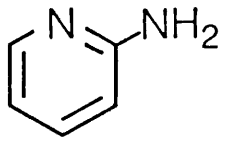
Of the number of chemicals that have been found to inhibit carotene cyclisation (Fig. 1:4:6) nicotine and 2-(4-chlorophenylthio)- triethylamine hydrochloride (CPTA) are perhaps the best known. Nicotine was found to inhibit cyclisation in a *Mycobacterium* sp. (Howes and Batra, 1970b) and in various other non-photosynthetic bacteria [*Flavobacterium* spp. (Goodwin, 1972), *Micrococcus* sp., (Lewis and Kumta, 1973); *Myxococcus fulvus* (Kleinig and Reichenbach, 1973)] such that lycopene accumulated at the expense of β-carotene.

When various fruits (citrus, apricot, peach and tomato) were sprayed with CPTA solutions, it was found that lycopene accumulated (Coggins *et al.*, 1970). Similarly, fruits of *Capsicum annuum* sprayed with CPTA accumulated lycopene, and to a lesser extent neurosporene and γ-carotene, at the

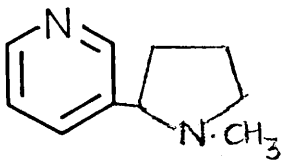
FIG. 1:4:6 INHIBITORS OF CAROTENE CYCLISATION



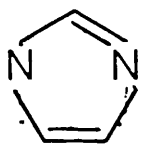
CPTA



2-aminopyridine



Nicotine



Imidazole

*Please see Table 1:4 for systematic name.

expense of more cyclised carotenoids (Simpson et al., 1974). However, it was found that neither nicotine nor CPTA had any affect on cyclisation in a carotenogenic cell-free system from C.annuum fruit plastids (Camara and Monéger, 1982), that CPTA had no affect on the carotenogenic system from tomato fruit plastids (Bucholtz et al., 1977) and that nicotine had no affect on the Narcissus flower plastidal system (Beyer et al., 1980).

A Brevibacterium sp. cultured in CPTA or nicotine was found to accumulate lycopene, neurosporene and γ-carotene in place of β-carotene. Removal of the inhibitor resulted in resumption of β-carotene synthesis with loss of accumulated neurosporene (Hsieh et al., 1974). Lycopene is accumulated through the simultaneous inhibition of lycopene hydration (to rhodopin) and cyclisation when Rhodomicrobium vanielli is cultured in CPTA or nicotine (cited by Goodwin, 1980). Nicotine and CPTA both inhibit canthaxanthin synthesis in Micrococcus roseus where it has been postulated that CPTA inhibits hydroxylation, nicotine inhibits oxygenation and both inhibit the second cyclisation process of a novel carotenogenic pathway (Cooney and Berry, 1981).

Various amines with a similar structure to CPTA, triethylamine and tributylamine hydrochlorides have also been reported to inhibit cyclisation in B.trispora (Hsu et al., 1974) as have certain derivatives of imidazole and pyridine (Ninet et al., 1969). Pyridine derivative pKa values were compared to the phytoene and lycopene accumulated and β-carotene absent from inhibited cultures of

P.blakesleeanus; it was observed that those derivatives with pK*a* 5-7 were the most active and it was postulated that these were the most easily assimilated (Elahi *et al.*, 1973).

1:5 DESCRIPTIONS OF PHYCOMYCES BLAKESLEEANUS and APHANOCAPSA 6714

1:5:1 PHYCOMYCES BLAKESLEEANUS

Fungi and yeasts are classified as outlined in table 1:5:1 according to the definitions used by Burnett (1968) and Ainsworth (1971). The genus Phycomyces belongs to the sub-division Zygomycotina, the fungal group defined as lacking the dikaryotic phase in the sexual cycle.

The life cycle of P.blakesleeanus as outlined in Fig.1:5:1.consists of asexual and sexual phases. In the sexual phase single unbranched aerial hyphae (sporangiophores) emerge from the mycelium after 2-3 days growth, the tips of which swell and develop into sporangia where spores are formed. When mature sporangia come into contact with water or solid objects, the outer wall ruptures and the spores are released. In the sexual cycle specialised hyphae (zygophores) are produced that grow towards mycelium of opposite mating type. Once zygophores of opposite 'sex' make contact, they swell, grow into the air and through a series of specialised structures are transformed into zygophores that contain the products of sexual-genetic interaction. Then follows a period of dormancy (at least three months) after which the zygospore germinates and a single germsporangiophore emerges that develops a sporangium from which spores are eventually released.

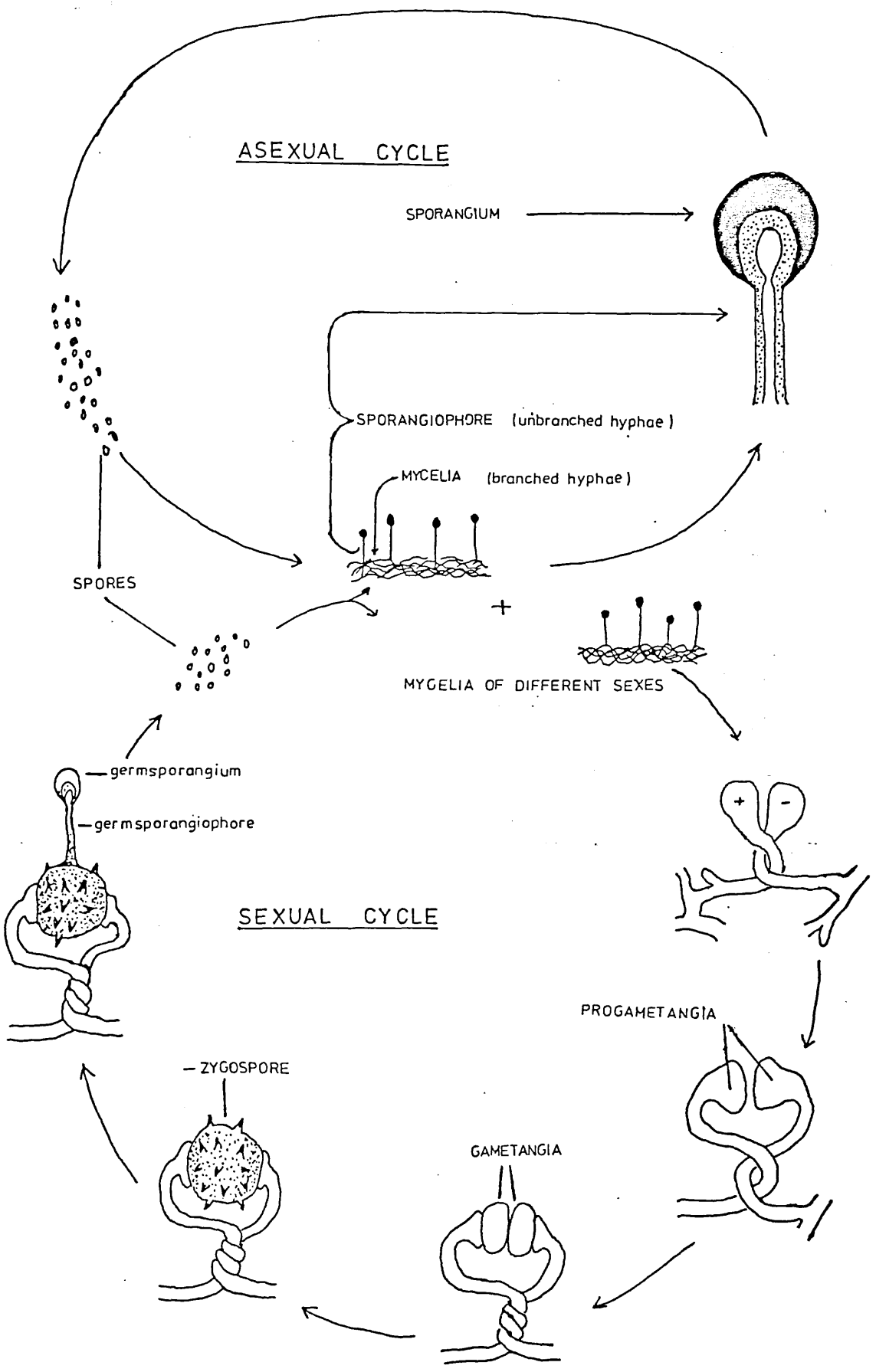
The physiology of P.blakesleeanus has been well studied and reviewed by Bergman et al (1969) and the suitability of this fungus for various fields of research reviewed by Cerda-Olmedo (1975).

TABLE 1:5:1 TAXONOMY OF FUNGI AND YEASTS

DIVISION	SUB-DIVISION	CHARACTERISTICS	REPRESENTATIVE GENERA
Myxomycota		Slime Fungi - Free living amoebae that unite to form plasmodia	Dictyostelium
Eumycota			
	Mastigomycotina	No Dikaryotic phase in Life Cycle. Motile zoozpores produced.	Achyla Blastocladiella Phytophthora Pythium
	Zygomycotina	No Dikaryotic phase in Life Cycle. Non-motile spores produced.	Blakeslea Mortierella Mucor Phycomyces Rhizopus
	Ascomycotina	Mycelium usually septate. Vegetative reproduction often by conidia (or by budding). Sexual reproduction involves ascospores. Ascospores produced within asci.	Aspergillus* Cephalasporium* Ciliaria Cochliobolus Fusarium* Neurospora Ophiobolus Sclerotinia Verticillium* Whetzelinia YEASTS
	Basidiomycotina	Mycelium usually septate. Sexual reproduction often by Basidiospores. Basidiospores borne in basidia that are often clumped together as fruiting bodies.	Clothrus Coprinus Dacryopinax Sclerotium* Ustilago

*These Fungi are Fungi Imperfecti, fungi that have lost the sexual cycle and are properly classified in the sub-division Deuteromycotina. However, because of similarities with certain organisms possessing a full sexual cycle, these organisms have been grouped alongside, in the sub-divisions Ascomycotina and Basidiomycotina.

FIG. 1:5:1 LIFE CYCLE OF PHYCOMYCES BLAKESLEEANUS



1:5:2 APHANOCAPSA 6714

1:5:2:1 Taxonomy. Cyanobacteria (blue-green algae) are Gram negative, oxygen evolving photosynthetic prokaryotes that are able to synthesize chlorophyll *a* and contain phycobilin-protein complexes.

Taxonomy of the cyanobacteria is complex. Traditionally these organisms have been associated with algae and have thus been classified according to observations made by phycologists on field material. Rippka and co-workers (1979) proposed that certain genera be redefined "in such a way that simple and clear-cut generic assignments" could be made for cultured cyanobacteria. The generic definitions used by phycologists (Bourelly, 1970; Geitler, 1932) were maintained as far as possible, but species names were replaced by culture numbers as unambiguous identifiction was considered difficult (Stanier *et al.*, 1971; Rippka *et al.*, 1979). In this taxonomic scheme, the cyanobacteria were subdivided into five large groups based on structural differences, as defined in table 1:5:2. These groups do not precisely correspond to the major taxa proposed by phycologists.

The Section I bacteria, to which *Aphanocapsa* 6714 belongs, are unicellular organisms that reproduce by binary fission (or by budding in one genus only) and are the most primitive cyanobacteria. The 57 cultured strains associated with Section I were re-assigned to the six genera (Rippka *et al.*, 1979) defined in table 1:5:3. The genus *Synechocystis*, as defined above, encompassed the five traditional genera of

TABLE 1:5:2 MAJOR SUB-GROUPS OF CYANOBACTERIA

Unicellular Cells: single or forming aggregates held together by additional outer cell wall layers	Reproduction by budding or binary fission		SECTION I
	Reproduction by multiple fission giving rise to small daughter cells (baeocytes) or by both multiple fission and binary fission.		SECTION II
Multicellular	Reproduction by random trichome breakage, by formation of hormogonia and (Sections IV and V only) sometimes by germination of akinetes.	Trichome always composed only of vegetative cells.	Division in only one plane. SECTION III
		In the absence of combined nitrogen trichome contains heterocysts; some also produce akinetes.	Division in only one plane. SECTION V
			Division in more than one plane. SECTION V

Drawn from Ripka *et al* (1979).

TABLE 1:5:3 GENERA OF SECTION I CYANOBACTERIA

MODE OF REPRODUCTION	THYLAKOIDS PRESENT?	PROMINENT SHEATH?	DIVISIONAL PLANES	PROPOSED GENUS
Binary Fission	Yes	Yes	1	*Gleothece*
			2-3	*Gleocopsa*
		No	1	*Synechococcus*
			2-3	*Synechocystis*
	No	Yes	1	*Gleobacter*
Budding	Yes			*Chamaesiphon*

Redrawn from Rippka *et al* (1979)

Synechocystis, Aphanocapsa, Microcystis, Merismopedia and Eucapsis. Traditionally such unicellular, poorly sheathed, spherical blue-green algae have been discriminated on the basis of aggregate format (Table 1:5:4). However, in culture these organisms do not aggregate and so it was proposed that they all be described by one genus (Komarek, 1976; Rippka et al., 1979).

A comparison of T1 ribonuclease digests of 16S rRNA from eight species of cyanobacteria (Bonen et al, 1979), suggested that Aphanocapsa (Synechocystis) 6714 was more closely related to the more advanced Section IV and V cyanobacteria than to most other Section I organisms; the phylogenetic tree implied by this experiment is given in Fig. 1:5:2.

1:5:2:2 Physiology and Cell Division The cellular organization of Aphanocapsa 6714 and other cyanobacteria is prokaryotic and similar to Gram negative bacteria (Stanier and van Niel, 1962). The cell is encapsulated by a composite envelope that consists of a semi-permeable plasmalemma, a rigid peptidoglycan layer and an outer membrane surrounded by a slime layer (Drews and Weckesser, 1982 for review). Typically, cyanobacteria possess a protoplasm composed of a pigmented, membranous periphery (chromatoplasm) and a colourless fibrillo-granular centre (centroplasm). The centroplasm is the site for protein synthesis and contains ribosomes and genetic material, whilst the chromatoplasm contains thylakoid membranes and is the site for photosynthesis. In Aphanocapsa 6714, as in most other cyanobacteria, thylakoids are arranged in layers

TABLE 1:5:4 TRADITIONAL GENERA OF UNICELLULAR, UNSHEATHED, SPHERICAL, BLUE-GREEN ALGAE

AGGREGATE TYPE	FURTHER DISCRIMINATION	PROPOSED GENUS
No Aggregation		*Synechocystis*
Irregular Aggregation: caused by slime layer uniting cells	Gas Vacuoles	*Microcystis*
	No Gas Vacuoles	*Aphanocapsa*
Regular Aggregation; caused by regular cell division	Rectangular Aggregates	*Merismopedia*
	Cubical Aggregates	*Eucapsis*

FIG. 1:5:2 TENTATIVE PHYLOGENY OF CYANOBACTERIA AFTER BONEN ET AL., (1979)
(Redrawn from Doolittle, 1982)

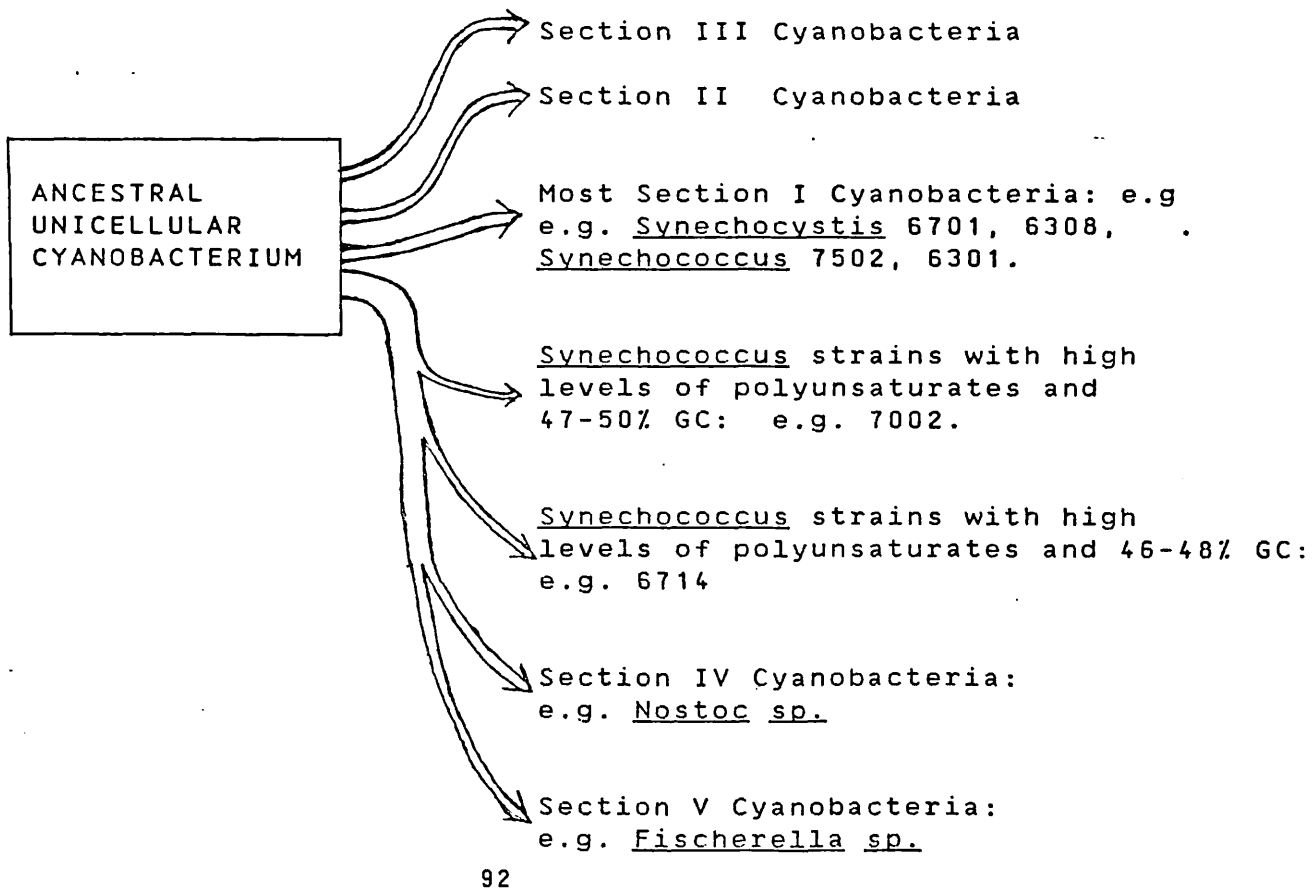


FIG. 1:5:3A STRUCTURE OF A TYPICAL CYANOBACTERIAL CELL

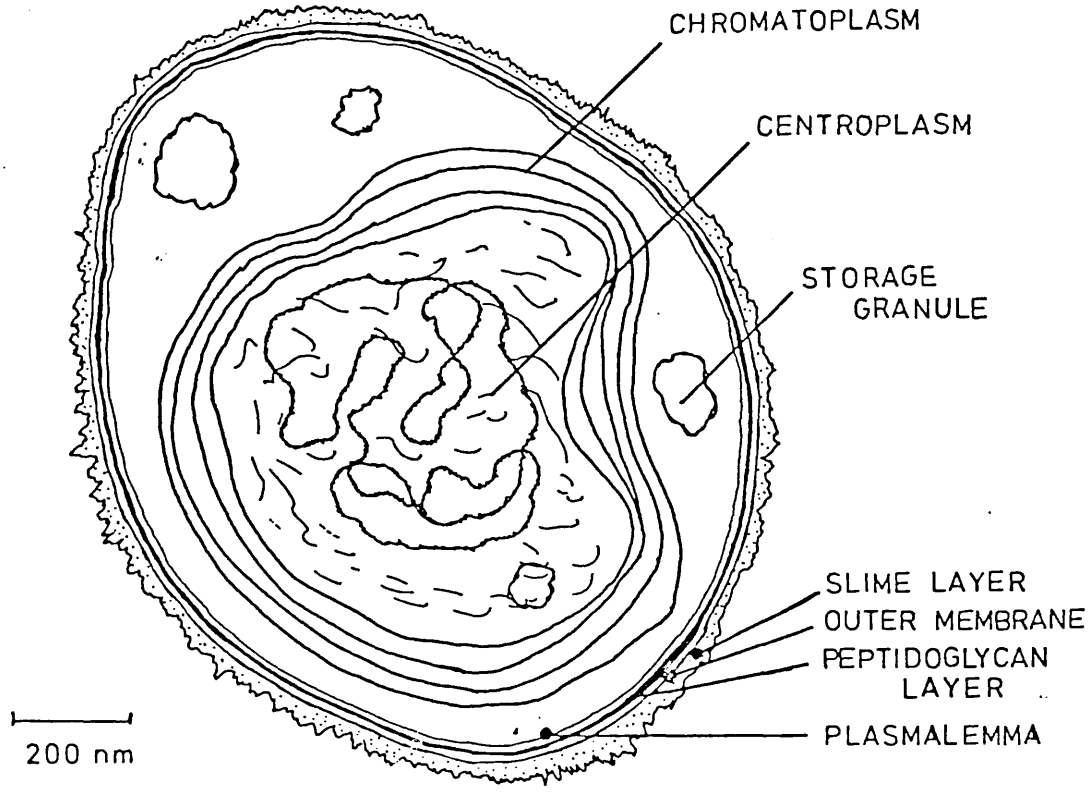
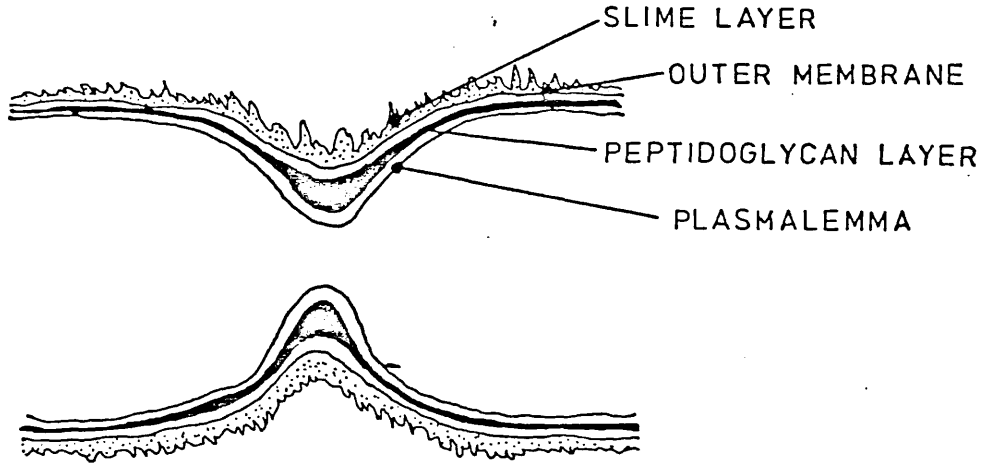


FIG. 1:5:3B CONSTRICTIVE BINARY FISSION



parallel to the envelope forming an anastomasing network of concentric shells (Fig. 1:5:3a). The thylakoids, although similar to those of higher plants, possess phycobilisomes- particles that contain accessory pigment-protein complexes (phycobiliproteins) for the cyanobacterial photosystem II (Cohen-Bazire and Bryant, 1982 for review). Also situated within the protoplasm are storage granules (e.g. polyphosphate bodies, cyanophycin granules and polyglucan granules) that accumulate toward the end of exponential growth and disperse during the stationary phase.

Aphanocapsa 6714 divides by simple constrictive type binary fission, as observed with Gram negative bacteria. Division is initiated by the centripetal growth of the plasmalemma and cell wall (Fig. 1:5:3b). This grows inwards like the closing of a lens diaphragm until the dividing wall is complete and the daughter cells separate (Allen, 1968). A mutant isolated from Synechococcus 7002 has thylakoids grouped into discrete bundles without any of the envelope, suggesting that distribution of the thylakoids between daughter cells is not passive (Ingram and Thurston, 1970)

1:6 AIMS OF THE PROJECT

In view of the complex reaction by plants to bleaching herbicides (see 1:4 and 1:2), it was suggested that chemicals suspected of inhibiting carotene biosynthesis be evaluated, using carotenogenic cell-free systems (Britton, 1979). Chemicals that inhibit carotene

biosynthesis have potential commercial value in weed control. For cell-free systems to give a reliable guide to herbicide activity in the field it is desirable that they originate from photosynthetic tissue, thus a novel system was developed from the cyanobacterium Aphanocapsa 6714. Ideal test systems, however, would be those from weeds themselves.

Together with the well-established cell-free system from the fungus P.blakesleeanus (Yokoyama et al., 1962) the Aphanocapsa system was used in this project to investigate:-

i) cofactor requirements of carotene biosynthesis;

ii) the structure-inhibitor activity relationship of phenyl-pyridazinones and phenylfuranes with respect to phenyl ring substitution;

iii) the structure-inhibitor activity relationship of phenyl-alkyl-benzamides with respect to phenyl ring substitution;

iv) the effect of diphenyl compounds, particularly derivatives of benzophenone, on in vitro carotenogenesis.

CHAPTER 2

MATERIALS AND METHODS

2:1 MATERIALS

2:1:1 SOLVENTS

All solvents were of the highest purity available and were purchased from various suppliers.

2:1:2 CHEMICALS

General labaoratory chemicals were purchased from Merck, Darmstadt, W. Germany, and were of analytical grade wherever possible. Nucleotide cofactors were obtained from Boehringer, Mannheim, W. Germany and reduced glutathione from Sigma, Munich, W. Germany.

Thin layer plates, both silica gel G and neutral alumina, were supplied ready made by Merck, Darmstadt, W.Germany.

Lysozyme was obtained from Sigma, Munich, W.Germany.

Organic chemicals used in the synthesis of various N-alkyl-phenoxybenzamides (Chapter 5) were all bought from Aldrich-Europe, Nettetal, W. Germany, with the exception of 3-(4-chloro-) phenoxybenzoic acid, which was a generous gift from B.A.S.F., Ludwigshafen, W. Germany.

Nutrient agar and yeast extract were supplied by Oxoid Limited, Basingstoke, Hants.

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

2:1:3 RADIOCHEMICALS

DL - [2-^{14}C]Mevalonic acid lactone (10mCi/mmol) and [1-^{14}C] isopentenyl pyrophosphate (55mCi/mmol) were supplied by Amersham-Buchler, Braunschweig, W.Germany. The mevalonic acid was converted into the sodium salt prior to use by the addition of a requisite amount of sodium hydroxide.

[2-^{14}C]Geranylgeranyl pyrophosphate (2-4.22 mCi/mmol) was obtained from J.Soll and G.Schultz of the Hanover Veterinary College and was prepared from all-trans-farnesyl acet and [2-^{14}C]ethyl bromoacetate (Soll and Schultz, 1981).

2:1:4 HERBICIDES

Various experimental herbicides were kindly supplied by the following companies:-

Substituted phenyldihydrofuranes were generous gifts from Celamerck, Ingelheim, W.Germany, who also supplied the following 3-phenoxy-N-alkyl benzamides; 3-(4'-chloro-)-phenoxy-N-methyl benzamide (CUR 5025), 3-(4'-chloro-)-phenoxy-N-isopropyl benzamide (CUR 5026), 3-phenoxy-N-methyl benzamide (CUR 4850) and 3-phenoxy-N-propenyl benzamide (CUR 4914).

All substituted phenylpyridazinones, with the exception of SAN 9789 (norflurazon), were kindly supplied by B.A.S.F., Ludwigshafen, W.Germany; SAN 9789 was a generous gift from the Sandoz Company, Basel, Switzerland. B.A.S.F. also supplied the following 3-phenoxy-N-alkyl benzamides; 3-(2'-chloro-,4'-trifluoromethyl-)-phenoxy-N-ethyl benzamide

(BAS 177 183) and 3-(2',5'-dimethyl-)-phenoxy-6-nitro-N-ethyl benzamide.

The following 3-phenoxy-N-alkyl benzamides were generous gifts from Sumitumo Company Limited, Takarazuka, Japan; 3-phenoxy-N-ethyl benzamide (S-3141) and 3-(2',5'-dimethyl-)phenoxy-N-ethyl benzamide (S-3422).

The diphenylethers, oxyfluorfen and RH1939, were kindly supplied by Rohm and Hass Limited, Springhouse, Pennsylvania, U.S.A. Diphenyl compounds used for inhibitors were purchased from Aldrich-Europe, Nettetal, W.Germany.

2:1:5 ORGANISMS

Phycomyces blakesleeanus C115 [carS42, mad107(-)] a superyellow mutant, was a gift from Professor E. Cerda-Olmedo, Departamento de Genetica, Universidad de Sevilla, Sevilla, Spain.

Aphanocapsa 6714 (ATCC 27178) was obtained from the University of Göttingen, W. Germany.

2:2 METHODS

2:2:1 GROWTH AND MAINTENANCE CONDITIONS

2:2:1:1 Phycomyces blakesleeanus. Phycomyces was maintained on nutrient agar slopes and stored at 4° C. The nutrient agar medium contained, per litre distilled water:

L-Asparagine	2g
D-Glucose	20g

Nutrient Agar	20g
50x - Concentrate	20ml

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

KH_2PO_4	25g
$MgSO_4$	2.5g
14% W/W $CaCl_2$ Solution	1ml
Thiamine Hydrochloride	10mg
Trace Element Solution	0.5ml

The trace element solution contained, per 100ml distilled water:

Citric Acid (Monohydrate)	2g
$Fe(NO_3)_3 . 9H_2O$	1.5g
$ZnSO_4 . 7H_2O$	1.0g
$MnSO_4 . H_2O$	0.3g
$CuSO_4 . 5H_2O$	0.05g
$Na_2MoO_4 . 2H_2O$	0.05g

Slopes were prepared by pouring aliquots (2 ml) of hot (60° C) sterile medium into sterile test tubes and allowing to cool at approximately 15° to the horizontal. When cool, slopes were aseptically inoculated with sporangia from a mature culture, plugged with cotton wool and grown for seven days at 24°C under constant illumination (35 W/m^2). Mature slopes were then sealed with paraffin wax and stored at 4°C.

Mycelium was grown in the following liquid medium:

D-Glucose	25g	per litre distilled H_2O
L-Asparagine	2.5g	
$MgSO_4 . 7H_2O$	0.5g	

KH_2PO_4	1.5g	per litre distilled H_2O
Yeast Extract	0.5g	
Thiamine hydrochloride	0.25mg	

The above medium (2.5 l) was autoclaved (120 C for 15 min.) in a large, flat (3.5 l) 'medicine bottle' flask (made by Schott, Mainz, W.Germany) that had been adapted for air percolation. When cool, the flask was inoculated by aseptically washing spores from three agar slopes into the medium, incubated for 60h at 20 °C under constant illumination (35W/m^2) and agitation (100 agitations per minute). The cultures were percolated with filtered air (50ml/min) throughout growth.

The myceliumwas harvested by passing the culture through two layers of clean nylon gauze. The mycelium was thoroughly washed with tap water, then with distilled water, and finally squeezed dry and weighed.

One 2.5 l. flask typically yielded 40-60g mycelia (wet weight).

2:2:1:2 Aphanocapsa 6714. Aphanocapsa cells were grown in 1 litre Fernbach flasks with percolation by filtered air (10ml/min), under constant illumination (15W/m^2) at 35 C°in a sterile medium devised for the autotrophic growth of Scenedesmus acutus (Sandmann and Boger, 1980). It was subcultured regularly, typically after seven days growth.

The incubation medium contained per litre:-

KNO_3	100mg
$NaH_2PO_4.2H_2O$	234mg

KH_2PO_4	735mg
$MgSO_4.7H_2O$	49mg
$CaCl_2.2H_2O$	1.03mg
$Fe(NO_3)_3.9H_2O$	1.45mg
Titriplex III	13.5mg
$Al_2(SO_4)_3.16H_2O$	12.6g
$MnCl_2.4H_2O$	10µg
$CoSO_4.7H_2O$	2.8µg
$ZnSO_4.7H_2O$	6.3µg
H_3BO_3	31µg
$(NH_4)_6Mo_7O_{24}.4H_2O$	12.3µg
NH_4VO_3	2.9µg
$NiSO_4.7H_2O$	28µg
KI	250µg
KBr	240µg
LiCl	8.5µg
$CuSO_4.5H_2O$	5 g

This was adjusted to pH 7.8 with KOH.

2:2:2 PREPARATION OF CELL FREE EXTRACTS

2:2:2:1 Phycomyces blakesleeanus. Washed, hand dried, mycelium was cut into conveniently sized pieces and lyophilised under reduced pressure (100µM Hg, cold trap at -90° C) for 60h on a 10-148 MRBA continuous freeze drying apparatus (made by Virtis, New York, U.S.A.). The freeze dried material was weighed and stored in airtight containers at -20° C for periods of up to one month, or for longer under liquid nitrogen.

Freeze dried mycelium was rubbed through a 40-mesh sieve onto aluminium foil and weighed. The powder was poured into a 50ml centrifuge tube and mixed into a dough-like paste (1:5 w/v) with 400mM Tris-HC1 buffer, pH 7.8. The paste was then centrifuged for 30 min. at 4°C and 12000g (Sorvall, RC7-B) and the supernatant used as the cell-free extract.

2:2:2:2 Aphanocapsa. A four-day-old culture containing approximately 15µg chlorophyll/ml Aphanocapsa cell suspension was harvested by centrifugation for 10 minutes at 4°C and 440g. (Beckman, J2 - 21). The cells were resuspended in 10mm Tricine [N-tris-(hydroxymethyl)-methyl glycine] - NaOH buffer, pH 7.8 (400ml), supplemented with 10mM $MgCl_2$, 2.5mM Na_2HPO_4, 2.5mM K_2HPO_4 and 0.5M sucrose and recentrifuged under the same conditions.

The washed cells were resuspended in a small volume (approximately 20ml) of supplemented Tricine-NaOH, pH 7.8, and the suspension was divided between two 50ml centrifuge tubes. Spheroplasts were prepared from these cells by incubating with lysozyme (8mg/ml cell suspension) for two hours at 35°C with constant stirring (Spiller, 1980).

Spheroplasts were subsequently centrifuged at 4°C, 900g for five minutes, the supernatant was discarded and the pellets were pooled and resuspended in 400mM Tris-HC1-buffer, pH 7.8 (40ml). This suspension was centrifuged at 900g, 4°C for five minutes, the clear supernatant carefully decanted and the pellet resuspended in Tris-HC1, pH 7.8, to give a chlorophyll concentration of between 250-500 µg per ml suspension. This final suspension was used as a cell-free extract.

2:2:3 CELL FREE INCUBATION MIXTURES AND CONDITIONS

Cell-free incubations were performed in tapered, graduated (0-15ml), glass centrifuge tubes (Silberbrand, manufactured by Brand, Main, W.Germany). The standard incubation (0.5ml) mixture contained

ATP	5µmol	)
NAD^+	0.5µmol	) dissolved in 175µl
Glutathione	10 µmol	) Tris-HCl, pH 7.8
$MnCl_2$	3 µmol	)
$MgSO_4$	2 µmol	) dissolved in 25µl water
Radioactive substrate	-	as specified in text
Cell free extract	200µl	

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

NOTE

All the results presented in this thesis are average values obtained from duplicate incubations. They were verified by repeat experiments, again with duplicate incubations.

2:2:4 ANALYSIS OF REACTION MIXTURES

Extraction and purification procedures were adapted from those of Bramley and Davies (1974).

Methanol (3.5ml) was added to each incubation mixture, which was then gently agitated on a rotary mixer (10s). Light petroleum (b.p. 60°- 80°C, 9ml), together with a solution of carriers (20µl), containing phytoene (approx.

25µg) and traces of phytofluene, were then added. Incubations with cell extracts of P.blakesleeanus were extracted with carrier solution that also contained β-carotene (10µg) and squalene (15 µg). Tubes were sealed with a rubber cap, shaken vigorously (40s) and the contents washed into a partitioning funnel (100ml) that contained saturated salt solution (50ml) and light petroleum (b.p. 60°-80°, 10ml).

The aqueous hypophase was discarded and the epiphase delivered into a test tube containing a small amount (approx. 1g) of anhydrous sodium sulphate.

The dried petroleum solution was decanted into a round bottom flask (100ml), together with washings (approx. 10ml) and evaporated under reduced pressure. The solution (1 ml) was transferred to a tapered, graduated tube and the flask washed twice with diethyl ether (2x 1 ml).

The solution was then evaporated to near dryness under a stream of nitrogen, the tube plunged into ice and light petroleum (b.p. 60° - 80°, 50µl) applied around the rim. The resultant solution was then applied as a band (4 cm) onto a silica gel-G plate (5 x 20cm, 0.25mm thick), together with a diethyl ether (50µl) wash.

Silica gel plates were developed in petroleum (b.p. 100° -, 140°C)/toluene (9:1 to 14:1 v/v, depending on the humidity) and a 2cm band containing β-carotene and phytoene (typical Rf = 0.5 - 0.6) promptly scraped off into a plugged filter funnel. The pigments were eluted into a clean tapered tube by the immediate addition of diethyl

ether (5ml) and the eluate evaporated to near dryness under a stream of nitrogen.

Light petroleum (b.p. 60^{o}- 80^{o}, 50µl) was added to the ice-cooled tube, the solution applied onto an alumina plate (6.6 x 20cm, 0.1mm thick and impregnated with a fluorescent indicator) together with one wash (light petroleum, 50 µl) and the plate developed in petroleum (b.p. 100^{o} - 140^{o} C)-diethyl ether (32:1 by vol., or greater, depending on humidity).

Phytoene and phytofluene bands (typical Rf's 0.75 and 0.55, respectively) were located by viewing under near u.v. light (λ = 320nm) and, together with the β-carotene band (typical Rf = 0.4) were scraped off into separate mini-vials (Pico 'Hang-In' vials; Packard, Groningen, Holland) containing universal liquid scintillation fluid (Packard Instagel -2,3 ml). Squalene was located on the dried Silica gel plate by exposure to iodine vapour (typical Rf 0.75) and this was also scraped into a mini-vial.

Sample radioactivity was assayed in a liquid scintillation counter (Packard Tri-Carb, Model 574; 10 min. per vial). Correction for quenching was accomplished from a quench correction curve constructed from counting efficiency versus blue (E-∞, 50-1000): green (C-D, 30-300) channel ratios.

Autoradiography (spark chamber) of developed alumina plates revealed the discrete radioactive bands that coincided with the positions of phytoene and β-carotene.

2:2:5 PREPARATION OF PHYTOENE - PHYTOFLUENE CARRIER SOLUTION

2:2:5:1 Growth of P.blakesleeanus. The C115 strain of P.blakesleeanus was grown in liquid medium containing diphenylamine (approximately 70 μM; see Section 2:2:2:1). After 60h, mycelia was harvested, washed and squeezed dry as previously described (2:2:2:1).

2:2:5:2 Lipid Extraction and Saponification. Hand dry mycelium was re-suspended in acetone, homogenized (Sorvall-Omnimixer) and the lipid extract filtered by suction through a sintered glass funnel. This process was repeated once more with acetone, then three times with diethyl ether after which the filtrates were pooled and partitioned against water. The hypophase was discarded and the epiphase dried over anhydrous Na_2SO_4 (90 min.) before being filtered and the solvent evaporated under reduced pressure at 40° C.

The lipids were then saponified in methanolic KOH (6% w/v) in total darkness (60° C, 10 min.), cooled and the unsaponifiable lipids extracted by partition against diethyl ether (3x). The pooled diethyl ether extracts were washed by partition against water (3x), dried over anhydrous Na_2SO_4 (1h), and the solvent was evaporated under reduced pressure.

2:2:5:3 Separation of Phytoene and Phytofluene.

Phytoene and phytofluene were separated by chromatography on an aluminium oxide column (10g, Brockmann activity grade III) eluted with light petroleum (b.p. 60° - 80° C). The effluent was monitored by u.v. absorbtion (λ = 285 nm) and by spotting samples onto thin layers irradiated with u.v. light (λ = 325 nm). Fractions containing significant

absorbance at 285nm and/or showing bright green fluorescence were pooled and evaporated under reduced pressure to a small volume. The solution was transferred to an airtight dark bottle and stored at -20^{o}C.

2:2:5:4 Spectrophotometric Analysis. Absorbance of a sample of carrier solution (1 ml.) was measured from 250 - 450nm in a double-beam spectrophotometer (Pye-Unicam, SP 1800) against light petroleum (b.p. 60^{o} - 80^{o}C). Absorbance maxima were determined to be 285nm for phytoene and 345nm for phytofluene

2:2:6 PRECAUTIONS FOR WORKING WITH CAROTENOIDS

Since carotenoids are inherently unstable, it was essential that undue exposure to light or heat was avoided. Wherever possible, manipulations (after carotenoid extraction) were performed in dim (5mW/m^{2}) yellow-green light and all terpenoid solutions stored on ice.

Evaporation under reduced pressure was performed in round bottom flasks covered in aluminium foil.

Carotenoid solutions stored for prolonged periods (e.g. carrier solutions) were kept at -20^{o}C.

2:2:7 DETECTION OF RADIOACTIVITY ON THIN LAYER PLATES

Radioactivitiy from ^{14}C on thin layer plates of a density in excess of 1000dpm/cm^{2} were detected with a spark chamber (Birchover Instruments, Letchworth, Herts.)

CHAPTER 3

COFACTOR REQUIREMENTS OF THE CAROTENOGENIC CELL-FREE SYSTEMS

3.1 INTRODUCTION

Yokoyama and co-workers (1962) reported that a cell extract from P.blakesleeanus converted [2-^{14}C]MVA into β-carotene. Although these workers found some cofactor requirements for the overall conversion of MVA into β-carotene with this system, the requirements for each individual reaction were not elucidated (See Section 1:3:5:2).

The system from P.blakesleeanus was refined by Bramley and Davies (1974) who developed the use of sieved, freeze-dried, mycelia to produce the homogenate. Such a method enabled highly active, concentrated extracts to be prepared and enabled mycelia to be stored at -20° for up to a month. It was noted that for freeze dried material to efficiently convert MVA into terpenoids, it had to be absolutely dry. An attempt by these workers to assess the cofactor requirements of the cell-free system was unsuccessful since prolonged dialysis resulted in denaturation of the enzymes. No cofactor requirements for carotenogenesis with Aphanocapsa extracts have been reported.

In this chapter the results of investigations into the cofactor requirements of the carotenogenic systems from Phycomyces and Aphanocapsa 6714 are presented.

3:2 ADDITIONAL MATERIALS AND METHODS

3:2:1 REMOVAL OF ENDOGENOUS COFACTORS

A 2.5:1(v/w) extract prepared from lyophilised Phycomyces mycelia was prepared as described earlier

(Section 2:2:2:1). This was then passed through a column of Sephadex G25 superfine (1cm^{2}cross section area x 24 cm height) equilibrated with 400mM TrisHC1, pH 7.8 at a flow rate of approximately 300µl/min. The fraction eluting in the void volume was collected. These extracts were used in all experiments described in this chapter.

3:2:2 COFACTOR SOLUTIONS

In the final incubation mixture the concentrations of cofactors were 20mM GSH, 10mM ATP, 6mM $MnCl_2$, 4mM $MgSO_4$, and 1mM each of FAD^+, NAD^+ and $NADP^+$.

In experiments using high concentrations of ATP, the ATP stock solution was prepared in 400mM Tris adjusted and acidified to pH 7.8 with HC1.

3:2:3 ANAEROBIC INCUBATIONS

All anaerobic incubations were performed in Thunberg tubes, previously flushed with nitrogen and evacuated with a water pump until effervescence from the reaction medium had ceased.

3:3 RESULTS AND DISCUSSION

3:3:1 EXPERIMENTS WITH FILTERED EXTRACTS FROM PHYCOMYCES

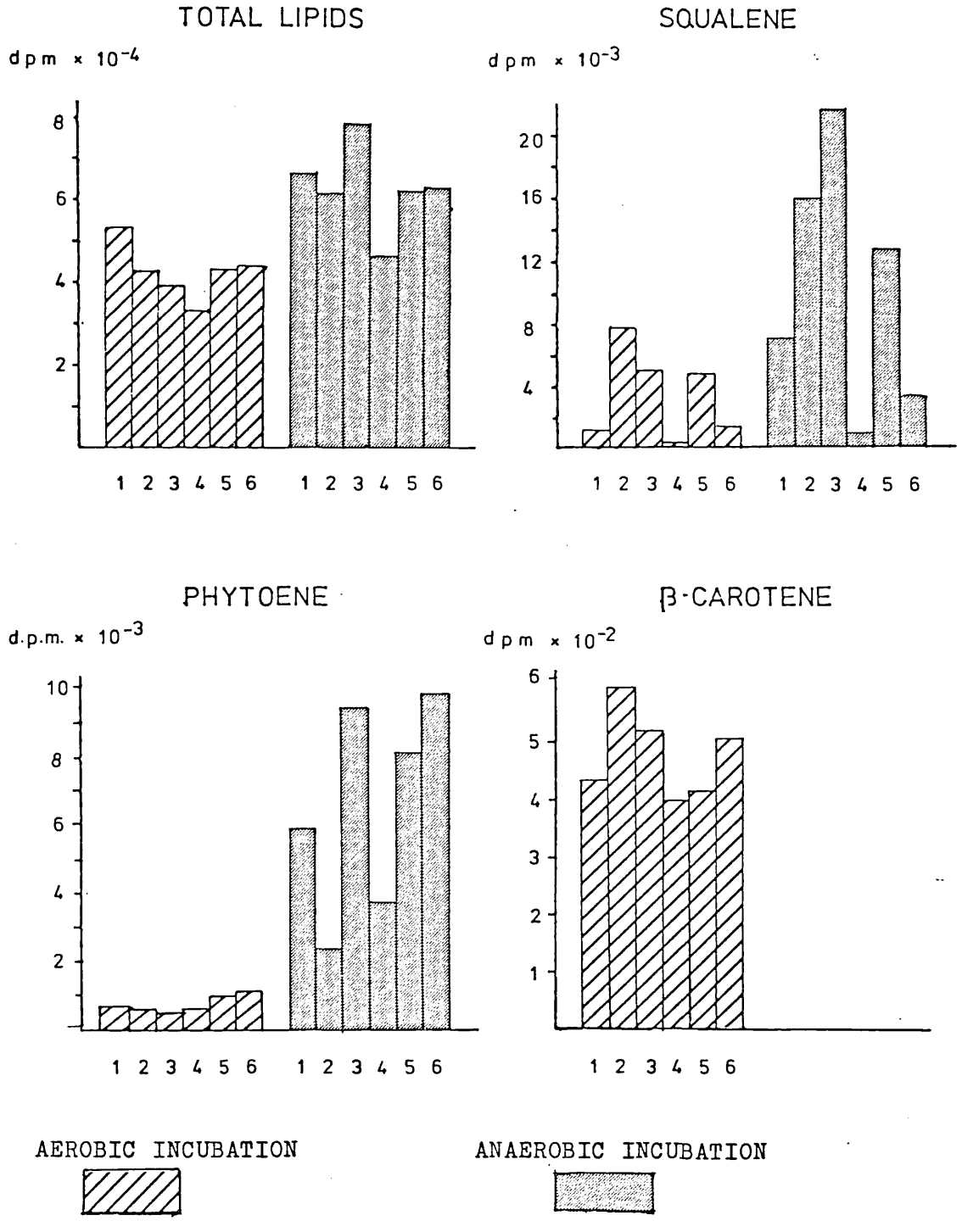
3:3:1:1 Preliminary Experiments. The results of experiments using filtered extracts incubated aerobically

and anaerobically in the presence of various cofactors (standard concentrations) with [2-^{14}C]MVA (Fig. 3:1) suggest that oxygen is necessary for phytoene desaturation, since significant incorporation into β-carotene was obtained only under aerobic conditions. Under anaerobic conditions, large incorporations into both squalene and phytoene were observed, suggesting that both of these compounds are normally metabolized through oxygen-dependent processes. It is also apparent that the filtered extract contains a NAD^+ phosphorylating enzyme, since incubations containing either nicotinamide cofactor had large incorporations of ^{14}C- label into squalene (Fig. 3:1). The effect of the nicotinamide cofactors on carotene biosynthesis was unclear from these experiments. The results from FAD^+-containing incubations suggest that this cofactor decreases incorporation into squalene and perhaps β-carotene, whilst increasing the incorporation into phytoene.

Terpenoid biosynthesis from [^{14}C]IPP was stimulated by ATP (Table 3:1). The results show that not only was the incorporation into total terpenoids higher (by x 1.9) in the presence of ATP, but also the incorporation into squalene, phytoene and β-carotene expressed as a percentage of total terpenoid incorporation. The effect of ATP is discussed further in Section 3:3:1:4.

3:3:1:2 Oxygen. The results presented in Table 3:2 (and Fig. 3:1) suggest that phytoene desaturation into β-carotene is oxygen dependent. In the absence of oxygen, the

FIG. 3:1 INCORPORATION OF [^{14}C]MVA INTO VARIOUS TERPENOIDS BY FILTERED EXTRACTS OF PHYCOMYCES



Filtered extracts incubated (2h, 20°) with 0.5μCi DL [2-^{14}C]MVA, standard concentrations (see 2:2:3) of GSH. ATP, Mg^{2+}, Mn^{2+} plus 1μM concentrations of 1)- , 2)$NADP^+$, 3)NAD^+, 4)FAD^+, 5)FAD^+ and $NADP^+$, 6)FAD^+ and $NADP^+$.

TABLE 3:1 THE EFFECT OF ATP ON TERPENOID BIOSYNTHESIS IN FILTERED EXTRACTS OF PHYCOMYCES

INCORPORATION FROM [^{14}C]IPP INTO	+ATP (10mM)	-ATP
Total Terpenoid Fraction	746,605 (100%)*	395,216 (100%)*
Squalene	162,422 (21.8%)*	31,442 (8.45%)*
Phytoene	39,961 (5.36%)*	11,104 (1.75%)*
β-Carotene	17,328 (2.35%)*	6,915 (1.75%)*

*Figures in brackets represent radioactivity as a percentage of the incorporation into the total terpenoid fraction.

Reaction mixtures contained standard concentrations of $MgSO_4$ $MnCl_2$, GSH and NAD^+ and were incubated for 2h, aerobically with 0.5μCi [1-^{14}C]IPP.

TABLE 3:2 THE EFFECT OF OXYGEN ON TERPENOID BIOSYNTHESIS IN FILTERED EXTRACTS OF PHYCOMYCES

INCORPORATION FROM [^{14}C]MVA INTO	$+O_2$	$-O_2$
Total Terpenoid Fraction	331,822 (100%)*	281,177 (100%)*
Squalene	107,563 (32.4%)*	101,349 (36.0%)*
Phytoene	26,651 (8.03%)*	62,990 (22.4%)*
β-Carotene	8,006 (2.41%)*	198 (0.07%)*

*Figures in brackets represent radioactivity as a percentage of the incorporation into the total terpenoid fraction.

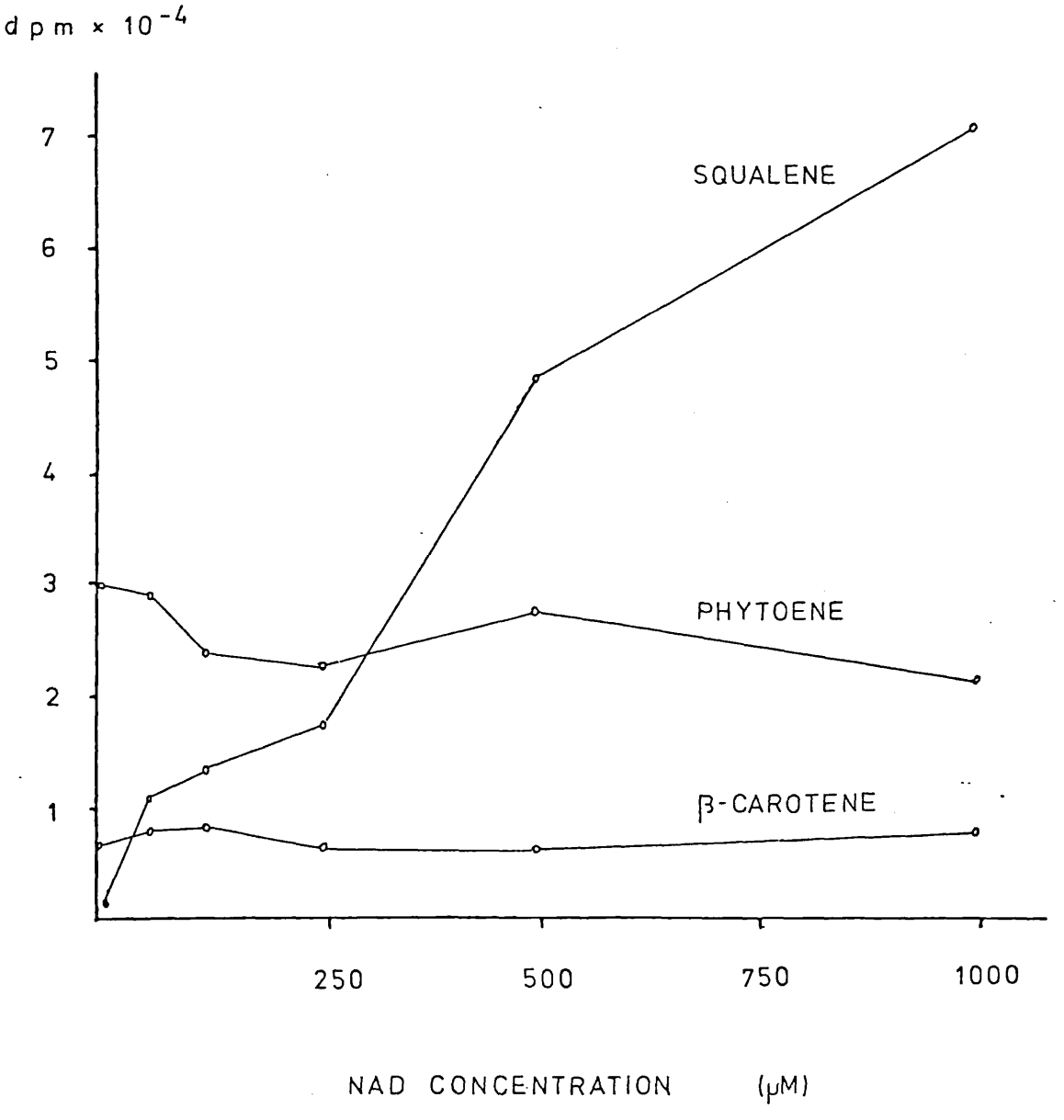
Standard reaction mixtures (see 2:2:3) incubated for 2h with 0.5μCi DL-[2-^{14}C]MVA

incorporation of label into β-carotene was found to be insignificant, whilst the incorporation into phytoene was massive. The high incorporation into phytoene cannot be explained by removal of end product regulation, since the mutant strain of Phycomyces used (C115, a carS mutant) lacks this regulatory mechanism. Such over-accumulation of phytoene was also observed when extracts of Phycomyces were incubated in the presence of inhibitors of phytoene desaturation (See Chapters 4, 5 and 6).

3:3:1:3 Nicotinamide Cofactors. The results of incubation of filtered cell-free extracts of Phycomyces with [2-^{14}C]MVA and a range of NAD^+ and $NADP^+$ concentrations (Figs. 3.2 and 3:3) show that only the synthesis of squalene is affected significantly by the nicotinamide cofactor concentration where $NADP^+$ appears to be the more effective cofactor. At low nicotinamide concentrations a small increase in the incorporation of ^{14}C- label into phytoene and β-carotene was detected. This was most likely due to the loss of squalene synthetase activity, thus making more isoprenyl pyrophosphates available to phytoene synthetase. It is therefore concluded that neither nicotinamide cofactor is essential for carotene biosynthesis in Phycomyces, or, that the cofactor is tightly enzyme-bound such that it cannot be removed by gel-filtration.

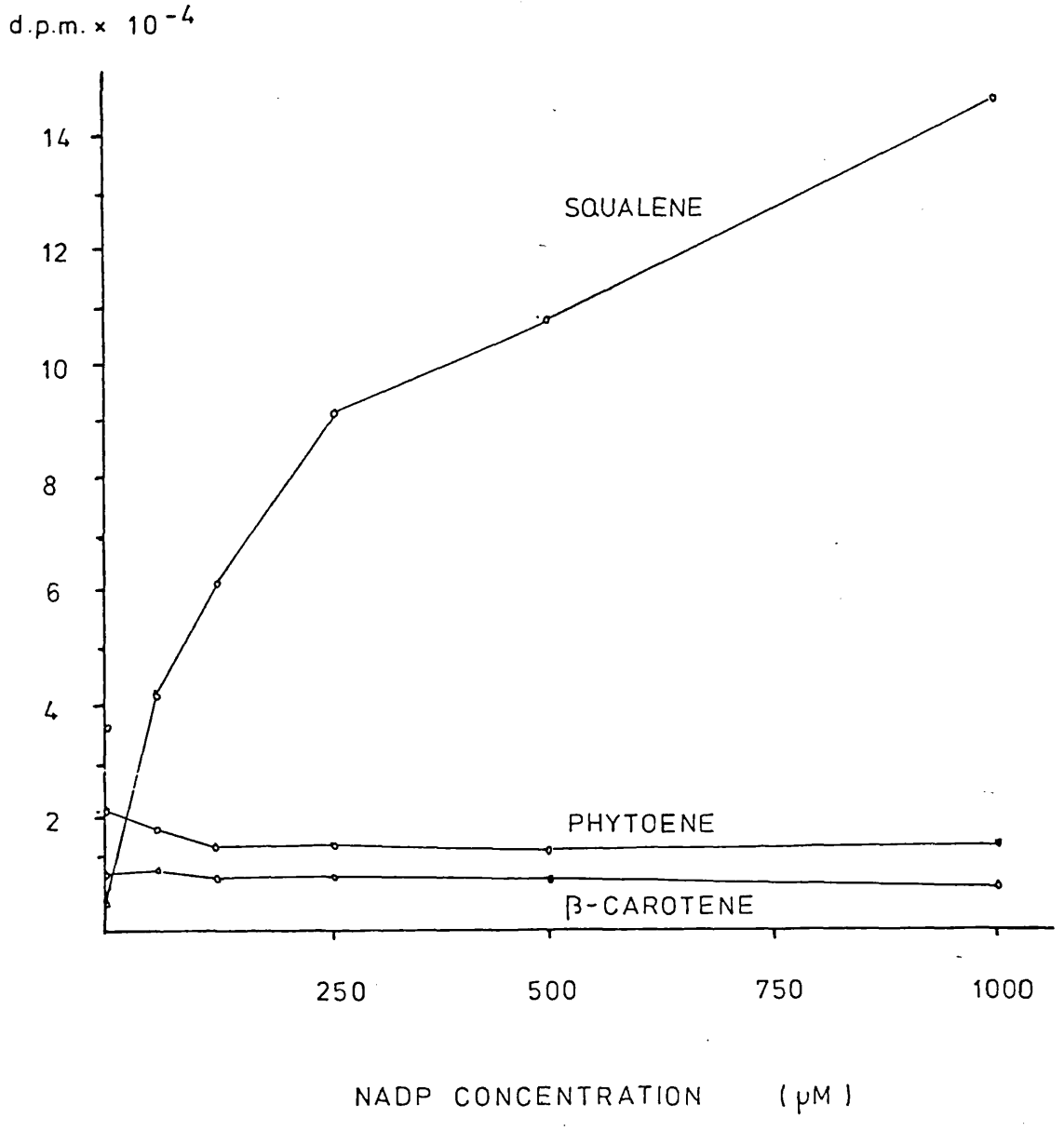
3:3:1:4 Adenosine Cofactors. The incorporation of [1-^{14}C]IPP into various terpenoids with respect to ATP concentration, suggests that the entire terpenoid pathway is stimulated by this cofactor up to a 20mM concentration

FIG. 3:2 THE EFFECT OF NAD^+ CONCENTRATION ON INCORPORATION OF [^{14}C]MVA INTO TERPENOIDS BY FILTERED PHYCOMYCES EXTRACTS



Filtered extracts were incubated aerobically (2h, 20°C) in the presence of 0.5μCi DL-[2-^{14}C]MVA and standard concentrations (see 2:2:3) of $MgSO_4$, $MnCl_2$, GSH and ATP.

FIG. 3:3 THE EFFECT OF $NADP^+$ CONCENTRATION ON INCORPORATION OF [^{14}C]MVA INTO TERPENOIDS BY FILTERED PHYCOMYCES EXTRACTS

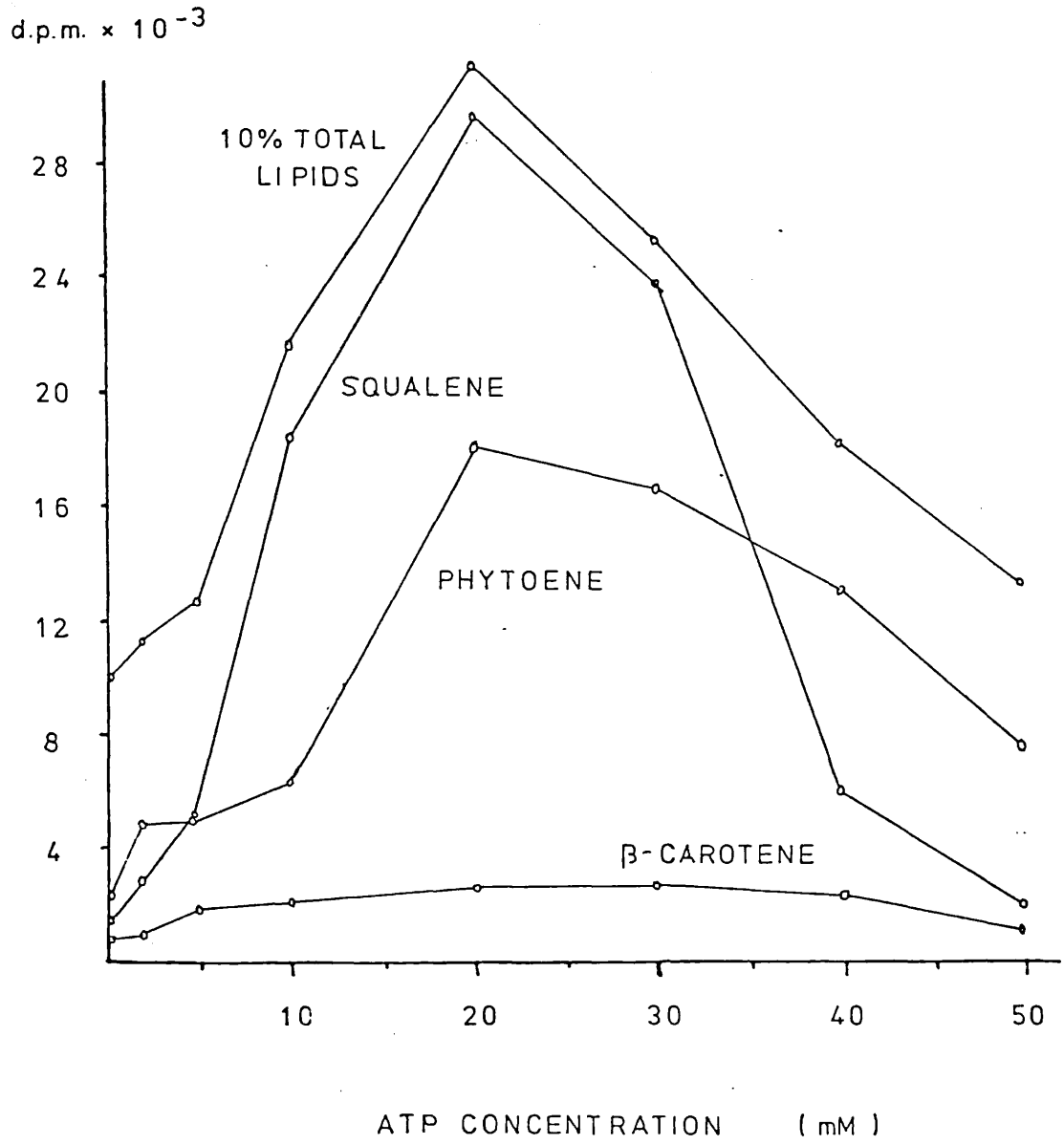


Filtered extracts were incubated aerobically (2h, 20°C) in the presence of 0.5μCi DL-[2-^{14}C]MVA and standard concentrations (see 2:2:3) of $MgSO_4$, $MnCl_2$, GSH and ATP.

(Fig. 3.4). The stimulation by ATP of a partially purified phytoene synthetase preparation has been reported (Maudinas et al., 1977; Section 1:3:5) and the modest increase in β-carotene incorporation, observed here with increasing ATP concentration is probably due to increased phytoene synthetase activity. The sharp rise of incorporation into squalene, with rising ATP concentration, may be explained in part by the phosphorylation of NAD^+ in the reaction medium into $NADP^+$, an essential cofactor for squalene synthetase. However, the close similarity of the relationships between ATP concentration and incorporation into squalene, phytoene and "total" terpenoids, suggests that this cofactor may affect the common isoprenoid pyrophosphate pathway.

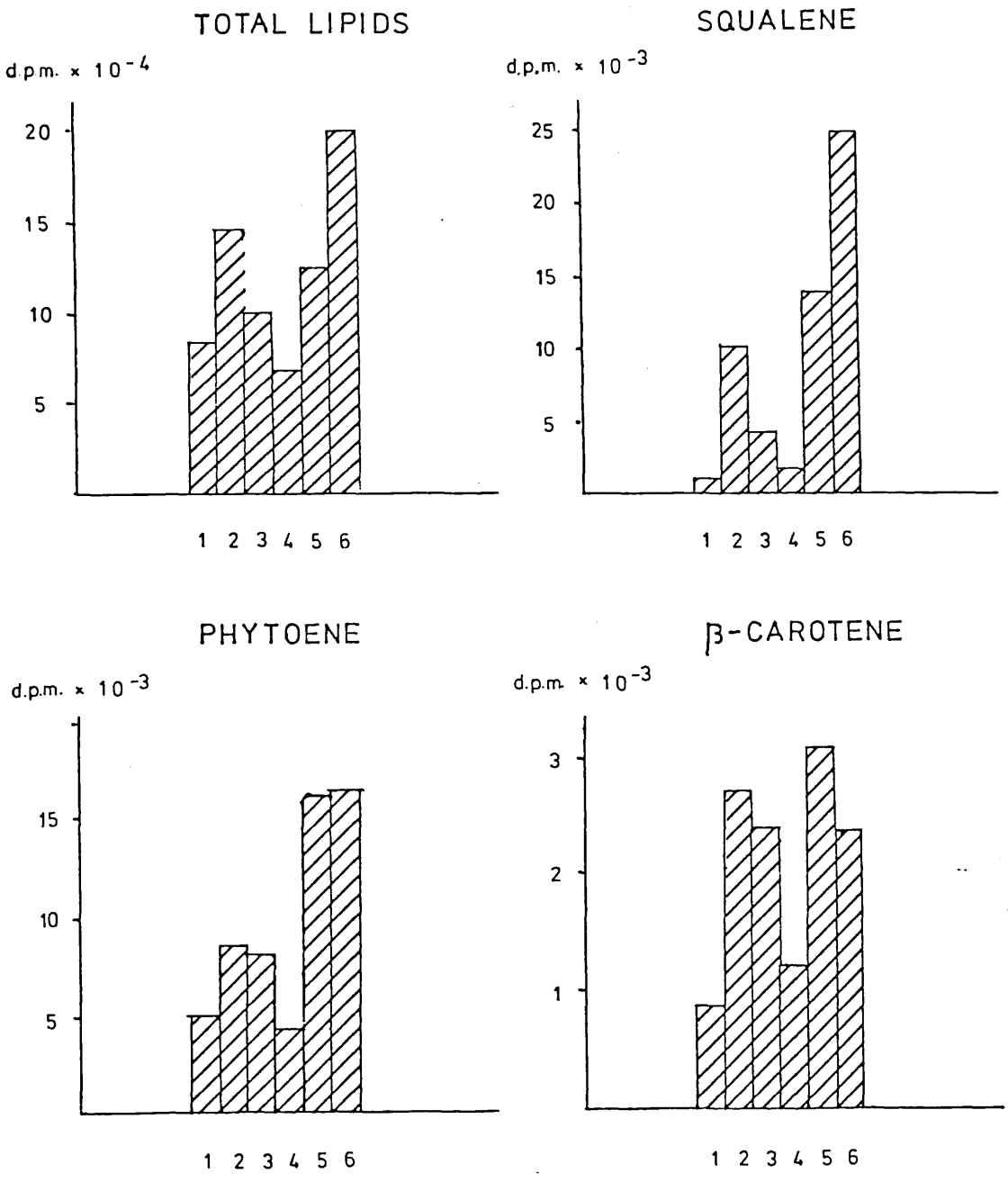
It was supposed that the apparent stimulation of terpenoid biosynthesis by ATP may have been caused through the protection of isoprenoid pyrophosphates from an alkaline phosphatase present in the cell extract. This hypothesis was investigated by comparing the effect on incorporation of [^{14}C]IPP into various terpenoids by filtered extracts in the presence of either AMP, ADP, ATP, ATP with KF and ATP without $MgSO_4$, against a control containing standard concentration of $MgSO_4$, $MnCl_2$, GSH and NAD^+. The results (Fig. 3:5) suggest that the degree of stimulation by the adenosine cofactor is related to the number of its phosphate groups, such that control values are similar to those with 10mM AMP. The addition of 10mM KF to ATP-containing media did not significantly affect incorporation, although the omission of Mg^{2+} generally increased incorporation,

FIG. 3:4 THE EFFECT OF ATP CONCENTRATION ON INCORPORATION OF [^{14}C]MVA INTO TERPENOIDS BY FILTERED PHYCOMYCES EXTRACTS



Filtered extracts were incubated aerobically (2h, 20°C) with 0.5μCi [1-^{14}C]IPP and standard concentrations (see 2:2:3) of $MgSO_4$, $MnCl_2$, GSH and NAD^+.

FIG. 3:5 INCORPORATION OF [^{14}C] IPP INTO TERPENOIDS IN THE PRESENCE OF VARIOUS ADENOSINE PHOSPHATES BY FILTERED EXTRACTS OF PHYCOMYCES



Filtered extracts incubated aerobically (2h, 20°) with 0.5µCi [1-^{14}C]IPP and standard concentrations (see 2:2:3) of $MnCl_2$, $MgSO_4$, GSH, NAD^+ plus 1)-, 2)ATP (10mM), 3)ADP (10mM), 4)AMP (10mM), 5)KF and ATP (10mM each, 6)ATP (10mM) without $MgSO_4$.

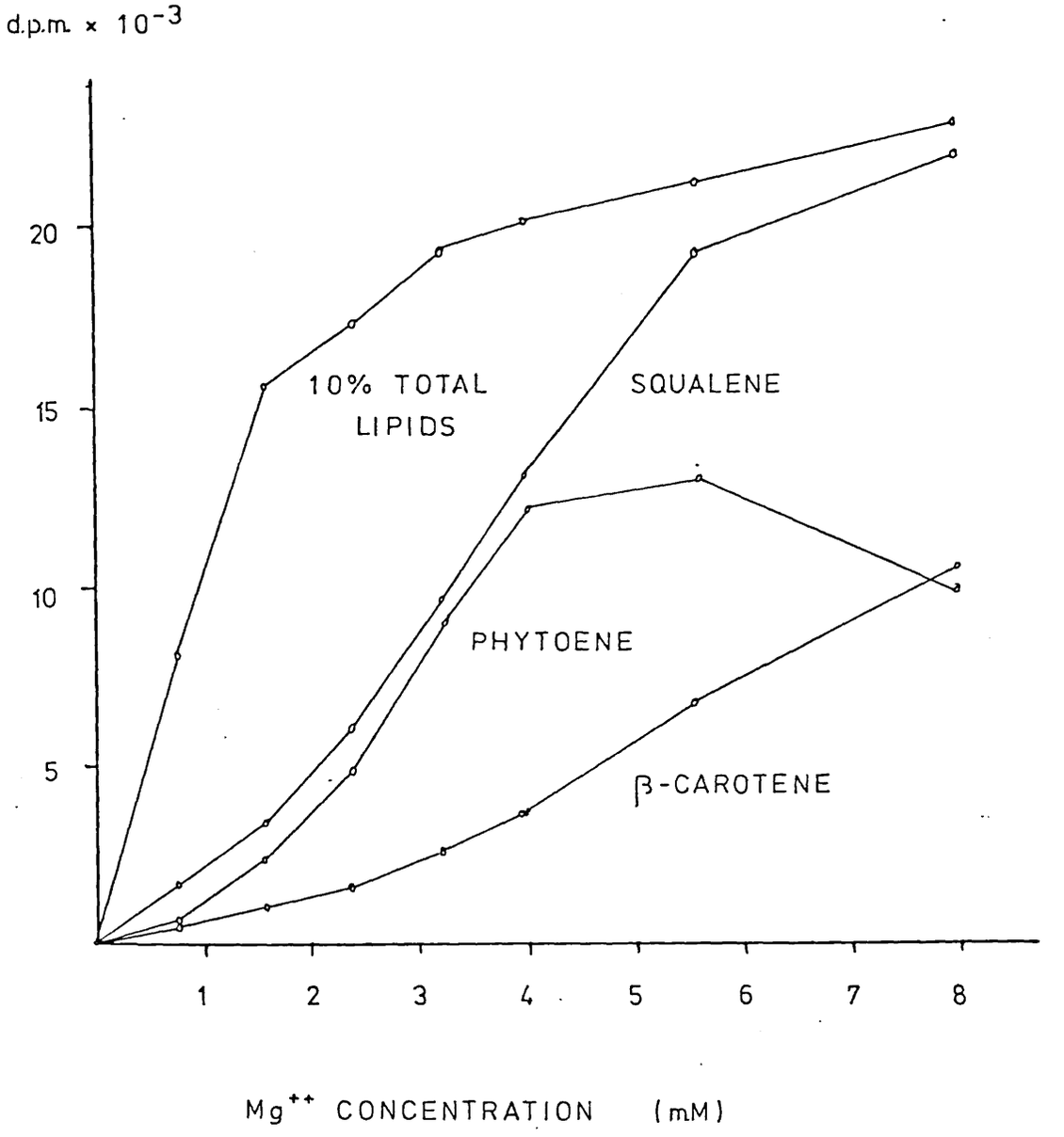
possibly through prevention of ATP metabolism. As 10mM ATP is below the concentration that caused maximum terpenoid incorporation, it was expected that addition of 10mM KF would have resulted in increased incorporation if the extract contained a fluoride-sensitive phosphatase.

It is consequently suggested that ATP stimulates both phytoene and squalene synthetases and/or the isoprenyl transferases in P.blakesleeanus

3:3:1:5 Divalent Ions. Terpenoid biosynthesis in filtered extracts was found to be dependent upon divalent ions (Fig. 3:6). This requirement, although absolute, is not specific for carotenoids since incorporation into various terpenoids was found when filtered extracts were incubated with either Mn^{2+} (Fig. 3:7) or Mg^{2+} (Fig. 3:8).

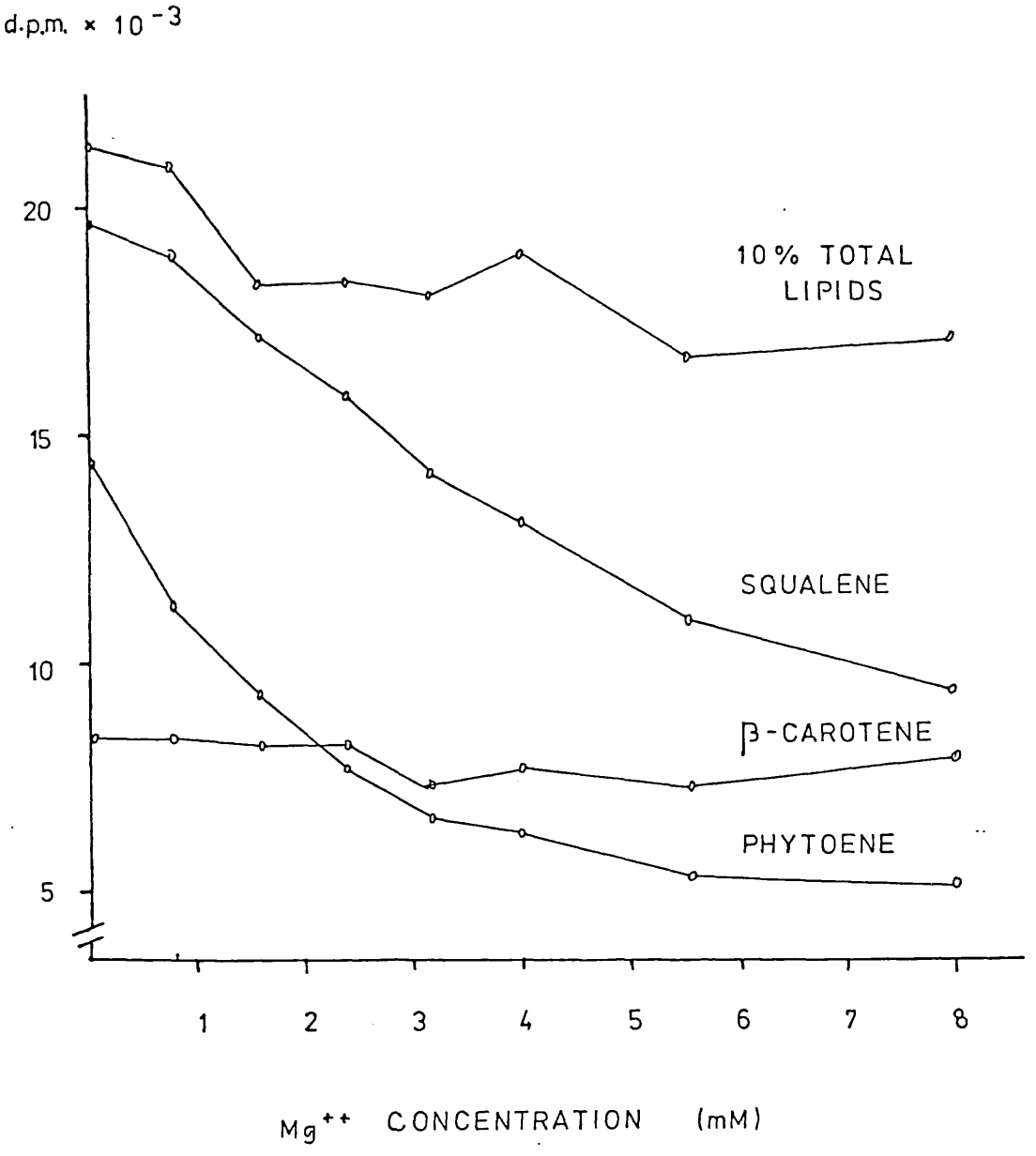
In the presence of $MnCl_2$ (6mM), increasing concentrations of $MgSO_4$ resulted in decreasing incorporations from [^{14}C]IPP into squalene and phytoene (Fig. 3:8), whilst in the absence of $MnCl_2$ the reverse was observed (Fig. 3:7). In the presence of $MgSO_4$ (4mM), increasing concentrations of $MnCl_2$ up to 3.6mM caused increasing incorporations from [2-^{14}C]MVA into phytoene and squalene (Fig. 3:8); higher concentrations, however, were inhibitory. The apparent inhibition of terpenoid biosynthesis by Mg^{2+} in the presence of Mn^{2+} has been discussed above with respect to ATP stimulation (Section 3:3:1:4). The IPP=phytoene synthetase complex, isolated from red tomato fruit plastids, has a strict requirement for

FIG. 3:6 THE EFFECT OF MAGNESIUM ION CONCENTRATION ON [^{14}C]IPP INCORPORATION, IN THE ABSENCE OF MANGANESE BY FILTERED EXTRACTS OF PHYCOMYCES



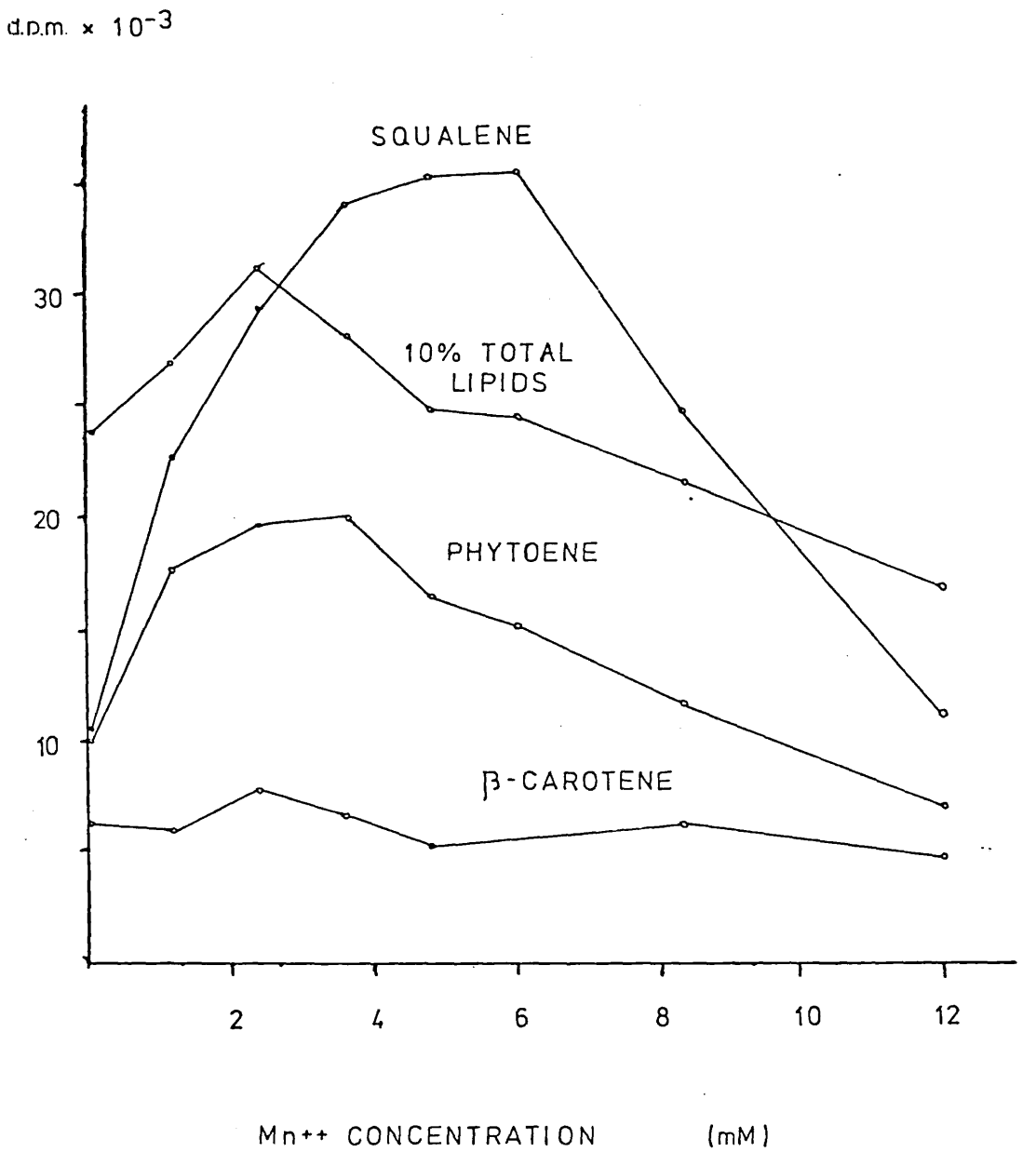
Each incubation contains 0.5μCi [^{14}C]IPP, standard concentrations (see 2:2:3) of GSH, ATP and NAD^{+}; no $MnCl_2$.

FIG. 3:7 THE EFFECT OF MAGNESIUM ION CONCENTRATION ON [^{14}C]IPP INCORPORATION, IN THE PRESENCE OF MANGANESE, BY FILTERED EXTRACTS OF PHYCOMYCES



Filtered extracts incubated (2h, 20°C) with 0.5μCi; [^{14}C]IPP and standard concentrations (see 2:2:3) of $MnCl_2$, GSH, ATP and NAD+.

FIG. 3:8 THE EFFECT OF MANGANESE CONCENTRATION ON [^{14}C]MVA INCORPORATION INTO TERPENOIDS BY FILTERED PHYCOMYCES EXTRACTS



Filtered extracts incubated with 0.5μCi D1 - [^{14}C]MVA and standard concentrations of GSH, ATP, NAD^+ and $MgSO_4$.

Mn^{2+} (Maudinas *et al.*, 1977) whilst a phytoene synthesising system from the fungus *N.crassa* was reported to require Mg^{2+} which could be replaced, at low concentrations, by Mn^{2+} (Spurgeon, *et al.*, 1979). From the results presented here, it is possible to suggest that Mn^{2+} is the preferred divalent ion where Mg^{2+} is less efficient but competes with manganese *in vitro* as the prosthetic group on an enzyme.

The increasing incorporations with high Mg^{2+} concentrations (Fig. 3:8) may be partly due to the "enzymic and non-enzymic stimulation" of mevalonate decarboxylation (Yokoyama *et al.*,1962), in addition to the stimulation of the post-IPP enzymes suggested above.

3:3:2 EXPERIMENTS WITH A WASHED MEMBRANE PREPARATION FROM *APHANOCAPSA* 6714

A membrane suspension prepared from *Aphanocapsa* 6714 spheroplasts was found to incorporate radioactivity from [2-^{14}C]GGPP into various carotenoids. This system was optimised with respect to nucleotide cofactor requirements and incubation conditions. Removal of endogenous cofactors by gel filtration was not possible with such a membranous system, so that cofactors were removed by washing in the buffer used in the preparation procedure (see Section 2:2:2:2).

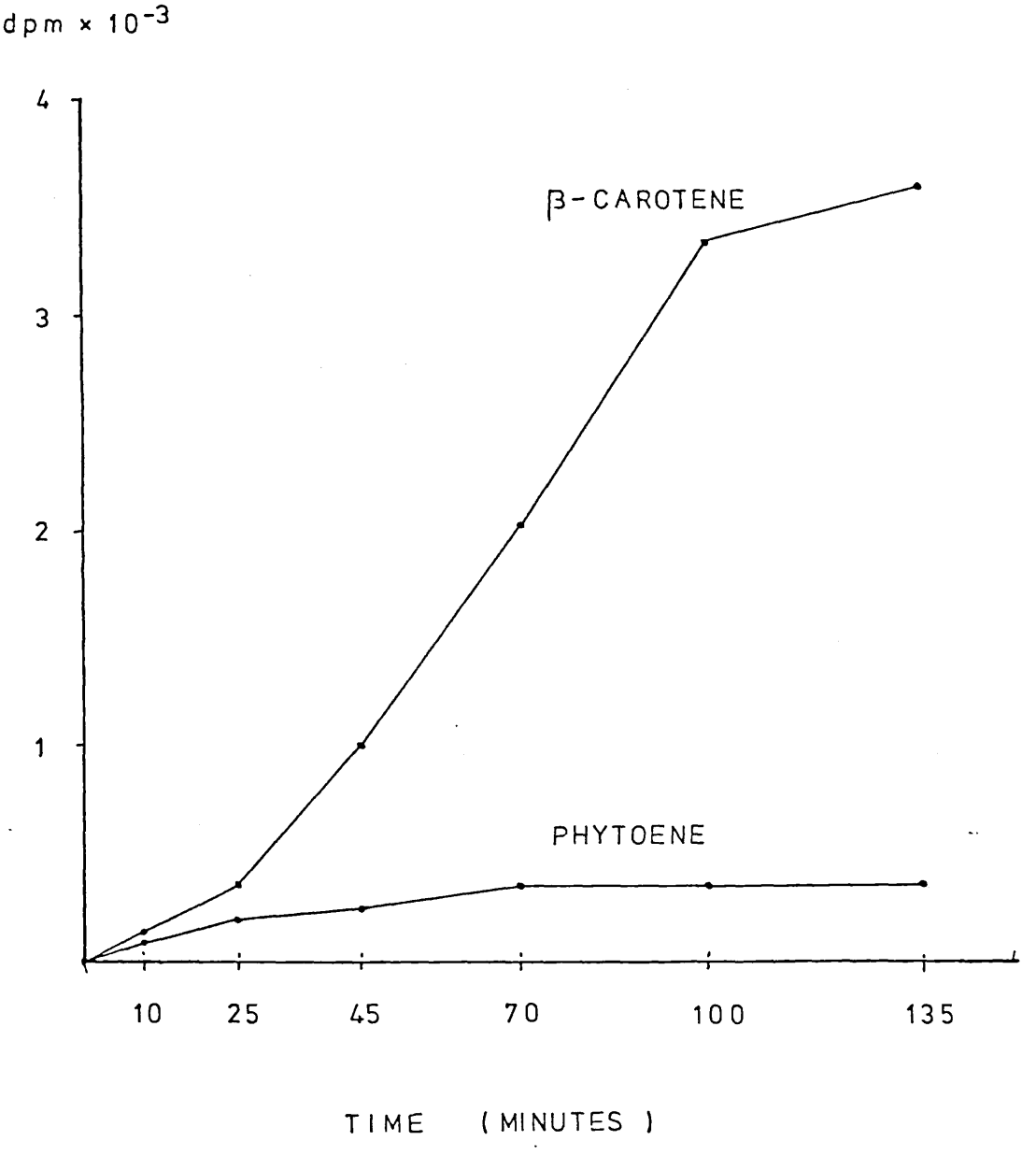
3:3:2:1 Time Course of Incorporation of [^{14}C]GGPP. A stock reaction medium (3.5ml) containing washed *Aphanocapsa* membranes, incubated in the presence of 3.5μCi[^{14}C]GGPP and

standard concentrations (see Section 3:2:2) of $MgSO_4$, $MnCl_2$, GSH, ATP, NAD^+, $NADP^+$ and FAD^+ was sampled (0.5ml aliquots) at various times and analysed with respect to incorporation of ^{14}C into phytoene and β-carotene.

The time course (Fig.3:9) shows that phytoene and β-carotene are synthesised after only 10 min. Incorporation into β-carotene reached a linear rate before 45 min. and this continued until over 100 min. after the start of the reaction. Incorporation into phytoene increased only slightly during the 135 min. incubation period and did not exhibit an early peak of incorporation, coinciding with the start of incorporation into β-carotene, as has been reported for the Narcissus chromoplast system (Beyer et al., 1980). The time course for the Aphanocapsa system suggests that phytoene is promptly desaturated after its formation, i.e. phytoene synthesis is rate limiting in vitro and possibly in vivo, since this carotene is not present in cells grown under normal conditions.

3:3:2:2 Oxygen and Light. Experiments designed to test the effect of light and oxygen on this system revealed that carotene biosynthesis was disrupted only when incubations were both anaerobic and in the dark (Table 3:3). Further investigations demonstrated that the membrane preparation was photosynthetically active, producing 80-100µg O_2/h/mg chlorophyll in the light (Dr. G. Sandmann, personal communication). The results (Table 3:3) suggest that phytoene desaturation in this system as in the Phycomyces system (Section 3:3:1:2) is dependent upon oxygen. Anoxia

FIG. 3:9 TIMECOURSE OF PHYTOENE AND β-CAROTENE FORMATION FROM [^{14}C]GGPP BY AN APHANOCAPSA MEMBRANE PREPARATION



Standard reaction mixtures (see 2:2:3) plus $NADP^+$ (1μm) and FAD^+ (1μm) incubated with 0.5μCi [^{14}C]GGPP in light. Each mixture contained 70μg chlorophyll per 200μl aliquot of membrane preparation.

TABLE 3:3 THE EFFECT OF LIGHT AND OXYGEN ON (*IN VITRO*) CAROTENOGENESIS BY A MEMBRANE PREPARATION FROM *APHANOCAPSA* 6714

INCUBATIONS CONDITIONS	RADIOACTIVITY (dpm) INTO PHYTOENE	INCORPORATED β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE
Light, $+O_2$	284	996	0.28
Light, anaerobic	273	962	0.28
Dark, $+O_2$	239	1,164	0.21
Dark, anaerobic	1,528	119	12.8

Reaction media contained *Aphanocapsa* washed membrane preparation (100µg chl.) incubated with 0.5µCi [^{14}C]GGPP and standard concentrations (see 3:2:2) of $MgSO_4$, $MnCl_2$, GSH, ATP, NAD^+, $NADP^+$ and FAD^+.

results in an accumulation of ^{14}C label into phytoene, at the expense of β-carotene.

3:3:2:3 Nucleotide Cofactors. Results of experiments with washed membrane preparations incubated in the presence of various nucleotide combinations (Table 3:4) suggest that the cofactor requirement of this system is similar to that of Phycomyces.

In the absence of ATP, incorporation from [^{14}C]GGPP into β-carotene was 43% of the control, whilst incorporation into phytoene was only 22%. These results suggest that ATP stimulates phytoene synthesis rather than the syntheses of coloured carotenoids, perhaps by direct stimulation of the phytoene synthetase as reported by Maudinas et al., (1977), or through protection of GGPP and PPPP from phosphatase action as discussed in Section 3:3:1:4.

Whilst the independent omission of either FAD^+ or the nicotinamide cofactors from reaction media had no adverse effects, the absence of both FAD^+ and nicotinamide cofactors resulted in lower carotenoid incorporation (60-70% of control) without significantly affecting the phytoene = β-carotene incorporation ratio. Washed membrane preparations incubated in the absence of nicotinamide cofactors, FAD^+ and ATP incorporated very little label from [2-^{14}C]GGPP into phytoene or β-carotene (6.5% of control). The role of nicotinamide cofactors and FAD in this system is not clear, but the results suggest that these also stimulate phytoene synthesis. A partially purified IPP=phytoene synthetase

TABLE 3:4 THE EFFECT OF VARIOUS NUCLEOTIDE COFACTORS ON CAROTENOGENESIS BY AN *APHANOCAPSA* MEMBRANE PREPARATION.

	RADIOACTIVITY INCORPORATED INTO		INCORPORATION RATIO PHYTOENE= β-CAROTENE
	PHYTOENE	β-CAROTENE	
Control 1	935	3977	0.23
Without Nicotinamide Cofactors	772	4244	0.18
Without FAD^+	1097	5174	0.21
Control 2	2067	8931	0.23
Without ATP	452	3883	0.12
Without Nicotinamide Cofactors and FAD^+	1263	6242	0.20
Without Nicotinamide Cofactors, FAD^+ and ATP	<100	576	-

All mixtures were incubated aerobically, in light with 0.5μCi [2-^{14}C]GGPP. Controls contained washed membrane preparations from *Aphanocapsa* 6714 (100μg chlorophyll per aliquot) and standard concentrations of $MgSO_4$, $MnCl_2$, GSH, ATP, NAD^+, $NADP^+$ and FAD^+ (see 3:2:2).

complex (200,000 dalton) isolated from tomato fruit plastids, is also stimulated by $NADP^+$ (Maudinas et al., 1977).

3:3:4: CONCLUSIONS

The two carotenogenic systems investigated were found to have similar cofactor requirements. In both cases phytoene synthesis was stimulated by ATP, and phytoene desaturation was oxygen dependent. These systems differ from those described by Porter and co-workers (Section 1:3:5:1 for discussion) where $NADP^+$ and FAD^+ were found to be necessary for both carotene desaturation and for cyclisation. This may reflect differences in the preparation of cell extracts as both systems investigated here are crude and may still contain significant amounts of tightly bound cofactors. The impurity of these extracts also complicates any interpretations of the data, since it is impossible to ascertain whether or not a cofactor directly or remotely (i.e. through other processes) influences carotene/terpenoid biosynthesis. Thus the true cofactor requirements for these carotenogenic systems cannot be unambiguously ascertained until the enzymes have been isolated and purified.

As a result of these experiments, however, a standard incubation mixture containing 6mM $MnCl_2$, 4mM $MgSO_4$, 20mM GSH, 10mM ATP and 1mM NAD^+ was adopted for use with both systems (Chapters 4 - 6). Reaction mixtures were routinely incubated for 2h @ 20°C aerobically in the light .

CHAPTER 4

INHIBITION OF CAROTENOGENESIS BY SUBSTITUTED PHENYLPYRIDAZINONES AND PHENYLFURANES

4:1 INTRODUCTION

Certain substituted phenylpyridazinones such as norflurazon (see Fig. 1:4:1 for structure, Table 1:4 for nomenclature) and substituted phenylfuranes, e.g. difunone (see Fig. 1:4:2 and Table 1:4) have been reported to inhibit phytoene desaturation (see 1:4:1 and 1:4:2). The relationship between structure and inhibitor (bleaching) activity has been studied with both these types of herbicide on cultures of the green alga Scenedesmus acutus.

It was concluded that for substituted phenylpyridazinones bleaching activity improved with:-

i) substitutions on the pyridazinone ring that encouraged electron withdrawal at position 4 and electron insertion at position 5 (Sandmann et al., 1981);

ii) substitutions in the phenyl ring at position 3 that were both lipophilic and encouraged electron withdrawal, the latter parameter being more important (Sandmann and Böger, 1982).

Similar studies with various substituted phenylfuranes revealed that the 2-oxo, 3-carbonitrile, 4-phenyl and 5-dimethylamino-methylene moieties were all essential for bleaching activity, although the 5-dimethylamino-methylene group could be replaced by 5-cyclobutylamino-methylene , but with reduced activity.
(B. Boehler-Kohler and P. Böger, unpublished results). Substitutions into the 4-phenyl ring were reported by these

workers to either increase or decrease phenylfurane bleaching activity according to the nature of the substitutent.

In this chapter details are presented of experiments using the Aphanocapsa system to investigate the effect on bleaching activity of various substitutions into the phenyl rings of both phenylpyridazinone and phenylfurane herbicides. The activities of these derivatives in the Aphanocapsa system are compared to those in Scenedesmus cultures and the effect of substitution on bleaching activity discussed in relation to the properties of the substituent.

4:1:2 INCORPORATION RATIOS

The herbicides tested here (and those described in chapters 5 and 6) all inhibit phytoene desaturation, thus reducing incorporation of ^{14}C- label into β-carotene whilst increasing incorporation into phytoene. To take account of both of these effects, it is convenient to calculate the ratio of the label incorporated into phytoene to that incorporated into β-carotene as a parameter for the degree of inhibition of phytoene desaturation. Due to the variation between different cell extracts in absolute carotenogenic activities and sensitivity to inhibitors, it was not possible to quantitatively compare data from different experiments. However, the phytoene: β-carotene

incorporation ratio for a particular herbicide relative to the control incorporation ratio was similar for different preparations. Thus relative incorporation ratios provide a valid index to the bleaching activity of a particular herbicide at a defined concentration.

4:1:3 SUBSTITUENT CONSTANTS

Since a number of bleaching herbicides differing only in phenyl ring substitution were compared with respect to inhibtor activity, it is appropriate to relate a change in inhibitor activity to a physical property of the substituent. The physical properties examined were substituent lipophilicity, bulk and the effects of the substituent on the electronic nature of the aromatic ring. Parameters for these properties were taken from 'Aromatic Substituent Constants for Structure-Activity Correlation' (Hansch *et al.*, 1973) and include the substituent lipophilicity factor 'π', molecular weight and molar refraction as parameters of substituent bulk and the Hammett parameters σ_m (substituent in *meta*-position) and σ_p (substituent in *para*- or *ortho*- positions) as parameters for substituent electron withdrawing capacity. The relevant physical data for the substituents discussed in this chapter are given in Table 4:7.

4:2 REACTION MIXTURES AND SOLVENTS

4:2:1 REACTION MIXTURES

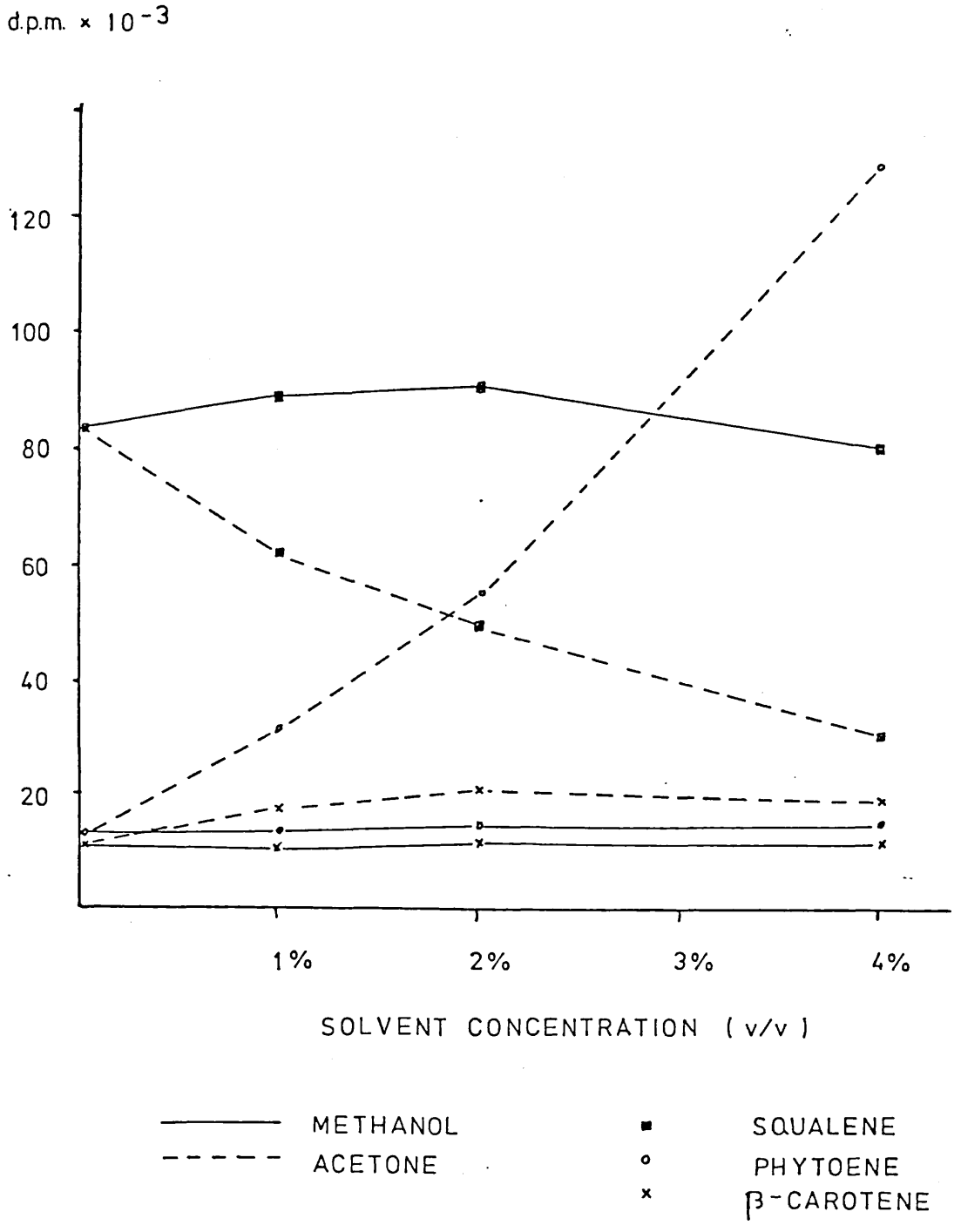
Cell-free preparations were incubated aerobically (2h, 20°C) in the standard reaction mixture (see 2:2:3) with either 0.5µCi DL-[2-^{14}C]MVA (for experiments with *P.blakesleeanus*) or 0.5µCi [2-^{14}C]GGPP (specific activity 4.22mCi/mmol, for experiments with *Aphanocapsa*) as substrate.

Phenylfuranes were dissolved in acetone whilst phenylpyridazinones were dissolved in methanol (stock solutions 0.02mM herbicide) or N,N'-dimethylformamide (stock solutions 0.2mM). The herbicides were added to reaction mixtures dissolved in solvent as 5µl aliquots prior to addition of cell-free material.

4:2:2 SOLVENT CONCENTRATION AND CAROTENOGENESIS

The effect of various concentrations of the above solvents on *in vitro* terpenoid biosynthesis was investigated with *Phycomyces*. Results show that whilst methanol had no effect on terpenoid biosynthesis (Fig. 4:1), increasing concentrations of acetone appeared to stimulate phytoene biosynthesis and to inhibit the synthesis of squalene. The possible effect of acetone is to stimulate phytoene biosynthesis such that phytoene synthetase competes with squalene synthetase for isoprenoid pyrophosphate substrates.

FIG. 4:1 THE EFFECT OF SOLVENT CONCENTRATION ON [^{14}C]MVA INCORPORATION INTO TERPENOIDS BY PHYCOMYCES EXTRACTS



Standard reaction mixture (see 2:2:3) incubated for 2 hours at, 20° C with 0.5μCi DL-[2 ^{14}C]MVA.

A small increase in β-carotene synthesis was also noted in the presence of acetone.

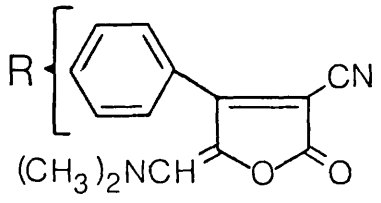
For concentrated solutions of substituted phenyl pyridazinones, N,N'-dimethyl formamide was used as solvent; this solvent was found to have no effect on terpenoid biosynthesis in the Phycomyces system at 1% (v/v) concentration.

4:3 RESULTS AND DISCUSSION

4:3:1 BLEACHING ACTIVITY OF SUBSTITUTED PHENYLFURANES IN THE APHANOCAPSA CELL-FREE SYSTEM

The relative inhibitory activities of substituted phenylfuranes with the Aphanocapsa system compare well with those found for cultures of Scenedesmus acutus (Boehler-Kohler and Böger, unpublished results). In both systems the unsubstituted phenyl derivative (difunone) was found to have moderate inhibitor activity (Tables 4:1 - 4:3) which was reduced on replacing the 5-dimethylamino group with a cyclobutylamino function (Tables 4:2 - 4:3). Reduction of the difunone 4-phenyl moiety to 4-cyclohexyl resulted in loss of inhibitory activity (Tables 4:2, 4:3). Certain substitutions that improved inhibition by the phenylfurane, particularly the halogenated (3,4-Cl_2 and 3-CF_3) derivatives, were found to be more effective in Scenedesmus cultures than in the Aphanocapsa system. This may be due to a difference in herbicide permeability across the cell wall of Scenedesmus.

TABLE 4:1 THE EFFECT OF VARIOUS DIFUNONE DERIVATIVES(0.1μM) ON CAROTENOGENESIS BY AN APHANOCAPSA MEMBRANE PREPARATION

DIFUNONE DERIVATIVE	D.P.M. INCORPORATED INTO PHYTOENE	D.P.M. INCORPORATED INTO β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
				
R=				
3- SCH_3	10,235	652	16	*(110)
3- C_6H_5	7,785	1,297	6.0	(41)
3,4- Cl_2	6,610	1,175	5.6	(39)
3- OCH_3	5,734	1,439	4.0	(27)
3-CF_3	5,534	1,905	2.9	(20)
Unsubstituted (Difunone)	2,411	2,260	1.1	(7.3)
Control	372.5	2,568	0.14	(1.0)

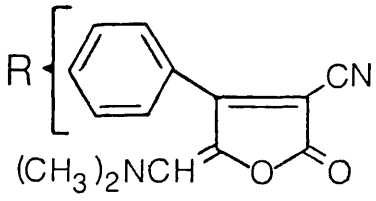
Aphanocapsa membrane preparation incubated in standard reaction media (see 2:2:3)

0.1μM herbicide, 0.5μCi [^{14}C]GGPP for 2h 20^{o}C.

Chlorophyll content = 52μg per 200μl aliquot.

*Figures in brackets represent the incorporation ratio relative to the Control.

TABLE 4:2 THE EFFECT OF VARIOUS DIFUNONE DERIVATIVES (1μM) ON CAROTENOGENESIS BY AN *APHANOCAPSA* MEMBRANE PREPARATION

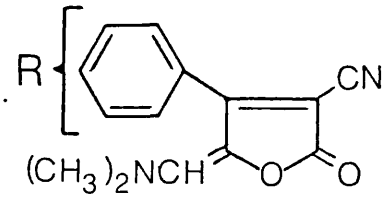
DIFUNONE DERIVATIVE	D.P.M. INCORPORATED INTO PHYTOENE	D.P.M. INCORPORATED INTO β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
				
R=				
Phenyl (difunone)	5,427	477.7	11.3	(67)
2-Cl- Phenyl	4,414	1,029	4.3	(25)
*(Phenyl; (5-cyclobutylamino	1,515	1,330	1.1	(6.7))
2,4-Cl_2-Phenyl	1,118	1,723	0.64	(3.8)
4- C_6H_5O-Phenyl	272	1,953	0.14	(0.82)
Cyclohexyl	275	2,160	0.12	(0.75)
Control	367.1	2,159	0.17	1.0

Aphanocapsa membrane preparation incubated in standard reaction media in the presence of 1μM herbicide, 0.5μCi [^{14}C]GGPP for 2h at 20°C. Chlorophyll content = 100 μg per 200 μl aliquot.

Figures in brackets represent the incorporation ratio relative to the Control.

*This derivative has the 5-dimethylaminomethylene group of difunone replaced by cyclobutylamino-methylene.

TABLE 4:3 THE EFFECT OF VARIOUS DIFUNONE DERIVATIVES (10μM) ON CAROTENOGENESIS BY AN *APHANOCAPSA* MEMBRANE PREPARATION

DIFUNONE DERIVATIVE	D.P.M. INCORPORATED INTO PHYTOENE	β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
				
R=				
Phenyl (Difunone)	11315	308	36	(300)
2-Cl- Phenyl	12218	681	18	(150)
*(Phenyl; (5-cyclobutylamino	5444	1538	3.5	(29)
2,4-Cl_2-Phenyl	3265	2127	1.5	(13)
4- C_6H_5O-Phenyl	502	3082	0.16	(1.3)
Cyclohexyl	637	4188	0.15	(1.3)
Control	416	3447	0.12	(1.0)

Aphanocapsa membrane preparation incubated in standard reaction media (see 2:2:3) in the presence of 10μM herbicide, 0.5μCi (2-^{14}C]GGPP for 2h at 20°C. Chlorophyll content = 80μg per 200μl aliquot.

Figures in brackets represent the incorporation ratio relative to the Control.

*This derivative has the 5-dimethylamine function of difunone replaced by 5-cyclobutylamino.

Those derivatives that are more active than difunone (Table 4:1) are all substituted in the meta-position, whilst those with less activity are ortho- or para-substituted. The chlorinated derivatives provide an illustration of this effect; the 3,4-Cl_2 derivative is more active than difunone whilst the 2-Cl derivative is less active than difunone and the 2,4-Cl_2 derivative even less active. It is possible that the chlorine group in the para position of the 3,4-Cl_2 substituted phenylfurane hinders the full effect of the chlorine atom in the meta-position. Such a difference in activity between meta substituted and ortho- or para- substituted derivatives suggests the difference may be related to conflicting substituent-induced electronic effects. However, both σ_m and σ_p values for chlorine (+0.37 and +0.23 respectively) indicate that this group induces electron withdrawal in both positions. Similarly, there is very little difference with respect to structure, lipophilicity or electron-withdrawing capacity between a phenyl group substituent in the meta-position and a phenoxy group in the para-position. The 3-C_6H_5 substituted phenylfurane is very active, (Table 4:1), whilst the 4-C_6H_5O- derivative has no significant inhibitor activity even at a concentration of 10μM (Table 4:3). It is therefore tentatively suggested that substitution into the meta-position of the phenylfurane has some steric advantage whilst substitution elsewhere is sterically disadvantageous.

With the meta-substituted phenylfuranes there appears to be no simple link between inhibitor activity and any single physical parameter. Since most of the substituents

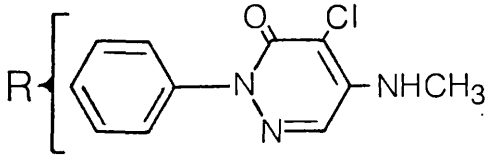
tested here are lipophilic and mostly electron-withdrawing, it would be useful to test the inhibitory activity of meta-substituted phenylfuranes with substituents of widely differing physical properties.

4:3:2 BLEACHING ACTIVITY OF SUBSTITUTED PHENYLPYRIDAZINONES IN THE APHANOCAPSA CELL PREPARATION

The substituted phenylpyridazinones had less phytoene desaturase inhibitory activity than the phenylfuranes, in the Aphanocapsa system (Tables 4:4, 4:5). The results show that only the 3-SCF_3 and 3-CF_3 phenylpyridazinone derivatives had a considerable effect on carotenogenesis at 1μM (Table 4:4). Other derivatives, such as the 4-CF_3 and 3-OSO_2CH_3 phenylpyridazinones were found to be poor inhibitors, whilst the 3-CN, 3-NO_2, 3-OCH_3 and unsubstituted derivatives had no significant effect at this concentration. Although the 4-CF_3 derivative was thought to be slightly inhibitory at 1μM, when tested at 60μM (Table 4:5) it was found to be inactive.

With the exception of the 3-OSO_2CH_3 derivative, the results with substituted phenylpyridazinones were similar in both Scenedesmus cultures and the Aphanocapsa cell – free system. Activity of substituted phenylpyridazinone inhibitors in cultures of Scenedesmus was improved by substitution in the meta-position with groups that were both lipophilic and electron-withdrawing (Sandmann and Böger, 1982). Whilst these results generally support this conclusion, the relatively high activity of the

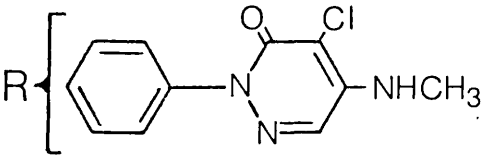
TABLE 4:4 THE EFFECT OF VARIOUS PHENYLPYRIDAZINONES (1μM) ON CAROTENOGENESIS BY AN *APHANOCAPSA* MEMBRANE PREPARATION

	NORFLURAZON DERIVATIVE	D.P.M. INCORPORATED INTO PHYTOENE	D.P.M. INCORPORATED INTO β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
					
	R=				
(1)	3-SCF_3	8087	1072	7.5	(38)
	3-CF_3 (norflurazon)	2968	1947	1.5	(7.6)
	3-OSO_2CH_3	915	2544	0.36	(1.8)
	3-CN	886	3246	0.27	(1.4)
	Unsubstituted	727	3605	0.20	(1.0)
	3-OCH_3	504	2677	0.18	(0.94)
	Control I	635	3169	0.20	(1.0)
(2)	3-SCF_3	12297	591	21	(210)
	3-CF_3	5468	2656	2.1	(21)
	4-CF_3	529	2353	0.22	(2.3)
	3-OSO_2CH_3	550	3496	0.15	(1.6)
	3-NO_2	474	3978	0.12	(1.2)
	Control II	460	4639	0.1	(1.0)

Aphanocapsa membrane preparation incubated in standard reaction media (see 2:2:3) in the presence of 1μM herbicide and 0.5μCi [^{14}C]GGPP for 2h at 20°C. Chlorophyll content = 50μg (1) and 65μg(2) per 200μl aliquot.

Figures in brackets represent the incorporation value relative to the Control.

TABLE 4:5 THE EFFECT OF VARIOUS PHENYLPYRIDAZINONES (60μM) ON CAROTENOGENESIS BY AN *APHANOCAPSA* MEMBRANE PREPARATION

NORFLURAZON DERIVATIVE	D.P.M. INCORPORATED INTO PHYTOENE	β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
				
R=				
3-OSO_2CH_3	1561	714	2.19	(26)
3-CN	650	949	0.68	(8.1)
3-NO_2	242	1012	0.24	(2.8)
3-OCH_3	232	1048	0.22	(2.6)
4-CF_3	145	1199	0.12	(1.4)
Unsubstituted	138	1223	0.11	(1.3)
Control	101	1201	0.08	(1.0)

Aphanocapsa membrane preparation incubated in standard reaction media in the presence of 60μM herbicide and 0.5μCi [^{14}C]GGPP for 2h at 20 °C. Chlorophyll content = 60μg per 200μl aliquot.

Figures in brackets represent the incorporation ratio relative to the Control.

3-OSO_2CH_3, derivative with Aphanocapsa in vitro is anomalous. According to the parameters discussed above, the 3-OSO_2CH_3 function compares unfavourably with respect to lipophilicity and electron-withdrawing capacity to both 3-CN and 3-NO_2 functions (Table 4:7).

The anomalous behaviour of the 3-OSO_2CH_3 derivative in the Aphanocapsa system, may be explained by two hypotheses:-

(i) the Aphanocapsa system metabolizes the 3-OSO_2CH_3 phenylpyridazinone in vitro into a derivative that is more lipophilic ;

(ii) the activity of the substituted phenylpyridazinone in the Aphanocapsa system is not fully explained by only two parameters.

The first hypothesis implies that such a transformation must be rapid and produce a substituent that is more lipophilic , whilst retaining similar electron-withdrawing properties to the 3-OSO_2CH_3 function. With respect to the second possibility, all substituents have molar refractance (MR) values of between 5.02 cm^3/mol ($-CF_3$) and 7.87cm^3/mol ($-OCH_3$), with the exception of the $-SCF_3$ (MR=13.81cm^3/mol and -OSO_2CH_3 (MR=16.99 cm^3/mol) functions (Table 4:7). The high molar refractance values for these substituents may suggest that inhibitory phenylpyridazinones are improved with large substituent bulk in Aphanocapsa. If so, this may explain the inhibitory activity in the

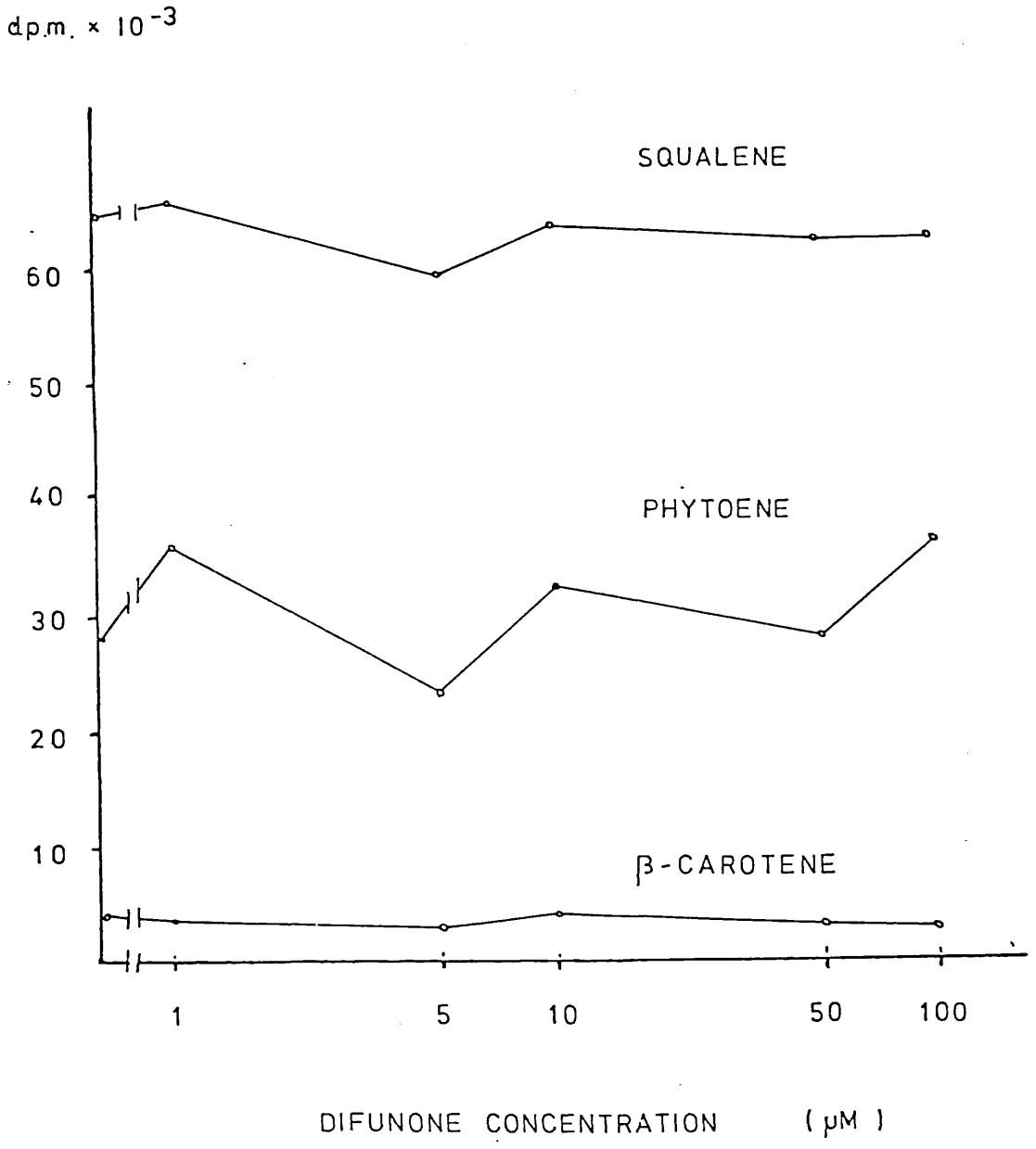
Aphanocapsa system of the 3-OCH_3 derivative relative to the unsubstituted compound.

In both Scenedesmus cultures and Aphanocapsa preparations the 3-CN phenylpyridazinone is more active than the 3-NO_2 derivative, yet in terms of lipophilicity, electron-withdrawing capacity and bulk, the latter should be the more potent inhibitor. Thus, there appears to be no simple, obvious relationship between the properties of substituents and apparent phenylpyridazinone activities. It should be noted that substituted phenylpyridazinones are multi-functional herbicides (see 1:4:1) and have been reported to inhibit the evolution of photosynthetically produced oxygen (I_{50} for norflurazon = 33μM). Since carotene desaturation in the Aphanocapsa system is dependent to a certain extent on photosynthetic oxygen, it is possible that part of the inhibition observed with phenyl pyridazinones at 60μM (Table 4.5) is due to reduced oxygen tension rather than direct herbicide inhibition of desaturation.

4:3:3 BLEACHING ACTIVITY OF SUBSTITUTED PHENYLFURANES AND PHENYLPYRIDAZINONES IN EXTRACTS FROM PHYCOMYCES

Experiments with the Phycomyces system show that incorporation from [2-^{14}C]MVA into carotenes is not significantly affected by difunone over a range of concentrations from 1μM to 100μM (Fig. 4.2). Furthermore, the 3-CF_3 phenylfurane derivative (100 μM) was not inhibitory in this cell-free system. These results

FIG. 4:2 THE EFFECT OF DIFUNONE CONCENTRATION ON INCORPORATION OF [^{14}C]MVA INTO TERPENOIDS BY PHYCOMYCES EXTRACTS



Phycomyces extract incubated (2h, 20°) in standard mixture (see 2:2:3) with 0.5μCi DL -[2-^{14}C]MVA.

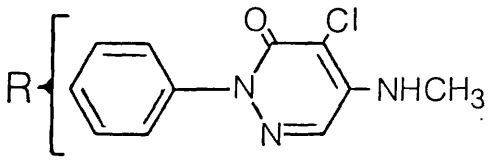
contrast with a report that difunone reduces the β-carotene content of Phycomyces cultures (Sandmann et al., 1979)

The 3-CF_3 phenyl pyridazinone derivative (norflurazon) was reported to inhibit phytoene synthesis by some 90% at 10μM in the Phycomyces system (Sandmann et al., 1980). This result, however, could not be repeated in the present investigation and no inhibitory activity was found with 10μM norflurazon, or other substituted phenylpyridazinones. At higher concentrations (50μM) however, norflurazon, the 3-SCF_3 and the 4-CF_3 phenylpyridazinones were found to inhibit phytoene desaturation rather than phytoene synthesis (Table 4:6a).

Since Phycomyces extracts were incubated anaerobically in the experiments of Sandmann and co-workers, similar anaerobic incubations were repeated in the presence of the 3-CF_3 (norflurazon) and 3-SCF_3 derivatives (Table 4:6b). The data show that even under these conditions the phenylpyridazinones did not inhibit phytoene synthesis.

The extracts from Phycomyces as prepared by Sandmann and co-workers used 0.2M Tris-HCl containing 30% (v/v) glycerol and 2mM mercaptoethanol, whereas the extracts prepared here used 0.4M Tris-HCl, without additives. It is possible that the former extraction technique preserved an inhibition site that is lost with the latter method of cell-free preparation.

TABLE 4:6 THE EFFECT OF VARIOUS PHENYLPYRIDAZINONES (50μM) ON CAROTENOGENESIS BY AN EXTRACT FROM PHYCOMYCES

NORFLURAZON DERIVATIVE		D.P.M. INCORPORATED INTO PHYTOENE	D.P.M. INCORPORATED INTO β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
					
A: AEROBIC INCUBATION					
R=	4-CF_3	42534	2781	15	(4.6)
	3-SCF_3	42314	4607	9.2	(2.8)
	3-CF_3	36676	5003	7.3	(2.2)
	Control	26651	8006	3.3	(1.0)
B: ANAEROBIC INCUBATION					
R=	3-SCF_3	68826	130		
	3-CF_3	65646	171		
	Control	62990	198		

Phycomyces extract incubated (2h, 20° C) anaerobically (Thunberg tubes) and aerobically in the presence of 50μM herbicide, 0.5μCi DL-[2-^{14}C] MVA in standard reaction media.

TABLE 4:7 I_{50} VALUES FOR INHIBITION OF CAROTENE SYNTHESIS IN *SCENEDESMUS* CULTURES AND PHYSICAL DATA FOR SUBSTITUENTS

DERIVATIVE	[1] I_{50}	[2] LIPOPHILICITY (π)	ELECTRONIC PARAMETERS σm	σp	BULK PARAMETER MR (cm^3/mol.)
A: PHENYL FURANES					
3,4 -Cl_2	0.05µM	0.71	0.37	0.23	2x6.03
3 -C_6H_5	0.1µM	1.96	0.06	n.a	25.36
3 -CF_3	0.1µM	0.88	0.43	n.a	5.02
3 -SCH_3	0.12µM	0.61	0.15	n.a	13.82
3 -OCH_3	0.15µM	-0.02	0.12	n.a	7.87
Unsubstituted	0.7µM	0	0	0	1.03
(5-cyclobutylamino)	1µM	n.a	n.a	n.a	n.a
2 -Cl	1.2µM	0.71	-	0.23	6.03
2,4 -Cl_2	5.0 µM	1.25	0.37	0.23	2x6.03
4 - OC_6H_5	n.i.d	2.08	-	-0.03	27.68
(4-cyclohexyl)	n.i.d.	n.a.	n.a	n.a	n.a
B: PHENYL PYRIDAZINONES					
3 -SCF_3	4 nm	1.44	0.40	n.a	13.81
3 -CF_3	10 nm	0.88	0.43	n.a	5.02
3 -CN	80 nm	-0.57	0.56	n.a	6.33
3 -NO_2	400 nm	-0.28	0.71	n.a	7.36
Unsubstituted	1.8µM	0	0	n.a	1.03
3-OSO_2CH_3	3.6µM	-0.88	0.39	n.a	16.99
3-OCH_3	14µM	-0.02	0.12	n.a	7.87

1. I_{50} values from Boehler-Kohler and Boger (unpublished results) for phenylfuranes and Sandmann and Boger (1982) for phenylpyridazinones.

2. Physical data for substituents from Hansch *et al.*, 1973.

n.a Value not applicable.

n.i.d. No inhibition detectable.

C H A P T E R 5

INHIBITION OF CAROTENOGENESIS BY DIPHENYL COMPOUNDS

5:1 INTRODUCTION

A variety of diphenyl compounds, including DPA, have been reported to inhibit carotene biosynthesis in many micro-organisms (see Sections 1:4:7 and 1:4:8 for discussion). Although Rilling (1965) proposed that such compounds might inhibit carotenogenesis through competition with partially desaturated carotenes for sites on the dehydrogenase, mechanisms involving these compounds in the inhibition of protein synthesis have not been discounted.

In this chapter the effect of diphenyl compounds on carotene biosynthesis *in vitro* is reported and structure-inhibitor activity relationships discussed, especially with respect to their possible role as competitive inhibitors (Rilling 1965).

5:2 REACTION MIXTURES

Cell-free preparations were incubated aerobically (2h, 20 ^{o}C) in standard reaction mixtures (see 2:2:3) with either 0.5µCi *DL*-[2-^{14}C]MVA, 0.5µCi [1-^{14}C]IPP (for experiments with *P.blakesleeanus*) or 0.7µCi [2-^{14}C]GGPP (specific activity 1.0 mCi/mmol, for experiments with *Aphanocapsa*) as substrate. Diphenyl compounds were dissolved in methanol and added to reaction mixtures in 5µl aliquots prior to addition of cell-free material.

5:3 RESULTS

Of all the diphenyl compounds tested, diphenylamine (Ph NH.Ph) and benzophenone (Ph CO.Ph) were found to be the strongest inhibitors of carotene desaturation in both cell-free systems (Tables 5.1 and 5.4). The effects of various aromatic ketones and benzophenone derivatives on carotene biosynthesis were investigated using both systems and a common structure-activity relationship was found. In both systems, inhibitor activity decreased as the ketone function became separated from either aromatic ring and, whilst benzophenone oxime (Ph C=NOH.Ph) was found to have a certain amount of inhibitory activity, benzophenone hydrazine (Ph $C=NNH_2$.Ph) had none (Tables 5.2, 5.3 and 5.4).

Carotene desaturation in the Phycomyces system was found to be moderately inhibited by diphenylmethane ($PhCH_2Ph$) and trans-stilbene (Ph CH=CH Ph, Table 5.3) and the peroxidizing diphenylether, oxyfluorfen (substituted PhOPh, Table 5.1) was also found to inhibit carotene biosynthesis in this system. Carotene biosynthesis was not significantly affected by benzoin ($PhCH_2CHOH$ Ph, Table 5.1) whereas deoxybenzoin ($PhCH_2CO$.Ph, Table 5.3) was found to inhibit desaturation.

The commercial substituted diphenyl compound 'Methoxyphenone' (3,3- dimethyl-4-methoxy-benzophenone,NK-049, see Section 1:4:7) was found to be a much stronger inhibitor of phytoene desaturation than norflurazon in the Phycomyces system (see Table 5:5).

TABLE 5.1 THE EFFECT OF VARIOUS DIPHENYL COMPOUNDS AND OXYFLUORFEN ON CAROTENOGENESIS BY AN EXTRACT FROM *PHYCOMYCES*

DIPHENYL INHIBITOR	INCORPORATION (D.P.M.) INTO PHYTOENE	β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
Benzophenone	127712	477	270	(38)
Diphenylamine	158603	304	520	(74)
Oxyfluorfen	96292	1989	48	(6.9)
Benzoin	48504	5426	8.9	(1.3)
Benzoyl methyl aniline	87351	1419	6.0	(8.8)
Control	43738	6240	7.0	(1.0)

Phycomyces extract incubated in standard reaction media (See 2:2:3) in the presence of 0.5μCi DL-[2-^{14}C]MVA and 100μM inhibitor for 2h at 20°C.

TABLE 5.2 THE EFFECT OF VARIOUS PHENYL KETONE INHIBITORS (100μM) ON CAROTENOGENESIS BY AN EXTRACT FROM *PHYCOMYCES*

DIPHENYL INHIBITOR	INCORPORATION (D.P.M.) INTO PHYTOENE	β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
Benzophenone	86862	1166	74	(48)
Benzophenone oxime	54353	3109	17	(11)
Benzophenone hydrazone	17054	8405	2.0	(1.3)
Diphenylacetone	40890	6875	5.9	(3.9)
Acetophenone	13492	7680	1.8	(1.1)
Control	11675	7609	1.5	(1.0)

Phycomyces extract incubated in standard reaction media (See 2:2:3) in the presence of 0.5μCi DL- [2-^{14}C]MVA and 100μM inhibitor for 2h at 20° C.

TABLE 5.3 THE EFFECT OF VARIOUS DIPHENYL COMPOUNDS ON CAROTENOGENESIS BY AN EXTRACT FROM *PHYCOMYCES*

DIPHENYL INHIBITOR	INCORPORATION (D.P.M.) INTO PHYTOENE	β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
Control 1	11,807	41,860	0.28	(1.0)
Benzophenone	75,342	3,600	21	(74)
Deoxybenzoin	47,294	20,946	2.3	(8.0)
Diphenylacetone	34,864	31,706	1.1	(3.9)
Control 2	8,922	31,281	0.28	(1.0)
Diphenylmethane	55,918	10,435	5.4	(19)
trans-Stilbene	45,834	5,973	7.7	(27)
Deoxybenzoin	42,657	22,825	1.9	(6.6)

Phycomyces extract incubated in standard reaction media in the presence of 0.25μCi [^{14}C]IPP and 100μM diphenyl inhibitor for 2h at 20°C.

TABLE 5.4 THE EFFECT OF VARIOUS DIPHENYL COMPOUNDS ON CAROTENOGENESIS BY AN *APHANOCAPSA* MEMBRANE PREPARATION

DIPHENYL INHIBITOR	INCORPORATION (D.P.M.) INTO PHYTOENE	β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
Benzophenone	1,495	500	3.0	(16)
Deoxybenzoin	257	593	0.43	(2.4)
Diphenylacetone	274	1,011	0.27	(1.5)
Benzophenone oxime	488	911	0.54	(2.9)
Benzophenone hydrazone	138	1,296	0.11	(0.6)
Diphenylamine	855	661	1.3	(7.0)
Control	177	970	0.18	(1.0)

Aphanocapsa membrane preparation incubated in standard reaction media in the presence of 0.7μCi [^{14}C]GGPP and 100μM diphenyl compound for 2h at 20°C. Chlorophyll content = 55μg/200μg aliquot.

TABLE 5.5 THE EFFECT OF NORFLURAZON AND METHOXYPHENONE (NK-049) ON CAROTENOGENESIS BY AN EXTRACT FROM PHYCOMYCES

INHIBITOR	INCORPORATION (D.P.M.) INTO PHYTOENE	β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
Norflurazon	48,959	3,122	16	(6.5)
Methoxyphenone	114,977	266	430	(180)
Control	26 400	1,093	2.4	(1.0)

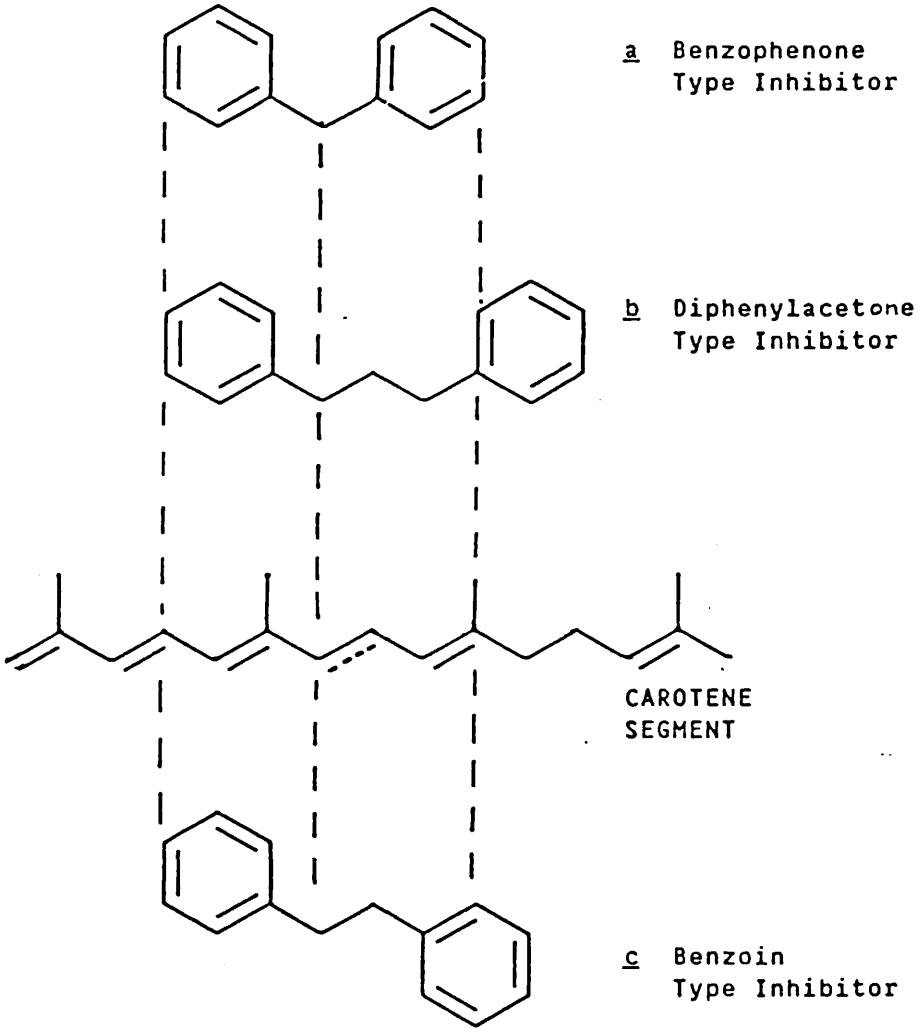
Phycomyces extract incubated in standard reaction media in the presence of 0.5μCi DL- [2-^{14}C]MVA and 100μM herbicide for 2h at 20 °C.

5:4 DISCUSSION

The results show that in both systems, inhibitory activity of diphenyl ketones decreases as the ketone group is isolated from either phenyl ring (Tables 5.3, 5.4). Thus deoxybenzoin ($PhCH_2COPh$) is not as effective an inhibitor as benzophenone (PhCOPh), and diphenylacetone ($PhCH_2COCH_2Ph$) is less effective than either. If such compounds inhibit through competition, then activity should be related to the degree of similarity with the carotenoid segment surrounding the desaturation site. It was suggested that diphenyl compounds such as benzophenone 'fit' the carotenoid segment well since the phenyl rings coincide with the desaturated bonds and methyl side chains of the carotenoid and the ketone coincides with the desaturation site (Fig. 5.1a; Rilling, 1965). Using the same argument, diphenylacetone may be seen to 'fit' the carotenoid segment, although the coincidence of one phenyl ring is not exact (Fig. 5:1b). This suggests that such compounds would not be located easily into the active site of the desaturase. On the basis of this hypothesis, diphenylacetone would be expected to be a more active desaturase inhibitor than deoxybenzoin.

It is possible that conjugation of the diphenyl molecule is an important factor in determining the inhibitory activity of these compounds in addition to shape. The ketone and amine functions that separate the aromatic rings of benzophenone and DPA, respectively make it possible for there to be complete conjugation throughout these molecules. Whilst diphenylmethane ($PhCH_2Ph$), or oxyfluorfen,

FIG. 5:1 VARIOUS DIPHENYL COMPOUNDS AS POTENTIAL COMPETITIVE INHIBITORS OF CAROTENE DESATURATION



has a similar shape to benzophenone/DPA, results show that neither is as active an inhibitor as the conjugated trans-stilbene (PhCH=CH Ph), a compound of similar shape to deoxybenzoin (Table 5.3). The results also show that the unconjugated diphenyl compound benzoin ($PhCH_2CHOH.Ph$) unlike deoxybenzoin, has no effect on carotene desaturation in the Phycomyces system (Table 5.1); it has been reported that diphenyl carbinol (PhCHOH.Ph, unconjugated but of benzophenone like shape) inhibits carotene desaturation in cultures of Mucor hiemalis (Herber et al, 1972). Thus it may be argued that deoxybenzoin is more active than diphenylacetone since the ketone group in the former compound is conjugated with a phenyl ring whereas in diphenylacetone it is isolated. To assess the contribution of shape towards inhibitor activity it would have been more appropriate to have compared the activity of deoxybenzoin ($PhCH_2CO.Ph$) to benzoyl methyl toluene ($PhCOCH_2CH_2Ph$) rather than to diphenylacetone. The results suggest however, on the basis of relative incorporation ratios, that the activity of benzoyl methyl aniline ($PhCOCH_2NHPh$) is not significantly greater than that of deoxybenzoin (Tables 5.1 and 5:3); the effect of the amine in benzoyl methyl aniline activity is debatable.

From these results it is suggested that diphenyl compounds inhibit desaturation by binding to the active site as proposed by Rilling (1965). Furthermore, it is tentatively concluded that the active site has great specificity for conjugated systems, but is plastic enough to accommodate structures with unfavourable shape if such

compounds are highly conjugated.

In both systems, benzophenone hydrazone ($PhC=NNH_2.Ph$) had no effect on carotene desaturation, whilst benzophenone oxime (PhC=NOH.Ph) was found to be a mild inhibitor (Tables 5.2 and 5.4). The chemical differences between these two derivatives is small, a hydroxyl group on the oxime compared with an amino group on the hydrazone, yet the difference in activity is significant; the reason for this is unclear. However, it is noted that whilst these two substituents have nearly equal molecular weights, the molar refractance values are significantly different (2.85cm^3/mol for -OH, 5.42 cm^3/mol for -NH). Both physical constants were proposed as parameters for substituent bulk in structure-activity correlation analysis (Hansch et al., 1973), where the indiscriminate use of molar refractance values were cautioned against since this parameter "contains an electronic contribution..... directly proportional to polarizability". It is possible, considering the near identical molecular weights, that the difference in activity between the oxime and hydrazone derivatives is due to the different electronic nature of the hydroxyl and amino functions.

CHAPTER 6

THE INHIBITION OF CAROTENOGENESIS BY 3-PHENOXY-N-ALKYL-BENZAMIDES

6.1 INTRODUCTION

Several substituted 4-nitrodiphenyl ethers have been reported as inhibitors of photosynthetic electron transport (Bugg *et al.*, 1980), inhibitors of photophosphorylation (energy transfer inhibition; Lambert *et al*, 1979) and as light-induced peroxidizing agents that damage thylakoid lipids (Kunert and Böger, 1982). The relationship between nitrodiphenyl ether substitution and phytotoxic activity has been investigated with spinach chloroplasts and cultures of *Scenedesmus acutus* (Lambert *et al*, 1982).

Certain 3-alkylamido diphenyl ethers (i.e. 3-phenoxy-*N*-alkyl-benzamides) inhibit carotene desaturation in cultures of *Scenedesmus acutus* (Lambert and Böger, 1982). These workers found that the potency of the phenoxybenzamide was increased by phenoxy ring substitution and that the most active compound was comparable in potency and action, to norflurazon (SAN 9789).

In this chapter the effects of various phenoxy-alkyl-benzamides on *in vitro* carotene biosynthesis are presented and a basic structure-activity relationship for these compounds is discussed.

6.2 ADDITIONAL MATERIALS AND METHODS

6:2:1 PHENOXY ALKYL BENZAMIDES

6:2:1:1 Industrial Sources. Various companies kindly supplied many 3-phenoxy-N-alkyl benzamides; these have been cited in Section 2:1:4.

6:2:1:2 Organic Syntheses. N-propyl-, isopropyl-, butyl-, sec-butyl- and tert-butyl-, 3-phenoxy-N-alkyl benzamide derivatives were prepared from 3-phenoxybenzoic acid andthe appropriate alkylamine via 3-phenoxybenzoyl chloride: the alkylamines and 3-phenoxybenzoic acid were purchased from Aldrich-Europe (Nettetal, W.Germany). Similarly, butyl and ethyl derivatives of 3-(4'-chloro-)-phenoxy-N-alkyl-benzamide and 2-phenoxy-N-butyl-benzamide were synthesized from the appropriate alkylamine and 3-(4'-chloro)-phenoxybenzoic acid (kindly supplied by B.A.S.F.) or 2-phenoxybenzoic acid (purchased from Aldrich-Europe).

3-Phenoxybenzoyl chlorides were prepared from the appropriate phenoxybenzoic acid and thionyl chloride (2x molar excess) refluxed together (1h, 80°, boiling chips) with continuous stirring in the presence of N,N'--dimethylformamide (2 drops) as a catalyst. After cooling, excess thionyl chloride was removed under reduced pressure (water pump, 30 mins) at room temperature. A sample of the acid chloride was applied to a silica gel plate (containing fluorescent indicator) and developed in petroleum ether = diethyl ether (1:1V/V), together with a free acid marker. No u.v. absorbing band was observed adjacent to the free acid marker (Rf 0.6) from the applied acid chloride.

Phenoxy-alkyl-benzamides were prepared by the dropwise-addition of acid chloride (approx.0.8ml) with continuous stirring onto an ice cold solution of the alkylamine (4x molar excess) dissolved in chloroform (50ml). After the mixture had cooled it was partitioned three times against water and the chloroform layer (hypophase) delivered through filter paper into a flask containing anhydrous sodium sulphate (2g). The dried solution was then filtered into a round bottom flask and the chloroform evaporated under reduced pressure (30^{o}).

If after chloroform evaporation the product solidified, it was recrystallized from a chloroform:petroleum ether solution. If not, the product was dissolved in chloroform, transferred to a test tube and the chloroform removed under a stream of nitrogen at $40^{o}C$.

2-Phenoxybenzoyl chloride was prepared from the acid dissolved in chloroform:diethyl ether (1:1 v/v, 50ml) refluxed (40^{o}, 1h) with thionyl chloride and pyridine as a catalyst. The pyridine hydrochloride was then filtered off and excess thionyl chloride (and pyridine) removed as described above.

The purity of these phenoxy-alkyl-benzamides was assessed by proton magnetic resonance (Bruker WH 250 MHz; $CDCl_3$ solvent). All the compounds, except one, produced the expected displacement profiles and proton ratio correlations and were thus judged to be at least 90% pure. The exception was that of 3-(4'-chloro-)phenyl-N-ethyl-benzamide, which produced two unexpected peaks at 3.35 p.p.m. and 4.00 p.p.m.

These peaks accounted for 38% of the aliphatic chain protons and were suggestive of an ethyl group bound to a strongly electron withdrawing moiety. The close correlation of proton ratios between the major aliphatic proton peaks and the aromatic and amide proton peaks ruled out the possibility of the contaminant being either aromatic or possessing other proton containing entities. The contaminant could not be identified from a library of proton NMR spectra. However, if the molecular weight of the contaminant is below 100 daltons (or 200 for a diethyl compound) then the 3-(4 -chloro-)-phenoxy-N-ethyl-benzamide is 85%(w/w) pure.

6:2:2 REACTION MIXTURES

Cell free preparations were incubated aerobically (2h, 20 ^{o}C) in the standard reaction mixture (see 2:2:3) with either 0.5 µCi [1-^{14}C]IPP (for experiments with P.blakesleeanus) or 0.7 µCi [2-^{14}C]GGPP (specific activity 1.0mCi/mmol, for experiments with Aphanocapsa) as substrate. Phenoxy-alkyl-benzamides were dissolved in methanol and added to reaction mixtures in 5µl aliquots prior to addition of cell free material.

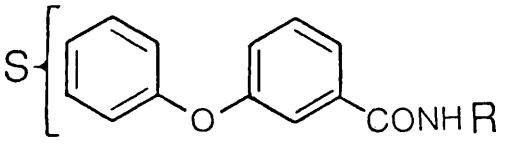
6:3 RESULTS AND DISCUSSION

6:3:1 EXPERIMENTS WITH MEMBRANE PREPARATIONS FROM APHANOCAPSA 6714

6:3:1:1 Preliminary Experiments. A number of phenoxy-alkyl-benzamides (PABs) that had been screened for herbicidal activity in cultures of Scenedesmus acutus, were tested as inhibitors of carotene biosynthesis in the Aphanocapsa system (Table 6:1). It had been found that carotene biosynthesis in Scenedesmus cultures was inhibited only by those PABs with phenoxy ring substitution and an alkyl moiety larger than a methyl group (Lambert and Böger, 1983). Whilst such PABs (i.e. S-3422 and CUR-5026) were amongst the most active inhibitors in the Aphanocapsa system, the unsubstituted propenyl derivative, CUR-4914, was just as effective and the unsubstituted ethyl derivative, S-3141, was also an inhibitor of carotenogenesis. These results suggest that in the Aphanocapsa system, the inhibition of carotene desaturation by PAB increases with alkyl group size and phenoxy ring substitution. Therefore the structure:activity relationship for PAB inhibition in the two systems is similar, but not identical. The apparent impotency of unsubstituted PABs (e.g. CUR-4914) in Scenedesmus cultures may be attributable to these compounds not being able to permeate the cell wall.

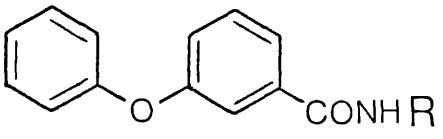
6:3:1:2 The Effect of Alkyl Chain Size on PAB Potency. Since the preliminary experiments (6:3:1:1) suggested that PAB potency increased with alkyl chain length, the role of the alkyl group in PAB inhibition of carotene desaturation was investigated by observing the effect of various unsubstituted PABs on the Aphanocapsa system. The results (Table 6:2) show that inhibitory action of PAB increases

TABLE 6:1 THE EFFECT OF VARIOUS PHENOXY-ALKYL BENZAMIDES ON CAROTENOGENESIS BY APHANOCAPSA MEMBRANE PREPARATIONS

PHENOXY ALKYL BENZAMIDE			D.P.M. INCORPORATED INTO		INCORPORATION RATIO	
			PHYTOENE	β-CAROTENE	PHYTOENE/β-CAROTENE	
S	R					
-	$-CH_3$	(CUR 4850)	226	768	0.30	(2.1)
-	$-CH_2CH_3$	(S- 3141)	533	437	1.2	(8.7)
-	$-CH_2CH=CH_2$	(CUR 4914)	1196	499	2.4	(17)
2',5'-$(CH_3)_2$	$-CH_2CH_3$	(S- 3422)	1081	475	2.3	(16)
4'- Cl	$-CH_3$	(CUR 5025)	355	803	0.44	(3.1)
4'- Cl	$-CH(CH_3)_2$	(CUR 5026)	1245	580	2.2	(15)
Control			101	717	0.14	(1.0)
Oxyfluorfen			108	762	0.14	(1.0)

Membrane preparation from Aphanocapsa incubated in standard reaction media (see 2:2:3) in the presence of 10μM herbicide, 0.7μCi [2-^{14}C]GGPP, for 2h at 20°C. Chlorophyll content = 50μg per 200μl aliquot.

TABLE 6:2 THE EFFECT OF THE ALKYL CHAIN ON PHENOXY ALKYL BENZAMIDE INHIBITION OF CAROTENOGENESIS IN <u>APHANOCAPSA</u> MEMBRANE PREPARATIONS

PHENOXY ALKYL BENZAMIDE  R		D.P.M. INCORPORATED INTO PHYTOENE	D.P.M. INCORPORATED INTO β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
- CH_3	(CUR 4850)	162	1557	0.11	(1.9)
- CH_2CH_3	(S- 3141)	492	1354	0.36	(6.7)
- $CH_2CH_2CH_3$		677	694	0.98	(18)
- $CH_2CH_2CH_2CH_3$		1019	886	1.2	(21)
- $CH(CH_3)_2$		403	1093	0.37	(6.8)
- $CH(CH_3)CH_2CH_3$		838	1004	0.84	(15)
- $C(CH_3)_3$		215	1300	0.17	(3.0)
- $CH_2CH = CH_2$	(CUR 4914)	925	1068	0.87	(16)
Control		82	1500	0.05	(1.0)

Membrane preparation from <u>Aphanocapsa</u> incubated in standard reaction media in the presence of phenoxy alkyl benzamide (10μM), 0.7μCi [2-^{14}C]GGPP, for 2h at 20°C. Chlorophyll content = 60μg per 200μl aliquot.

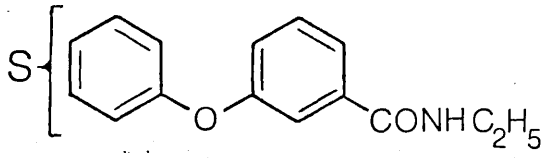
with alkyl chain length, rather than alkyl group size; the N-isopropyl derivative has comparable activity to 3-phenoxy-N-ethyl-benzamide rather than the N-propyl derivative and the N-sec-butyl derivative comparable activity to 3-phenoxy-N-propyl-benzamide. However, the effect of 3-phenoxy-N-tert-butyl benzamide on carotene desaturation was less than expected, by comparison with the activity of the N-ethyl derivative, suggesting alkyl groups with branching at the alpha-carbon may hinder the binding of the PAB to the enzyme.

Comparison of the inhibitory effects of the N-propyl and N-propenyl derivatives suggests that desaturation of the beta-gamma alkyl carbon bond has no significant effect on PAB activity.

6:3:1:3 The Effect of Ring Position and Substitution. A comparison of the inhibitory activities of 2-phenoxy- and 3-phenoxy- N-butyl-benzamides on carotene desaturation shows that the phenoxy group in the ortho position is not as effective as when it is in the meta position (Table 6:3). This difference may reflect either the different electron withdrawing capacity of the phenoxy group in these two positions, the steric factors, or a combination of both.

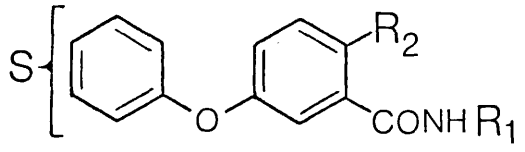
The effect of substitutions on the inhibitory activity of PABs was assessed by observing the action of various substituted 3-phenoxy-N-ethyl-benzamides on carotene desaturation by the Aphanocapsa system. Results (Table 6:4) suggest that substituent lipophilicity is the key factor in

TABLE 6:3 THE EFFECT OF PHENYL RING POSITION IN PHENOXY-N-BUTYL-BENZAMIDE INHIBITION OF CAROTENOGENESIS

PHENOXY ALKYL BENZAMIDE	D.P.M. INCORPORATED INTO PHYTOENE	D.P.M. INCORPORATED INTO β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
 S				
2- phenoxy	310	951	0.33	(4.5)
3- phenoxy	1025	539	1.9	(26)
Control	78	1080	0.07	(1.0)

Aphanocapsa membrane preparation incubated in standard reaction media in the presence of 0.7μCi [^{14}C]GGPP and 10μM phenyl alkyl benzamide for 2h at 20°C. Chlorophyll content = 50μg per 200μl aliquot.

TABLE 6:4 THE EFFECT OF PHENYL RING SUBSTITUTION IN PHENOXY N-ETHYL BENZAMIDE INHIBITION OF CAROTENOGENESIS

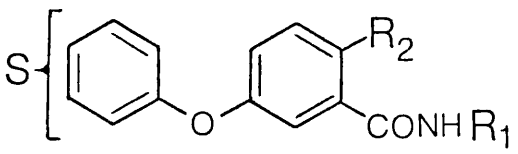
PHENOXY ALKYL BENZAMIDE  S R1 R2			D.P.M. INCORPORATED INTO PHYTOENE	D.P.M. INCORPORATED INTO β-CAROTENE	INCORPORATION RATIO PHYTOENE/β-CAROTENE	
4'-Cl	$-C_2H_5$	-H	960	722	1.3	(17.5)
2',5'- $(CH_3)_2$	$-C_2H_5$	-H	1428	640	2.2	(29)
2',CF_3,4'-Cl	$-C_2H_5$	-H	1562	494	3.2	(42)
2',5'-$(CH_3)_2$	$-C_2H_5$	$-NO_2$	139	1112	0.13	(1.6)
2'-CF_3,4'-Cl	$-C_2H_5$	$-NO_2$	123	1085	0.11	(1.5)
4'- Cl	$-CH_3$	-H	242	1169	0.21	(2.7)
Control			76	1003	0.07	(1.0)

Aphanocapsa membrane preparation incubated in standard reaction media in the presence of 0.7μCi [2-^{14}C]GGPP and 10μM phenoxy alkyl benzamide for 2h at 20°C. Chlorophyll content = 53μg per 200μl aliquot.

determining substituted PAB potency; no correlation was found between substituent bulk or substituent electron withdrawing capacity and the substituted PAB inhibitor activity. The most active PAB was found to be the 2'-trifluromethyl-4'-chloro derivative [lipophilicity factor (π), = 1.50] followed by the 2',5' dimethyl (π = 1.10) and the 4'-chloro (π = 0.71) derivatives of 3-phenoxy-N-ethyl benzamide. The potency of the substituted compounds, as measured by relative phytoene = β-carotene ratios, were all greater than that of the unsubstituted derivative 3-phenoxy-N-ethyl-benzamide (π = 0.00; see Tables 6:1 and 6:2). Lipophilicity factors (π) were calculated from octanol-water partitions of substituted and unsubstituted aromatic compounds (usually benzene and derivatives) as cited by Hansch et al., (1973).

Although 3- (2',5' dimethyl) and 3-(2'-trifluoro-methyl, 4' chloro)-phenoxy-N-ethyl-benzamides proved to be potent inhibitors of carotene desaturation, the 6-nitro derivatives of the same PABs were not (Table 6.4). The intro-duction of a nitro group next to the alkyl amido function apparently removes the ability of PABs to inhibit carotene desaturation. Thus 3-(2',5' dimethyl) and 3-(2'-trifluoromethyl, 4'-chloro)-phenoxy-6-nitro-N-ethyl benzamides proved to be weaker inhibitors than 3-(4'-chloro-) -N-methyl benzamide. In cultures of Scenedesmus acutus, however, the same 6-nitro PABs proved to be effective peroxidizing diphenyl ethers (Lambert and Böger, 1984). Peroxidation in vivo results in, amongst other symptoms, a loss of carotenoids that makes it

TABLE 6:5 THE EFFECT OF VARIOUS PHENOXY ALKYL BENZAMIDES ON CAROTENOGENESIS BY EXTRACTS OF *PHYCOMYCES*

PHENOXY ALKYL BENZAMIDE			D.P.M. INCORPORATED INTO		INCORPORATION RATIO	
			PHYTOENE	β-CAROTENE	PHYTOENE/β-CAROTENE	
S	R1	R2				
-	$-C_2H_5$	-H	18257	30838	0.59	(2.1)
-	$-(CH_2)_3CH_3$	-H	44822	14810	3.0	(10.6)
2'-CF_3,4'-Cl	$-C_2H_5$	-H	35944	17213	2.1	(7.3)
2'-CF_3,4'-Cl	$-C_2H_5$	$-NO_2$	21423	27795	0.77	(2.7)
Control			8922	31281	0.28	(1.0)

Extract from *Phycomyces* incubated in standard reaction media in the presence of (1-^{14}C]IPP and 60μM phenoxy alkyl benzamide for 2h at 20 °C.

difficult to determine the phytotoxic contribution due to inhibition of carotene biosynthesis of a possibly multi-functional peroxidizing herbicide. In such a case as this, a carotenogenic cell—free system is an ideal analytical tool.

6:3:2 EXPERIMENTS WITH CELL FREE EXTRACTS OF PHYCOMYCES BLAKESLEEANUS

Experiments with the Phycomyces system and selected PABs, revealed a similar structure-activity relationship for PAB inhibition of carotene desaturation as reported for the Aphanocapsa system (Table 6:5). It was noticeable that the Phycomyces system was not as sensitive as that of Aphanocapsa, making it necessary to use a 60μM standard concentration of PAB.

The N-butyl derivative was more effective than the N-ethyl derivative and the substituted 2'-trifluoromethyl, 4'-chloro derivative was more potent than the unsubstituted PAB (Table 6:5). Although the activity of the 6-nitro PAB was considerably less than the corresponding non-nitrated derivative, it was found to be inhibitory in this system.

None of the compounds affected the incorporation of ^{14}C label from [^{14}C]IPP into squalene or into total terpenoids (results not shown), thus indicating that the effect of PAB inhibition was restricted to the carotenoids.

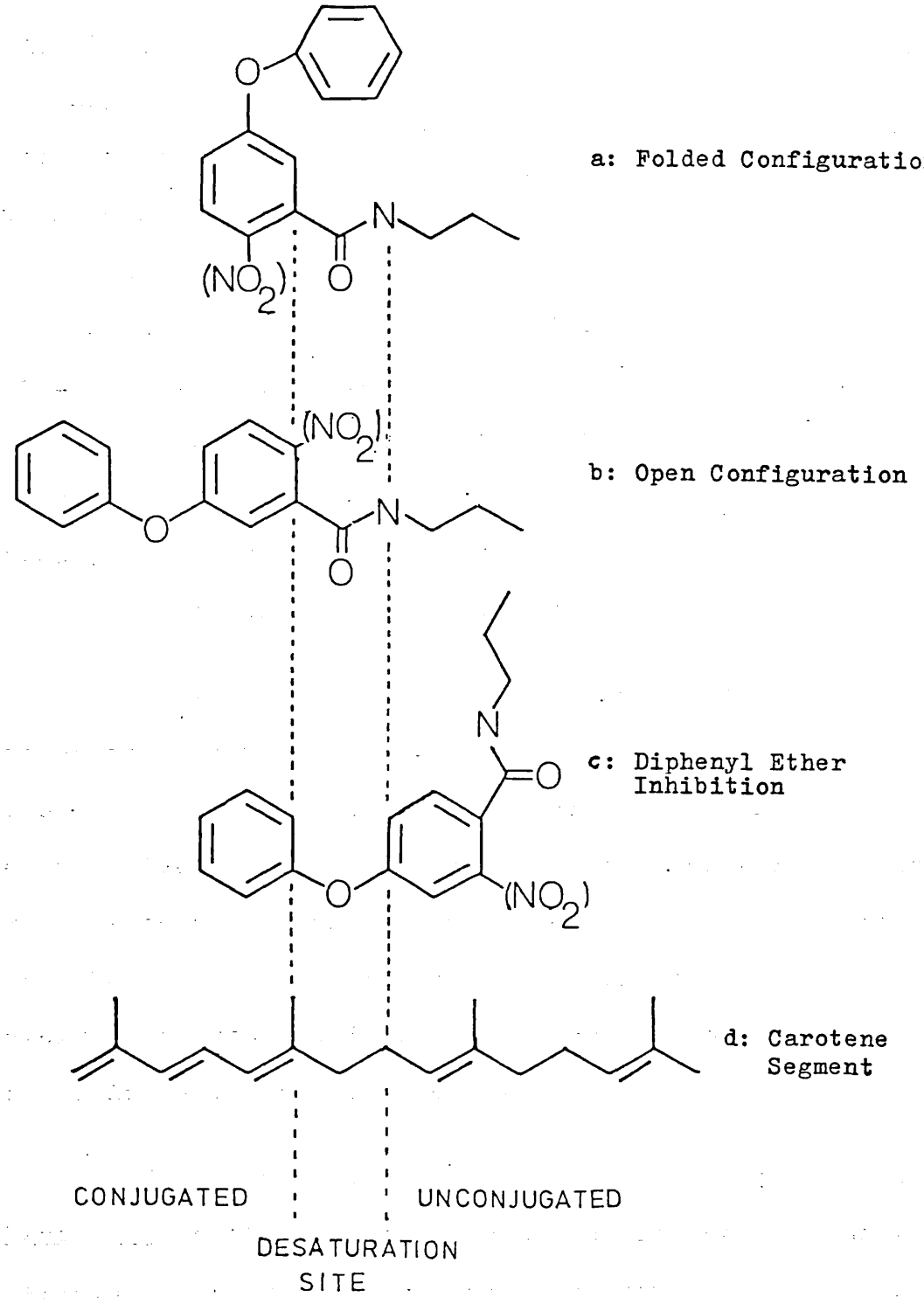
6:4 CONCLUSIONS

Rilling (1965) proposed that diphenyl compounds and phenazine dyes inhibited carotene biosynthesis in Mycobacterium spp. through competitive inhibition with partially saturated carotenes for the desaturase. This hypothesis may be extended to provide an explanation for the action of PABs on carotene desaturation in the two cell-free systems discussed above. The PAB can be thought of in terms of a partially saturated carotene (Fig. 6:1), possibly in transition state, such that the amide bond represents the bond that is to be desaturated, the benzamide ring the conjugated segment of the carotenoid and the alkyl chain the unconjugated region; the phenoxy ring is thought to give bulk and lipophilicity to the structure.

Whilst the results are compatible with this hypothesis, there is, as yet, little direct evidence in its support. However, such a model provides an explanation for the relatively poor potency of PABs possessing branched alkyl chains at the alpha carbon, but implies that branching at the beta-carbon should improve inhibitor activity. Unfortunately, no such PAB (e.g. 3-phenoxy-N-isobutyl-benzamide) was available to test this possibility.

The poor inhibitor activity of 6-nitro PABs in the Aphanocapsa system may be interpreted in these terms if it is assumed that the Aphanocapsa desaturase is inhibited only by PABs in the folded configuration (Fig. 6:1, a). If so, then any substitution in the 6-position of the benzamide

FIG. 6:1 PHENOXY ALKYL BENZAMIDES AS POTENTIAL COMPETITIVE INHIBITORS OF CAROTENE DESATURATION



ring would sterically hinder any association between enzyme and the conjugated aromatic ring. However, in the Phycomyces system such 6-nitro PABs have reasonable inhibitory activity. This may be due to the Phycomyces enzyme site being able to accommodate both folded and open PAB configurations (Fig.6:1, a,b) or to the enzyme being sensitive to the diphenyl ether (analogous to inhibition by DPA) in addition to the benzamide function (Fig. 6:1,c). These conjectures consider only the steric effects of the 6-nitro substitution on PAB inhibitor activity; substitution also affects the electronic nature of the ring and lipophilicity, both of which may play some role in this context.

CHAPTER 7

OVERALL COMMENTS AND CONCLUSIONS

7.1 THE CELL-FREE SYSTEMS

The cell-free systems discussed in this thesis may be considered as a first step towards the purification of the carotenogenic enzymes. In addition, these crude enzyme systems offer several advantages over whole cell cultures in assessing bleaching activity of herbicides. Results from the cell-free systems reflect the immediate effect of chemicals on existing enzymes, enabling true inhibitors of carotenogenesis to be distinguished from protein biosynthesis inhibitors and inhibitors that lead to the destruction of pigment molecules, e.g. by peroxidation. Since these systems are crude however, radioactive substrates are available to a number of terpenoid-synthesizing enzymes and the added cofactors available to many enzymes, any of which may influence carotene biosyntheses and be affected by various inhibitors. For these reasons, caution must be exercised when interpreting data on the effect of various chemicals on carotene biosynthesis, particularly when the effect is small and/or with respect to cofactor requirement.

Although these studies have not unequivocally shown that the system from Aphanocapsa is completely cell free, the amount of chlorophyll no longer present in whole cells after osmotic shock of the spheroplasts, is at least 90%: this suggests that a similar percentage breakage of cells has occurred (Bramley, personal communication). In addition there is evidence to suggest that the carotenogenically active component is cell free. Reaction media containing water or Tris-HCl (0.4M) washed

spheroplasts, procedures expected to promote lysis, were found to incorporate 6.5-7 times more radioactivity into carotenes than media containing spheroplasts that were suspended in stabilizing buffer prior to incubation (Clarke et al., 1982). Reaction media containing the whole spheroplast preparation would, at the end of incubation, be expected to contain a significant proportion of lysed spheroplasts. It is arguable that it is these lysed spheroplasts that are responsible for the modest carotenogenic activity of the 'intact' spheroplast preparation.

The experiments presented in this thesis indicate the cofactors and conditions necessary for maximum incorporation of radioactivity into β-carotene and the effect of three or four classes of inhibitor on carotene biosynthesis in the two in vitro systems. The cofactor requirements and the response to diphenyl compounds and phenoxy-alkyl-benzamides (PABs) proved to be essentially similar in both systems, although the response to phenylpyridazinone and phenylfurane herbicides was not the same. Thus it is presumed that whilst the carotenogenic apparatus in these two organisms is similar, there are some significant differences.

Experiments have shown that the PAB structure-activity relationship in cultures of Scenedesmus acutus is similar to that elucidated for the Aphanocapsa and Phycomyces cell-free systems (Lambert and Böger, 1984). This suggests that PABs may interact with a common, evolutionary stable site on the dehydrogenase. Whilst this is compatible

with the competitor hypothesis (see 6:4), recent kinetic experiments suggest that inhibition by S-3422 is non-competitive in the Aphanocapsa system (Sandmann and Bramley, personal communication). Therefore, the hypothetical evolutionary stable site appears not to be the active site of the dehydrogenase.

The difference in sensitivity between the two cell-free systems to the substituted phenylpyridazinones and phenylfuranes reflects differences in enzyme structure. It is possible that the phytoene desaturase of Phycomyces lacks (or has lost during preparation of the cell extract) the difunone binding site and that the phenylpyridazinone binding site is only partially present on this enzyme. Alternatively, both sites may be present, but not readily accessible to these herbicides.

7.2 PHYTOENE OVERACCUMULATION

The inhibitors discussed above all appear to act upon the carotene dehydrogenase, producing increased incorporation of [^{14}C] label into phytoene and a decreased incorporation into β-carotene. In both systems, particularly in that from Phycomyces, such inhibition causes more radioactivity to be incorporated into phytoene than is "lost" from β-carotene. Thus the question arises "Can it be assumed that all the accumulated phytoene *in vitro* is a result of dehydrogenase inhibition"? In the Phycomyces system it has been shown that acetone raises incorporation

of [^{14}C] label into phytoene at the expense of that into squalene, rather than β-carotene (see 4:2:2). Thus in Phycomyces at least there is another mechanism through which phytoene accumulates in vitro. There is no evidence in the results, however, to suggest that any other inhibitor of carotene formation in the Phycomyces system affects incorporation into squalene. Although there may be a little phytoene accumulated through inhibitor inter-action other than with the desaturase, the results suggest a relationship between the inhibition of β-carotene formation and the accumulation of phytoene. Thus it is concluded that with the inhibitors studied here, most if not all of the accumulated phytoene is a result of inhibition of desaturation.

Chemical inhibition of carotenogenesis is not the only mechanism through which phytoene is accumulated. In both cell-free systems incubation under anaerobic conditions resulted in phytoene over-accumulation and loss of β-carotene synthesis (see 3:3:1:2 and 3:3:2:2). When Phycomyces extracts were incubated in nitrogen no further phytoene accumulated in the presence of phenylpyridazinone inhibitors (see Table 4:7). Cell-free extracts from carA, carB, carR as well as carS mutants of Phycomyces were all found to accumulate a lot more phytoene in the presence of 100μM DPA (Clarke, et al., 1983). It was also found that cell-free preparations stored in ice for any period of time before incubation, or cell-free preparations from less than ideal material tended to incorporate more label into phytoene than

expected; incorporation into β-carotene was not significantly affected. Very little phytoene is detectable *in vivo*, yet the loss of two pyrophosphate groups from two molecules of GGPP as phytoene is synthesized makes this step thermodynamically very favourable.

The above observations suggest that under normal circumstances the activity of phytoene synthetase is somehow regulated and that this regulation is:

i) present in all *car*A, B, R, S mutants;
ii) is disrupted by DPA and (perhaps) other inhibitors;
iii) maybe oxygen dependent;
iv) is 'fragile' and prone to mechanical disruption and aging *in vitro*.

Considering these points, the following scheme has been constructed in an attempt to explain how phytoene over-accumulation results in cell-free extracts of *Phycomyces*.

It is assumed that phytoene synthetase exists in two states; i) a partially active form [I] that can convert GGPP into bound PPPP and ii) a fully active form that is capable of phytoene synthesis, [A]. Furthermore, it is suggested that bound to the desaturase complex is a transfer protein that activates phytoene synthetase [I] and transfers the nascent phytoene to the desaturase; the activity of this protein may be oxygen dependant. It is proposed that phytoene is synthesized and transferred to the desaturase as follows:-

i) phytoene synthetase-I binds two molecules of GGPP that are converted into bound PPPP;

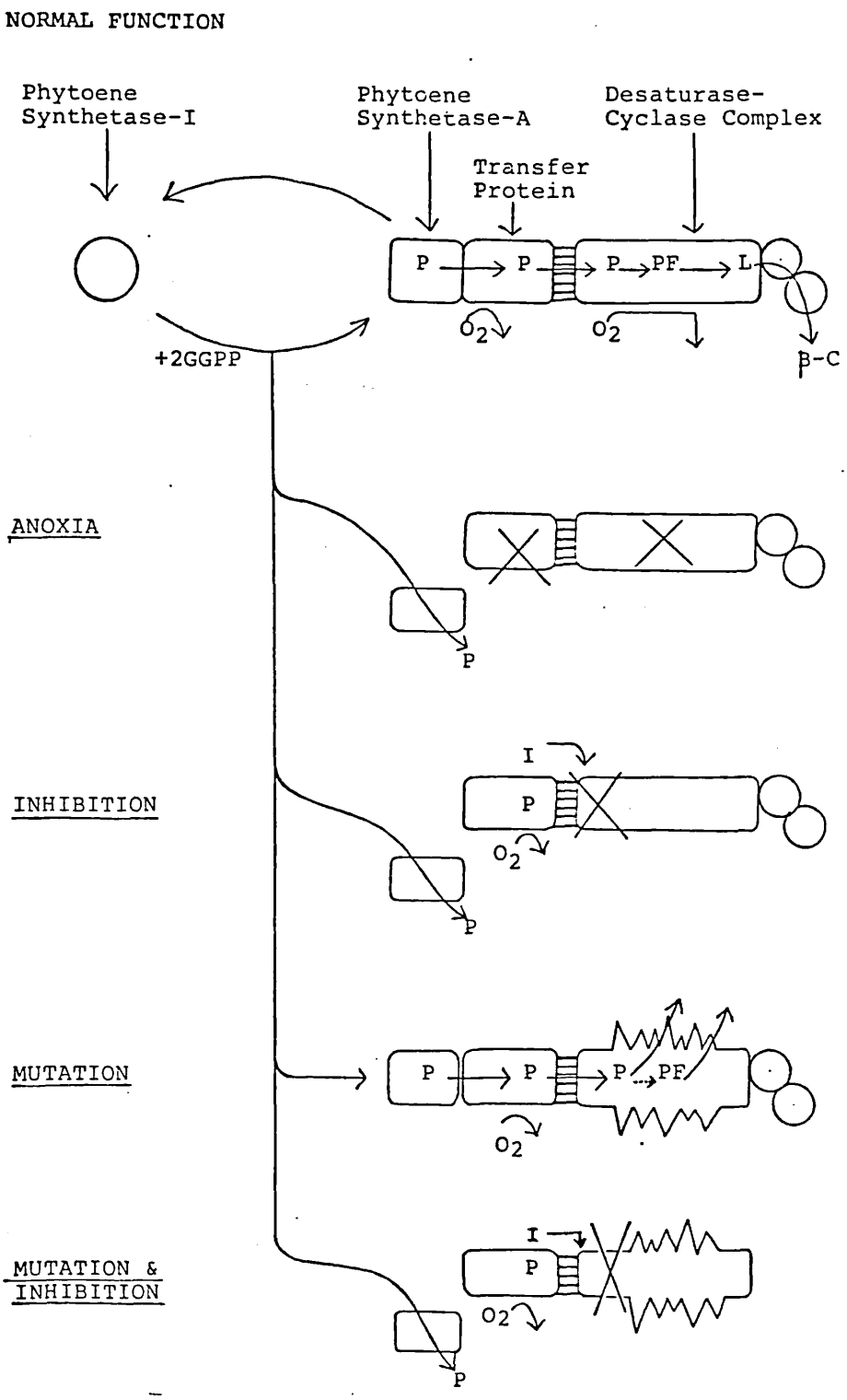
ii) the bound PPPP is attractive to the transfer protein and causes phytoene synthetase-I to bind. Upon binding conformational change produces the active phytoene synthetase-A;

iii) phytoene synthetase-A converts PPPP into phytoene and a more relaxed phytoene synthetase = transfer protein complex results from the loss of PPPP;

iv) phytoene is then transferred from the synthetase to the desaturase and the transfer results in further conformational change such that phytoene synthetase-I is reformed that dissociates from the transfer protein.

It is suggested that phytoene over-accumulation results when flux through the transfer protein is inhibited such that active phytoene synthetase eventually dissociates from the complex without the transfer of phytoene and consequently without the de-activating conformational changes. Inhibitors such as DPA are proposed to bind in such a way as to obstruct the passage of phytoene from the transfer protein to the desaturase and the transfer is easily hindered by mechanical shock. To explain the effect of anoxia on carotene synthesis it is necessary to postulate that the activity of the transfer protein is dependent upon oxygen. Quite what oxygen is needed for is not known and

FIG. 7:1. PROCESSES LEADING TO PHYTOENE OVER-ACCUMULATION



KEY : P=Phytoene L = Lycopene I = Inhibitor
PF = Phytofluene βC = β-Carotene

experiments designed to observe the quantity and type of phytoene produced from the various mutants in anoxic conditions would be most useful. The processes leading to phytoene over-accumulation are summarized in Fig. 7:1.

The hypothetical transfer protein may well be the carA product. Cell-free extracts of the C2 strain of Phycomyces (a carA mutant) incorporate comparatively little radioactivity into phytoene..... perhaps as a result of inefficient phytoene synthetase activation. However, if this is so then it would be expected that certain carA mutations would result in constitutive phytoene over-accumulation.

7.3 FUTURE WORK

Chemical inhibition of carotenogenesis represents a largely unexploited means of weed control. Whilst cell-free systems, such as those described in this thesis, and also various seedlings (see 1:4), have been used to screen potential bleaching herbicides, very little work has been reported using plants against which such herbicides would be employed. The net result is the development of 'herbicides' that are potent inhibitors of carotenogenesis in the test systems, but are not necessarily effective against crop pests: e.g., the experimental herbicides that Ridley (1982) found to be particularly effective against barley seedlings (see 1:4:5, 1:4:6) were not so against greenhouse cultivated weed species (ICI, personal communication). It would, therefore, be advantageous if research into novel herbicides

were not so divorced from target species. Whilst the preparation of carotenogenic cell-free extracts from weed species may not be possible for some time, a comparison of the effects of bleaching herbicides on the available cell-free systems and cultivated weed species would help to determine any similarities between them and also to assess how useful the cell-free systems are in screening.

Arguments have been forwarded to suggest the use of algae rather than higher plants to screen potential herbicides by Sandmann and co-workers (1979) who argue that:

1. Suitable species can be easily cultured under semi-sterile conditions in well defined liquid and simple media with high growth rates under continuous light.

2. Controlled growth conditions can be maintained.

3. Herbicides can be applied exactly via the culture medium.

4. Inhibitor effects develop quickly after application.

5. Absence of long-distance transport allows for small substance concentrations to be tested.

6. Absolute quantitative changes of cellular compounds can be rapidly and reproducibly determined when referred to the constant culture volume.

7. Determination of uptake, accumulation and excretion of herbicides (and metabolites) can be followed.

Similar arguments can be used to advocate algae (or other primitive organisms) as choice material for the preparation of cell-free extracts. The challenge thus lies in developing an algae system, or a combination of systems, appropriate to the target plant species.

Following the development of a crude cell-free system it is desirable to attempt to purify the component carotenogenic enzymes. Purer enzyme preparations would establish more accurately the effect of a herbicide on, or the cofactor requirements of, a particular reaction step, since the observed enzyme activity would be free from the influence of other enzymes. Enzyme purification would result in a better understanding of the organisation of carotenogenic enzymes in the various species as well as being a step towards elucidating enzyme reaction mechanisms. An understanding of both these aspects would in turn help not only to explain the herbicidal action of existing compounds, but help in the design of novel herbicides. Hopefully the nature of the carotenogenic enzymes of weed species (or higher plants in general) is sufficiently different from any mammalian enzyme to allow the development of a potent herbicide that is relatively non-toxic to man.

Having suggested how a deeper knowledge of carotenogenic enzymes may be useful in the design of novel herbicides, it is possible that herbicides can be used to

aid investigations of enzyme structures and mechanisms. A herbicide that is in competiton with the carotene substrate reflects the nature of the active site and possibly the nature of the substrate in a transition state. Structure activity studies on herbicides that can be shown to exert competitive inhibition could, therefore, provide clues as to how the enzyme operates. It would be useful to determine the type of inhibition each herbicide group exerts and whether herbicides exhibiting the same mode of inhibition compete for the same site. Such studies conducted with several cell-free systems will help identify specific herbicide target sites and give a clearer indication of differences in enzyme structure between systems: identification of target sites will aid herbicide design, whilst determination of differences in enzyme structure would help assess the validity of a particular system for screening.

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CAROTENE BIOSYNTHESIS WITH ISOLATED PHOTOSYNTHETIC MEMBRANES

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1. Introduction

There are several cell-free systems from eukaryotic organisms capable of in vitro carotenogenesis. The system from the fungus *Phycomyces*, converting mevalonic acid (MVA) into β-carotene has been extensively investigated [1,2]. A homogenate from *Neurospora* can synthesize phytoene from MVA and other prenyl pyrophosphates [3,4]. Isolated tomato [5], *Narcissus*-flower [6], and *Capsicum*-fruit [7] chromoplasts are reported to convert isopentenyl pyrophosphate (IPP) into β-carotene and be able to catalyze other reactions along this pathway. Recently, an *Amphidinium* homogenate has been found to synthesize xanthophylls and β-carotene from MVA [8].

We report here on a cell-free carotenogenic system from the blue-green alga *Aphanocapsa:* the first such system from an autotrophic prokaryote. The system is photosynthetically active and converts geranyl-geranyl pyrophosphate (GGPP) into β-carotene and xanthophylls. Data are presented showing the effects of various cofactors and 2-phenylpyridazinone analogs [9] on the carotenogenic ability of this system.

2. Materials and methods

Aphanocapsa (ATCC 6714) was cultivated for 4 days in 1-l Fernbach flasks at 35°C under 15 W/m² fluorescent light. Cultures were harvested with chlorophyll contents of 15 μg/ml. Growth medium and general conditions are described in [10]. Spheroplasts were prepared by lysozyme treatment (8 mg/ml; Sigma, Munich) according to [11] for 2 h at 35°C and then suspended in 10 mM tricine–NaOH buffer (pH 7.8), [*N*-tris-(hydroxymethyl)-methyl glycine] with 10 mM $MgCl_2$, 2.5 mM Na_2HPO_4, 2.5 mM K_2HPO_4, and 0.5 M sucrose. The untreated suspension was diluted 10-fold and washed with either water or 0.4 M Tris–HCl (pH 7.8), [tris-(hydroxymethyl)-amino methane]. Washed suspensions were spun down and the pellet resuspended in 0.4 M Tris–MCl (pH 7.8), to a chlorophyll concentration of 500 μg/ml (washed thylakoid suspension).

The incubation medium was essentially that used with *Phycomyces* extracts [12] in a final volume of 500 μl. Radioactive substrates were equivalent to 0.5 μCi of either GGPP, IPP or MVA (specific activities 4.22, 55 and 10 mCi/mmol, respectively). Unless stated otherwise, extracts having a pH of 7.5 were incubated for 90 min, at 20°C, under 35 W/m² white light, and contained 100 μg of chlorophyll. Reaction was stopped by adding methanol (3.5 ml) and 0.5% acetone (v/v) in petrol, b.p. 50–70°C (9 ml). The tubes were shaken vigorously and the contents poured into a mixture of petrol (10 ml) and saturated NaCl solution (50 ml). The petrol phase was collected and the solvent evaporated under nitrogen. The residue was redissolved in 50 μl of petrol, placed on silica-gel plates (Merck, Darmstadt) and developed in 7% (v/v) benzene in petrol. The yellow band was removed, the carotenes eluted with diethyl ether, reduced to 200 μl in volume under N_2, and applied to activated Al_2O_3 plates (fluorescent type: Merck, Darmstadt) together with phytoene marker. These were firstly developed for 5 min with 0.6% acetone (v/v) in petrol and subsequently in a chamber containing only petrol. The β-carotene and phytoene bands were scraped off and transferred into scintillation vials filled with 3 ml of Unisolve I (Zinsser, Frankfurt) and assayed for radioactivity by liquid scintillation. Data are means of four determinations: phytoene and β-carotene were mea-

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sured by two independent incubations each done in duplicate.

[2-^{14}C]Geranylgeranyl pyrophosphate was obtained from [2-^{14}C]geranylgeraniol which was chemically prepared from [2-^{14}C]ethylbromacetate (Amersham-Buchler, Braunschweig) and all-trans farnesyl acetone according to [13]. [2-^{14}C]mevalonic acid lactone and [1-^{14}C]isopentenyl pyrophosphate were purchased from the same company.

3. Results and discussion

Table 1 shows how carotenogenic activity of this system varies with preparation. Untreated spheroplast preparations (no. 1) convert less GGPP but to a lower phytoene:β-carotene incorporation ratio than do resuspended ones (no. 2–4). The increased phytoene incorporation in resuspended preparations could be due to phytoene synthase dissociating from the dehydrogenase complex. No phytoene accumulation is found in intact cells of *Aphanocapsa*. As yet, only carotenogenic systems from chromoplasts yield such an efficient phytoene conversion in vitro with a ^{14}C-ratio of phytoene:β-carotene incorporation of 0.5 [6,7]. Fungal systems show comparatively poor phytoene desaturation in vitro [2,4,12].

A time-course experiment (fig.1) showed significant incorporation into phytoene and β-carotene from labeled GGPP after only 10 min incubation. Incorporation into phytoene increased only slightly during the 135 min incubation with no significant deflection as was reported for the *Narcissus* chromoplast time course [6]. We suggest that in *Aphanocapsa*, a tight link exists between phytoene synthesizing and metabolizing enzymes allowing rapid phytoene dehydrogenation. β-Carotene formation showed a sigmoidal time course while saturation was not obtained within 2 h. It should be noted that this system also synthesizes xanthophylls, but this is the subject of another publication.

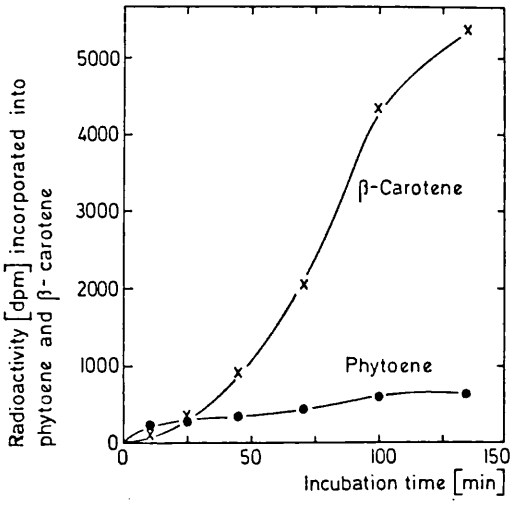


Fig.1. Time course of formation of phytoene and β-carotene from [^{14}C]geranylgeranyl pyrophosphate.

Cofactor requirements for carotenogenesis were assayed with water-washed spheroplasts. It is evident from the data (table 2, no. 2) that ATP stimulates phytoene synthesis from GGPP. A similar stimulating effect of ATP on the conversion of GGPP into phyto-

Table 1
Incorporation of radioactivity (dpm) from [^{14}C]substrates equivalent to 0.5 μCi by thylakoids from *Aphanocapsa* equivalent to 100 μg of chlorophyll

No.	Spheroplast treatment, substrates added	Radioactivity incorporated into			^{14}C Ratio of synthesized phytoene/β-carotene
		phytoene	β-carotene	phytoene + β-carotene	
(1)	Untreated + MVA[a]	0	0	0	–
	Untreated + IPP[a]	0	0	0	–
	Untreated + GGPP	50	1098	1148	0.05
(2)	Resuspended in Tris-buffer + GGPP (0.4 M, pH 7.8)	693	6953	7646	0.10
(3)	Washed with buffer, then resuspended + GGPP	873	6759	7632	0.13
(4)	Washed with water, then resuspended + GGPP	1033	7060	8093	0.15

[a] Incorporation of radioactivity into petrol phase (see section 2) from MVA was 1794 dpm and from IPP 1999 dpm

Table 2
Requirement of cofactors, oxygen, and light for the conversion of geranylgeranyl pyrophosphate into phytoene and β-carotene

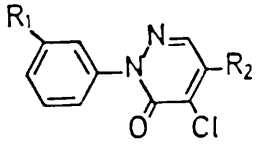
	Assay composition and conditions	Radioactivity (dpm) incorporated into			^{14}C Ratio of synthesized phytoene/β-carotene
		phytoene	β-carotene	phytoene + β-carotene	
(A)	All incubations aerobic and illuminated				
	(1) All nucleotides (ATP, NAD, NADP and FAD) or ATP and NAD/NADP only or ATP and FAD only	1033	4465	5498	0.23
	(2) All nucleotides except ATP	226	1942	2168	0.12
	(3) ATP only	631	3121	3752	0.20
	(4) No nucleotides	Backgrd. (75)	288	–	–
(B)	All incubations contain all nucleotides				
	(5) Aerobic and dark	832	4569	5401	0.18
	(6) Anaerobic and light	1162	4306	5468	0.27
	(7) Anaerobic and dark	5324	467	5791	11.4

Aphanocapsa spheroplasts washed with water (100 μg chlorophyll) were incubated with GGPP equivalent to 0.5 μCi over 90 min. For complete medium see section 2. Expt. no. 7,8 were done in Thunberg tubes which were evacuated by a water-jet pump until foaming had stopped

ene was reported for a tomato-plastid preparation [14]. Our studies suggest that *Aphanocapsa* requires only ATP plus either NAD, NADP, or FAD for carotenogenesis from GGPP (table 2, no. 1–4). A similar nucleotide requirement is found with gel-filtered cell-free extracts from the C115 strain of *Phycomyces* (I. E. C. et al., unpublished). The nucleotide requirement in tomato chromoplasts is more specific: phytoene dehydrogenation requires NADP whilst lycopene cyclization requires FAD [15].

Light had no effect on total carotenogenesis in aerobic and anaerobic preparations (table 2, no. 5–7).

Table 3
Influence of 2-phenylpyridazinone derivatives on the conversion of geranylgeranyl pyrophosphate into phytoene and β-carotene by a cell-free system from *Aphanocapsa*

Additions (1 μM)	Radioactivity (dpm) incorporated into		^{14}C Ratio of synthesized phytoene/β-carotene
	phytoene	β-carotene	
			
No. R_1 R_2			
(1) CF_3 $NHCH_3$	9907	1834	5.40
(2) CF_3 OCH_3	2256	3679	0.62
(3) H OCH_3	727	3824	0.19
(4) Control, no addition	624	4033	0.16

Spheroplasts washed with buffer (100 μg chlorophyll) were incubated with GGPP equivalent to 0.5 μCi for 90 min

As washed thylakoid preparations photosynthetically produce oxygen in the light, without electron donors or acceptors added (80–100 μmol/mg chlorophyll X h), it is suggested that phytoene desaturation is oxygen-dependent. In anaerobic dark samples, conversion of phytoene to β-carotene was drastically inhibited (no. 7).

In higher plants and green algae, certain 2-phenyl-pyridazinones inhibit phytoene desaturation [9,16]. The effect of such compounds on carotenogenesis in our cell-free system is presented in table 3. It is seen that norflurazon (compound no. 1), BAS 44521 (no. 2), and BAS 13761 (no. 3) exhibit a strong, intermediate and weak effect on phytoene desaturation, respectively. These results conform with whole-cell studies using *Scenedesmus* [17] and *Aphanocapsa* cultures. A good correlation between in vivo and in vitro studies in response to herbicides makes the *Aphanocapsa* cell-free system ideal for testing potencies of other bleaching herbicides on photosynthetic tissue.

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HERBICIDE ACTION ON CAROTENOGENESIS IN A PHOTOSYNTHETIC CELL-FREE SYSTEM

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INTRODUCTION

Herbicides such as norflurazon [SAN 9789, 4-chloro-5-methylamino-2-(3-trifluoromethylphenyl)-pyridazin-3(2H)one] (1) and difunon [EMD-IT 5914, 5-(dimethylaminomethylene)-2-oxo-4-phenyl-2,5-dihydrofurane-carbonitrile-(3)] (2,3) are known to inhibit phytoene desaturation preventing the biosynthesis of coloured carotenes capable of photoprotection. The phytotoxic effect is due to a decrease in β-carotene and accumulation of phytoene, the latter not being able to protect chlorophyll. Screening tests using cultures of the green alga Scenedesmus acutus have shown the change in potency in response to modifying these herbicides (4). Such structure-activity studies have been extended to an in-vitro carotenogenic (thylakoid) system from the blue-green alga Aphanocapsa that synthesizes labelled carotenes from [2-^{14}C]-geranylgeranyl pyrophosphate (GGPP) (5).

This communication reports on structure-activity studies using the in vitro Aphanocapsa system. The norflurazon and difunon derivatives investigated here are restricted to aromatic ring substitutions or to some replacements (difunon only) on the parent molecule (Fig.1). Results from this cell-free system are compared to those obtained with intact Scenedesmus (comp. ref.[6] for 2-phenylpyridazinones).

METHODS

Spheroplasts were prepared from 4 day old Aphanocapsa cultures by lysozyme treatment according to (7). This preparation was washed in excess 0.4 M tris-HCl, pH 7.8 [tris-(hydroxymethyl)-aminomethane], pelleted and resuspended in the same buffer to a chlorophyll concentration of between 250-300 µg/ml. Reaction -

mixtures containing 0.2 ml of the washed thylakoid suspension were incubated for 2 h at 20°C with the equivalent of 0.5 µCi [2-^{14}C] GGPP, 4 mM $MgSO_4$, 6 mM $MnCl_2$, 20 mM glutathione, 10 mM ATP, 1 mM NAD, and 5 µl herbicide solution in a final volume of 0.5 ml. Reactions were stopped with the addition of 3.5 ml methanol. The mixture was analysed for incorporation of label into phytoene and β-carotene and compounds soluble in petrol ether, as described in (5).

Herbicides were dissolved in methanol (norflurazon and derivatives) or acetone (difunon and derivatives) to a stock concentration of 10^{-3}M and diluted as required. The herbicides investigated are shown in Table 1 (norflurazon derivatives) and Table 2 (difunon derivatives).- Scenedesmus was grown according to (4).

RESULTS AND DISCUSSION

The results of experiments using the in vitro system of Aphanocapsa are presented in Tables 1 and 2 whilst the I_{50} concentrations deduced using intact cells of Scenedesmus are presented in Table 3.

Norflurazon derivatives

The results presented in Table 1 show that apart from the m-SCF_3-phenyl and m-CF_3-phenyl (norflurazon) derivatives, the Aphanocapsa thylakoid suspension was quite insensitive to the other compounds at a 10^{-6}M concentration. Carotenogenesis was significantly inhibited in this system when incubated with 10^{-5}M concentrations of the p-CF_3-phenyl, m-OSO_2CH_3-phenyl, and m-CN-phenyl derivatives. The remaining compounds (m-NO_2, m-OCH_3 and m-C_6H_5-phenyl derivatives) were not effective even at this concentration (data not shown).

A comparison of Tables 1 and 3 shows that, under the experimental conditions, cell-free preparations from Aphanocapsa were not as sensitive to norflurazon-type compounds as intact Scenedesmus. Whilst both systems were most sensitive to the m-SCF_3 and m-CF_3-phenyl derivatives, the Aphanocapsa cell-free system was comparatively more sensitive to the p-CF_3-phenyl and m-OSO_2CH_3-phenyl compounds than the m-CN- or m-NO_2-phenyl derivatives. In Scenedesmus cells, this sensitivity was reversed.

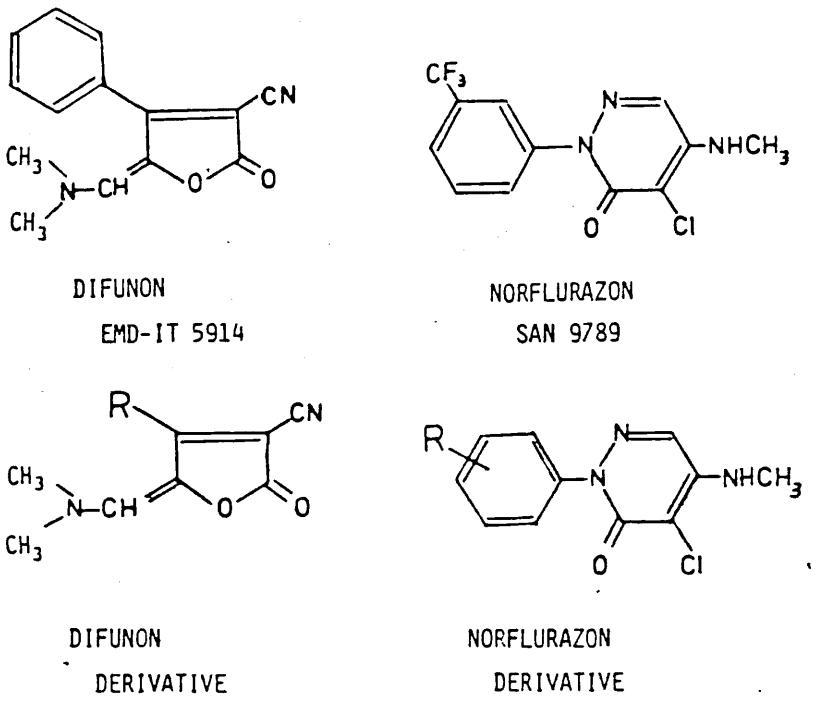


Fig. 1. Structure of difunon and norflurazon with the general structures of the derivatives reported in this communication.

TABLE 1

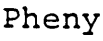
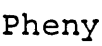
THE EFFECT OF NORFLURAZON DERIVATIVES ON CAROTENOGENESIS

Aphanocapsa thylakoid suspension incubated with 10^{-6}M herbicide and GGPP equivalent to 0.5 µCi for 2 h at 20°C.

Substituents at the phenyl ring	Radioactivity (dpm) incorporated into: phytoene	β-carotene	^{14}C ratio of synthesized phytoene/β-carotene
Control	635.7	3169	0.20
m-SCF_3	12540	737.8	17.0
m-CF_3[a)]	5264	1881	2.8
p-CF_3	731	1607	0.45
m-OSO_2CH_3	926	2446	0.37
m-CN	886.5	3246	0.27
m-NO_2	655.7	2718	0.24
H-	504	2978	0.18
m-OCH_3	727	3190	0.20

a) norflurazon

TABLE 2
THE EFFECT OF DIFUNON DERIVATIVES ON IN-VITRO CAROTENOGENESIS
Aphanocapsa thylakoid suspension incubated with various concentrations of herbicide and GGPP equivalent to 0.5 μCi for 2 h at 20°C.

Difunon derivative (R structure, see Fig.1)	Radioactivity (dpm) incorporated into: phytoene	β-carotene	^{14}C ratio of synthesized phytoene/β-carotene
A. Concentration: 10^{-7}M			
m-SCH_3-phenyl	9600	1112	8.7
m-C_6H_5-phenyl	8170	1509	7.2
m,p-Cl_2-phenyl	6274	1430	4.4
m-OCH_3-phenyl	6443	2024	3.2
m-CF_3-phenyl	6149	2216	2.8
Phenyl (difunon)	2426	2668	0.92
B. Concentration: 10^{-6}M			
Phenyl (difunon)	5427	478	11.2
o-Cl-phenyl	4414	1029	4.3
Phenyl;  N-CH=[a]	1515	1329	1.1
C. Concentration: 10^{-5}M			
Phenyl;  N-CH=[a]	5444	1538	3.5
o,p-Cl_2-phenyl	3265	2127	1.5
p-C_6H_5O-phenyl	502	3082	0.16
Cyclohexyl	637	4188	0.15
Control (no addition)	400	2743	0.15

[a] This group replaces $(CH_3)_2N$-CH= in difunon.

Difunon derivatives

It is observed from Tables 2 and 3 that cell-free thylakoid incubations are as sensitive to difunon derivatives as intact Scenedesmus cells. Although the potency order of the highly effective derivatives (i.e. those more potent than difunon) is somewhat different for the systems, both systems contain the same derivatives in that category. It is also noted that both systems show a marked difference in sensitivity between the highly effective

analogs and difunon. It is tempting to suggest that permeability across the membrane of Scenedesmus cells causes the halogenated derivatives (m,p-Cl_2-phenyl and m-CF_3-phenyl) to be comparatively more potent with intact cells (Table 3) and the m-OCH_3-phenyl and m-SCH_3-phenyl analogs to be less potent with respect to incubations with Aphanocapsa thylakoid suspensions (Table 2). Experiments are under way to test this hypothesis by cultivating Aphanocapsa in difunon-derivative-containing media.

The similar sensitivity of these two systems towards difunon derivatives holds for compounds that are less effective than difunon. The only discrepancy is the relative potency of the o-Cl-phenyl derivative which is more effective in the Aphanocapsa cell-free system. Even at high concentrations, both systems were insensitive to the p-phenoxyphenyl and the cyclohexyl analog.

TABLE 3

I_{50} CONCENTRATIONS FOR INTERFERENCE WITH CAROTENOID FORMATION OF SCENEDESMUS BY NORFLURAZON AND DIFUNON DERIVATIVES

Norflurazon derivatives (Substituents at the phenyl ring)	I_{50} [10^{-8}M] [a]	Difunon derivatives (R structure, see Fig.1)	I_{50} [10^{-8}M] [b]
m-SCF_3	0.4	m,p-Cl_2-phenyl	5
m-CF_3	1	m-C_6H_5-phenyl	10
m-CN	8	m-CF_3-phenyl	10
m-NO_2	10	m-SCH_3-phenyl	12
p-CF_3	71	m-OCH_3-phenyl	15
H-	180	H-(difunon)	70
m-OSO_2CH_3	360	N-CH=	100
m-OCH_3	1400	o-Cl-phenyl	120
		o,p-Cl_2-phenyl	500
		p-C_6H_5O-phenyl	i.n.d. [c]
		cyclohexyl	i.n.d. [c]

[a] taken from [6]
[b] Unpublished data from B.Boehler-Kohler et al., this laboratory
[c] Inhibition not detectable.

CONCLUSION

Whilst cell-free preparations from Aphanocapsa show comparable sensitivity to difunon derivatives as intact Scenedesmus cells, the same cannot be said of the sensitivity towards norflurazon derivatives. A Hansch equation, based on Hammett parameters and a lipophilicity factor, has been prepared predicting the potency of norflurazon derivatives in Scenedesmus cultures (6). In this equation, the lipophilicity factor is relatively unimportant. This is also apparent in the Aphanocapsa system as the potency of the norflurazon analogs does not correlate with the lipophilicity factor. The differing sensitivities probably reflect different protein structures of the desaturation enzymes between the eukaryote Scenedesmus and the prokaryote Aphanocapsa. We may conclude that the inhibition sites for these two herbicide types are different. The difunon site is of a similar nature in both eukaryote and prokaryote, whereas the norflurazon site appears to be only partially "realised" in the prokaryotic system.

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Inhibition of Phytoene Desaturase – the Mode of Action of Certain Bleaching Herbicides

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Bleaching Herbicides, Cell-Free System, Carotene Biosynthesis, Phytoene Desaturase, Inhibitors

Assay systems have been developed in order to differentiate between the modes of action of certain bleaching herbicides. These include inhibition of chlorophyll or carotenoid biosynthesis, and initiation of pigment degradation. Herbicidal compounds with phytoene desaturase as their primary target site were investigated in a cell-free carotenogenic system from *Aphanocapsa*. In a comparative study, the structural prerequisites for inhibition of phytoene desaturase were established for both benzophenone analogs and various *m*-phenoxybenzamides. This inhibitory action of the latter compounds is enhanced by lipophilic groups with certain steric properties.

Introduction

Herbicides are directed towards various processes in plants. A typical example of a plant-specific target is the (plastidic) photosynthetic apparatus. Here, the so-called bleaching herbicides interfere with the chlorophyll and carotenoid content of the organelle [1, 2].

The bleaching herbicides consist of a group of compounds which are heterogenous in their modes of action. The term "bleaching" refers to a decrease in the amount of pigment after a certain period of growth in the presence of a herbicide as compared to an untreated control. This pigment deficiency can be caused either by initiation of pigment degradation [3] or by inhibition of biosynthesis of either chlorophylls or carotenoids [4]. In this study, we report on several means to elucidate the primary mode of action of different types of bleaching herbicides. In addition, data are presented concerning the structural requirements of some groups of herbicidal compounds that inhibit phytoene desaturation.

Materials and Methods

The microalgae *Scenedesmus acutus* (276-3a, Algal Culture Collection of the University of Göttingen) and *Aphanocapsa* strain 6714 were grown in 1-l flasks under sterile conditions. Media and cultivation conditions for heterotrophic *Scenedesmus* and *Aphanocapsa* have been given elsewhere [5, 6]. Growth was determined as packed cell volume in graduated microcentrifuge tubes of 80 µl capacity.

Chlorophyll determination was carried out after hot methanol extraction (65 °C, 15 min) according to McKinney [7]. Carotenoids were extracted from cells with hot methanol containing 6% KOH (w/v). After transfer into petrol (b.p. 60 to 80 °C), the content of β-carotene was estimated from the 445-nm absorbance peak with an extinction coefficient $E^{1\%}_{1cm} = 2500$. The absorbance maximum at 287 nm was taken for calculation of the phytoene concentration.

Thylakoid membranes used for cell-free carotene biosynthesis were prepared by lysozyme treatment (3 mg/ml; 2-h incubation at 35 to 37 °C). The membranes were collected by centrifugation (3 min, $1000 \times g$), washed with 0.4 M Tris-HCl buffer, pH 7.8, and resuspended in the same buffer. Details of incubation with [2-^{14}C]geranylgeranyl pyrophosphate (GGPP) and herbicidal compounds, separation and purification of carotenes by TLC, as well as measurement of radioactivity incorporation have been previously described [8].

Lipid peroxidation was assayed by monitoring the degradation of plastidic sulfolipid of *Scenedesmus* cells in the presence of herbicides. The procedure of prelabeling the sulfolipid was described in a previous publication [9]. *Scenedesmus* was grown for

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2 days in a medium containing 10 μCi [^{35}S]sulfate per liter. Subsequently, the cells were transferred into media with unlabeled sulfate, then the herbicides were added and the cells kept under culture conditions for 48 h.

Herbicides (99% pure) were added from 10 mM stock solutions in methanol or methanol/dimethylformamide (1:1, v/v).

The herbicidal compounds used in this study were kindly provided by the following companies: BASF AG, Ludwigshafen/Limburgerhof, Germany, nos. 4, 5, 12, 14–24; Celamerck, Ingelheim, Germany, 4-chloro-m-phenoxy benzoic acids for the synthesis of no. 26; Ciba-Geigy, Basel, Switzerland, no. 9; Eli Lilly Co., Indianapolis, Indiana, USA, no. 6; ICI, Bracknell, UK, no. 12a; May and Baker Ltd., Dagenham, UK, no. 7; Mitsubishi Chemical Industries Ltd., Yokohama, Japan, no. 2; Rhône-Poulenc SA, Vitry-sur-Seine, France, nos. 1, 8; Rohm and Haas, Spring House, PA, USA, no. 11; Stauffer Chemical Company, Richmond, CA, USA, no. 10; Sandoz AG, Basel, Switzerland, no. 13; and Sumitomo Ltd., Takarazuka, Japan, nos. 25, 27.

Results and Discussion

The green alga *Scenedesmus* can be grown under heterotrophic conditions in the dark with a fully developed photosynthetic apparatus, thus preventing photooxidation of pigments. The effects of certain compounds on the contents of chlorophylls, colored carotenoids, and phytoene under these growth conditions are shown in Table I. Compounds of group (A) affect the chlorophyll concentration, but do not interfere with the carotene content. As can be seen from the applied concentrations, MK-616 (no. 2) is more effective than oxadiazon (no. 1) and DTP (no. 3). We tentatively assume that these three compounds are inhibitors of chlorophyll formation. Specific assay systems have to be developed to demonstrate the inhibitory site in the chlorophyll-biosynthetic pathway.

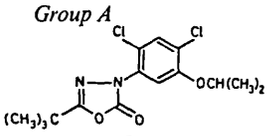
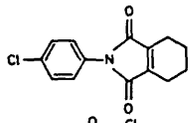
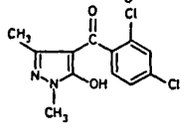
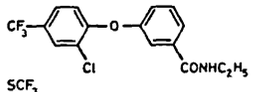
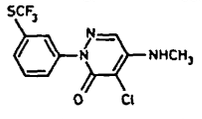
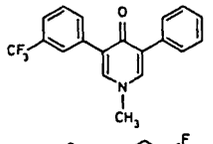
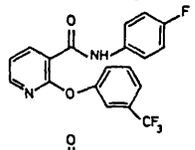
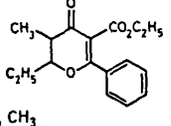
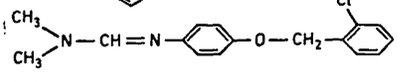
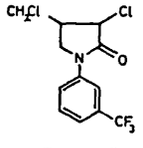
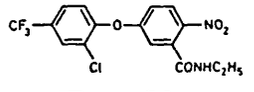
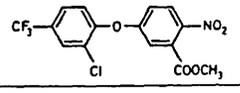
Compounds in group (B) and (C) decrease both chlorophylls and carotenoids. In the presence of the compounds (B), inhibition of carotenoid formation is about twice as strong as inhibition of chlorophyll formation. In contrast with (C), application of these compounds results in a similar decrease of *both* the chlorophyll and carotenoid content. Accumulation of phytoene was evident with compounds of group (B) only. This strongly suggests that these chemicals interfere with carotene biosynthesis as proposed previously for compounds nos. 4 [10], 5 [11], 6 [12], 8 [13], and 10 [14]. A concurrent decrease in chlorophyll content with chemicals of group (B) is regarded as a secondary response to carotenoid deficiency of the cells which is much smaller or nil using shorter herbicide treatments, *e.g.* 1 day instead of 2 [10, 15].

Acifluorfen methyl (no. 11) and the other nitrodiphenyl ether (no. 12) of group (C) are regarded as peroxidative compounds [16]. Specific assays for peroxidative action and for inhibitors of carotene biosynthesis are available [4, 17]. This allows a better discrimination of both types of bleaching herbicides. Therefore, the influence of oxadiazon, a substituted phenoxybenzamide, and its corresponding nitro derivative (no. 12), each taken from one of the three herbicide groups of Table I, on a cell-free carotenogenic enzyme system and on the content of a plastidic lipid was investigated.

As shown in Table II, [2-^{14}C]GGPP is efficiently converted into β-carotene, with comparatively low incorporations into phytoene and phytofluene. In the presence of the phenoxybenzamide (no. 4), however, the flow of radioactivity into β-carotene is inhibited and an accumulation of radioactivity into phytoene and phytofluene can be observed. A useful parameter for inhibition of cell-free carotene biosynthesis, the ratio of phytoene/β-carotene, increased over 30-fold. In contrast, oxadiazon, the nitrophenoxybenzamide (no. 12), and fomesafen gave no pronounced effect. The latter finding corroborates the results with intact *Scenedesmus* [10]: a p-NO_2 group apparently alleviates inhibition of carotenogenesis by a compound like no. 4.

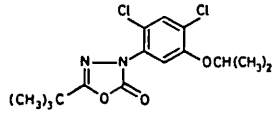
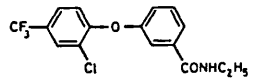
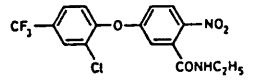
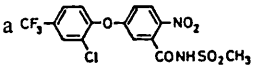
Table III shows the initiation of peroxidative degradation of prelabeled sulfolipid in *Scenedesmus* cells. Over a growth period of 2 days, the amount of sulfolipid was unchanged and no physiological degradation was observed [9]. Oxadiazon and the phenoxybenzamide exhibited no peroxidative properties at concentrations of 1 μM, whereas the nitro derivative (no. 12) is responsible for a 50% degradation of prelabeled sulfolipid. The peroxidative properties of compound no. 12, a nitrodiphenyl ether, were already demonstrated through light-induced formation of short-chain hydrocarbons by *Scenedesmus* cells [10].

Table I. Pigments in heterotrophic *Scenedesmus* grown for 2 days in the presence of inhibitors of chlorophyll (A) and carotenoid (B) biosynthesis or in the presence of peroxidative herbicides (C).

No.	Herbicides added	Concentration applied [μM]	Chlorophyll [mg/ml pcv], %	Total colored carotenoids [mg/ml pcv], %	Phytoene accumulation [mg/ml pcv]	Abbreviation or number of compound
	Control	–	4.8 (100)	0.58 (100)	n.d.	
	Group A					
1		1	3.5 (73)	0.55 (95)	n.d.	Oxadiazon
2		0.1	3.8 (79)	0.53 (91)	n.d.	MK-616
3		5	3.7 (77)	0.57 (98)	n.d.	DTP
	Group B					
4		1	3.8 (79)	0.273 (47)	0.041	BAS 174639
5		0.1	2.0 (41)	0.104 (18)	0.383	–
6		0.1	1.9 (39)	0.099 (17)	0.418	Fluridone
7		0.1	2.2 (47)	0.098 (17)	0.438	MB 38183
8		0.1	3.2 (66)	0.173 (30)	0.115	LS 80.707
9		0.1	2.1 (44)	0.158 (27)	0.123	CGA 22867
10		1	4.1 (86)	0.279 (48)	0.052	R-40244
	Group C					
11		1	1.1 (23)	0.15 (26)	n.d.	Acifluorfen-methyl
12		1	3.1 (64)	0.40 (69)	n.d.	BAS 177985

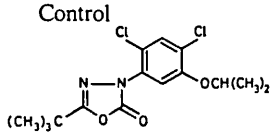
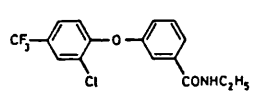
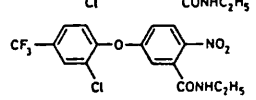
n.d. = not detectable.

Table II. Cell-free carotene synthesis in the presence of bleaching herbicides (10 μM).

No.	Addition	Incorporation of radioactivity [dpm] into			Phytoene/ β-carotene ratio
		Phytoene	Phytofluene	β-Carotene	
	Control	68	50	1004	0.07
1		89	59	986	0.090
	Oxadiazon				
4		1562	174	494	3.16
12		137	53	1112	0.12
12a		61	53	902	0.07
	Fomesafen				

Isolated *Aphanocapsa* thylakoids incubated with 0.7 μCi [2-^{14}C]GGPP, 2 h, at 20 °C (see Methods and [8]).

Table III. Degradation of ^{35}S-prelabeled sulfolipid in *Scenedesmus* cells by bleaching herbicides (1 μM).

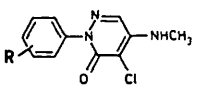
No.	Addition	Radioactivity in sulfolipid (10^3 dpm/l suspension)
	Control	223
1		239
	Oxadiazon	
4		226
12		104

Incubation time was 2 days, with herbicides added, under culture conditions.

From the results of Tables II and III it can be seen that the mode of action of bleaching herbicides is strongly dependent on certain substituents of the molecule. The insertion of a nitro group into the phenoxybenzamide (no. 4) alters an inhibitor of carotene biosynthesis into a compound with peroxidative properties. A substantial additional contribution of carotene-biosynthesis inhibition to bleaching activity can be excluded with our cell-free carotenogenic system.

Table IV compares inhibition of carotene biosynthesis either in intact cells or in cell-free preparations of *Aphanocapsa* by *meta*-substituted 2-phenylpyridazinones [11]. Column (2) shows a decrease in β-carotene formation by the cells. Concurrently, an accumulation of phytoene and phytofluene was observed with all active analogs. For compounds nos. 5 and 13 these precursors were determined quantitatively. With *Aphanocapsa* cells phytofluene is always formed with these inhibitors in contrast to *Scenedesmus*, where phytoene alone is seen. Column (1) demonstrates the degree of inhibition in the cell-free *Aphanocapsa* system by the "inhibition index" which is defined as the ratio of ^{14}C incorporated into phytoene to ^{14}C-labeled β-carotene as referred to the same ratio of the untreated control. Thus a figure higher than 1 indicates inhibition. The 2-phenylpyridazinones are arranged in the order of decreasing inhibition of cell-free carotenogenesis. This arrangement of compounds also matches inhibition of β-carotene formation in *Aphanocapsa* cells except for the m-NO_2 (no. 21) and p-CF_3 (no. 23) analogs which show higher relative activity in intact *Aphanocapsa* cells than in its cell-free system.

Table IV. Inhibition of carotene synthesis by norflurazone derivatives (phenyl-ring substituents) in both the cell-free and cellular system of *Aphanocapsa*.

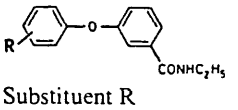
No.		Concentration	(1) Inhibition index[d] of the cell-free system	(2) Inhibition of β-carotene formation in intact *Aphanocapsa* cells % control
		Control	1	100[a]
5	*m*-SCF_3		85	11[b]
13	*m*-CF_3		14	33[c]
14	*m*-OSO_2CH_3		1.9	90
15	*m*-CN	1 µM	1.4	72
16	*m*-NO_2		1.2	61
17	*m*-OCH_3		1	96
18	*m*-H		1	92
19	*m*-OSO_2CH_3		27.3	48
20	*m*-CN		8.6	67
21	*m*-NO_2	60 µM	3.0	21
22	*m*-OCH_3		2.8	77
23	*p*-CF_3		1.5	47
24	*m*-H		1.4	75

[a] Equivalent to 620 µg β-carotene/ml pcv; [b] additional accumulation of phytoene (1330 µg/ml pcv) and phytofluene (247 µg/ml pcv); [c] phytoene 1026 µg/ml pcv and phytofluene 297 µg/ml pcv; [d] for definition see Fig. 1; here, the index is referred to 1 µM or 60 µM of herbicide, respectively.

Apparently, most inhibitors of carotene biosynthesis affect the same target – the phytoene-desaturase complex. Three potential sites of herbicide interference, all resulting in phytoene accumulation, can be postulated. As desaturation of phytoene to phytofluene is an oxidative reaction in *Aphanocapsa* [8] which may involve an electron-transfer system [18], inhibition of either the dehydrogenation reaction or of electron transfer to oxygen is possible. In addition, isomerization has to occur somewhere between 15-*cis* phytoene, the product of phytoene synthase, and the colored carotenoids which have the all-*trans* configuration. Further experiments will have to show whether isomerization of *cis*- to *trans*-carotenes occurs at phytoene or phytofluene. If this takes place at the level of phytoene, the isomerase may be another target. However, herbicide-induced accumulation of phytoene together with phytofluene as reported for difunones [19] and shown here for *m*-phenoxybenzamides (Table II) or 2-phenylpyridazinones (Table IV) excludes inhibition of phytoene isomerization for these compounds, since phytofluene is also accumulated.

The structures of the inhibitors of phytoene desaturation are very different (see Table I, group B). For diphenylamine- and benzophenone-type compounds Rilling [20] suggested competition with phytoene for the catalytic site of the phytoene-desaturase complex. In Figure 1, we have tried to establish a structural relationship between inhibitors and phytoene. A phytoene segment is shown, and the arrow indicates the position where the double bond is inserted by desaturation. The benzophenone compounds chosen in Fig. 1 exhibit maximum inhibition activity in our cell-free system, when parts of their structure resemble the phytoene segment. For example, it can be assumed that the

Table V. Inhibition of carotenogenesis by *m*-phenoxybenzamides (10 µM): influence of phenoxy substituents on inhibition of phytoene desaturase (cell-free *Aphanocapsa* system).

No.	 Substituent R	Lipophilicity π*	Inhibition index
25	H	0	14.6
26	4-chloro	0.71	19.0
27	2,5-dimethyl	1.12	29.4
12	2-chloro, 4-trifluoro-methyl	1.59	45.1

* Values for π were taken from [22]; for definition of the inhibition index see legend of Fig. 1.

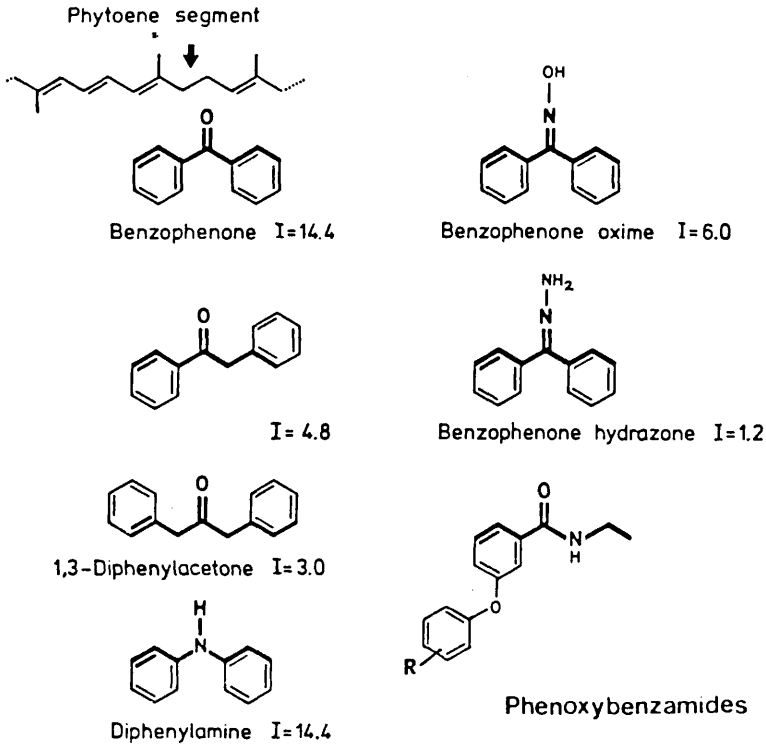


Fig. 1. Structural relationship of inhibitors of phytoene desaturase. Inhibition index *I* is defined as the ratio of ^{14}C incorporated into phytoene to β-carotene in the presence of 10 μM inhibitor as referred to the same ratio of the untreated control. See Table V for inhibition indices of *m*-phenoxybenzamides.

left-hand benzene ring of benzophenone mimics the chromophore of phytoene, while the central portion fits into the active site which is then blocked by the oxo group. When the right-hand benzene ring is brought out of phase by insertion of an additional CH_2-group, thus hindering the attachment of the inhibitor, inhibition decreases as indicated by lower values of the inhibition index *I*. When both benzene rings are out of phase, inhibition is found to be even lower. Substitution of the carbonyl group by an amine results in a similarly effective inhibition, whereas replacement by an oxime or hydrazone decreases the activity since, apparently, these substituents are too bulky to allow a close contact between the inhibitor and the desaturase complex. These differential inhibitory properties can also be observed with a fungal carotenogenic enzyme system [21]. By analogy to the benzophenone derivatives it can be assumed for m-phenoxybenzamides that part of the molecule resembling the phytoene structure (bold-drawn part) is essential for inhibition of phytoene desaturation. In addition, the phenoxy moiety apparently enhances the inhibitory activity by increasing the lipophilicity of the molecule (Table V). The lipophilicity of substituents and inhibition index of the corresponding compounds increase concurrently.

Phytoene desaturation in cell extracts from *Phycomyces* is under negative control of neurosporene, lycopene, β-zeacarotene, and γ-carotene which are all intermediates of β-carotene biosynthesis [23]. Therefore, we cannot exclude the possibility that some compounds of Fig. 1 might act in a similar way on phytoene desaturase at an allosteric site. If this were the case, non-competitive inhibition by these compounds would be expected. Experiments are under way to show whether inhibition is of a competitive or non-competitive type. However, these data are difficult to obtain, since (^{14}C-labeled) phytoene has to be solubilized with detergents and these – in turn-decrease the activity of the carotenogenic system.

Detailed knowledge about targets of phytotoxic chemicals and their effective structural elements should be helpful in the development of new herbicidal compounds. These data represent a first biochemical approach towards elucidating the mode of action of bleaching herbicides which inhibit "phytoene desaturase". The recent development of a cell-free system including photosynthetically active

membranes provides a means of characterizing the enzyme complex responsible for formation of β-carotene and of predicting structures of potential inhibitors directed towards the enzymes of carotene biosynthesis.

Acknowledgment

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[1] Many relevant articles are compiled in a special issue, no. 11/12, of Z. Naturforsch. **34c**, pp. 893–1074 (1979).
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Inhibition of Carotene Biosynthesis in Cell Extracts of *Phycomyces blakesleeanus*

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Carotenoid Biosynthesis, Bleaching Herbicides, Inhibitors of Phytoene Desaturation, *Phycomyces blakesleeanus*, Cell Extracts

Cell extracts of the C115 (β-carotene-accumulating) strain of *Phycomyces blakesleeanus* were incubated with either [2-^{14}C]MVA or [1-^{14}C]IPP and a range of possible inhibitors of carotenogenesis, including bleaching herbicides, biphenyl compounds and geranylacetone. Several of these compounds were potent inhibitors of β-carotene formation and caused the accumulation of phytoene. No other carotenes were found to accumulate, *in vitro*. The structures of these inhibitors, compared to that of phytoene, suggest that they affect the enzymic activity of "phytoene dehydrogenase", possibly by competitive inhibition.

Introduction

A wide range of compounds are known to inhibit the biosynthesis of carotenoids. These include bleaching herbicides [1], algal excretion products [2], diphenylamine and related compounds [3, 4] and a large number of onium derivatives [5]. In most cases, especially in plant tissues, the desaturation of carotenes is affected, resulting in the accumulation of phytoene and/or β-carotene [6]. Most studies on the mode of action of inhibitors of carotenoid formation have used whole cells or tissues, mainly because of the lack of effective carotenogenic enzyme systems from photosynthetic tissues. Although this problem has been somewhat alleviated with the development of an active preparation of thylakoids from *Aphanocapsa* [7, 8], cell extracts of the fungus *Phycomyces blakesleeanus* represent one of the best characterized cell-free systems yet available for *in vitro* studies on carotene biosynthesis. In this publication we describe the effects of a range of compounds, including bleaching herbicides, on carotenogenesis by this model system.

Abbreviations: MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; J852, 4-isobutoxy-2-isopropylamino-6-methyl-pyrimidine.

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Materials and Methods

The C115 *car S42 mad 107* (–) strain of *Phycomyces blakesleeanus* was obtained from the culture collection of the Departamento de Genética, Universidad de Sevilla, Spain. Its growth and maintenance conditions have been described previously [9].

The preparation of cell-free extract, incubation conditions, and the extraction and purification of radioactive carotenes have been described in detail in earlier publications [10, 11]. Radioassay of the purified carotenes was by liquid scintillation counting [12]. Inhibitors (10 µl) were added in methanol solutions prior to addition of cell extracts to the incubations. Methanol (10 µl) was similarly added to control incubations.

Results and Discussion

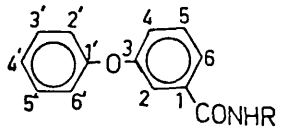
As inhibitors of carotene formation, neither fluridone nor J852 were effective *in vitro*, as they are in photosynthetic tissues. In both cases, their I_{50} values are significantly (–) lower in higher plants than the concentrations used in this study. Difunon was more inhibitory and caused a significant accumulation of phytoene, while amitrole prevented the formation of all carotenes, but apparently stimulated squalene biosynthesis (Table I). None of these four herbicides caused an accumulation of ζ-carotene, in contrast to reports of the effect of both

Table I. The effect of certain bleaching herbicides on carotene formation in cell extracts of *Phycomyces*.

Treatment	% of Total lipid incorporation		Ratio phytoene/ β-carotene	Reported effect in plants [see ref. 6]
	phytoene	β-carotene		
Control	10.3	10.4	0.99	–
I Fluridone				
10^{-6} M	10.8	9.3	0.87	accumulation
10^{-5} M	14.6	10.6	10.3	of phytoene
10^{-4} M	25.1	4.9	12.0	
II Difunon				
10^{-6} M	35.2	11.5	3.07	accumulation of phytoene
III Amitrole				
10^{-5} M	0.31	0.26	0.06*	accumulation of acyclic carotenes
IV J852				
10^{-5} M	12.7	11.0	1.15	accumulation of
10^{-4} M	20.6	12.5	1.65	ζ-carotene

All incubations were carried out with 1 μCi DL-[2-^{14}C]MVA.
* Incorporation into squalene increased 2.5-fold compared to the control.

Table II. Structure-activity relationships of N-alkylphenoxybenzamides (60 μM) on carotenogenesis in *Phycomyces* cell extracts.



Substituent	Incorporation [dpm]*		Ratio phytoene/β-carotene
	phytoene	β-carotene	
Control	8922	31281	0.29
R = $-CH_2CH_3$	18257	30838	0.59
R = $-CH_2CH_2CH_2CH_3$	44822	14810	3.0
R = $-CH_2CH_3$; 2'-Cl; 4'-CF_3	35944	17213	2.1
R = $-CH_2CH_3$; 2'-Cl; 4'-CF_3; 6-NO_2	21423	17795	0.77

* From 0.25 μCi [1-^{14}C]IPP.
Data are averages of 3 experiments, s.d. < 10%.

amitrole [13] and J852 [6] in higher plants. These differences between *in vitro* and *in vivo* sensitivites may be either a result of the carotenogenic enzymes being fundamentally different in *Phycomyces* and photosynthetic tissues, or because the principal target site of these herbicides is other than binding to the enzyme responsible for the metabolism of phytoene.

Another group of bleaching herbicides, the N-alkylphenoxybenzamides, were found to inhibit *in vitro* carotenogenesis (Table II). Their relative effectiveness, however, was related to the substi-

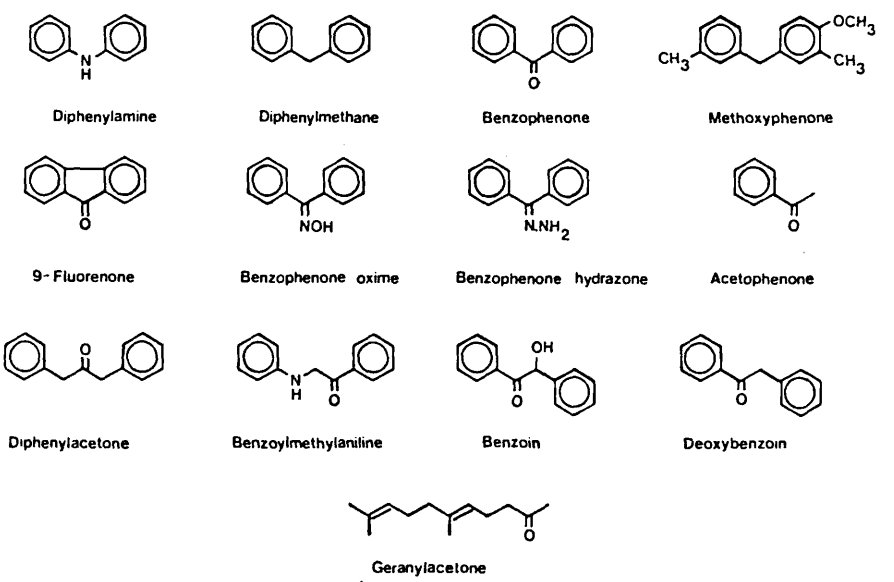


Fig. 1. Structures of biphenyl compounds, acetophenone and geranylacetone.

Table III. Inhibition of *in vitro* carotenogenesis by biphenyl derivatives and geranylacetone.

Compound [100 μM]	Incorporation [dpm]*		Ratio phytoene/β-carotene
	phytoene	β-carotene	
Control	43738	6240	7
Methoxybenzophenone	182825	144	1270
Diphenylamine	158603	304	522
Geranylacetone	175630	406	433
Diphenylmethane	144900	420	345
Benzophenone	127712	478	267
9-Fluorenone	172327	1373	126
Benzophenone oxime	223349	1110	93
Benzoylmethyl aniline	87351	1419	62
Deoxybenzoin	188869	3777	50
Diphenylacetone	168024	5310	32
Benzoin	48504	5426	9
Benzophenone hydrazone	70078	6492	9
Acetophenone	50386	6298	8

* From 0.5 μCi DL-[2-^{14}C]MVA.
See Fig. 1 for structures.

tuents attached to the amide group and to the aromatic rings. A butyl moiety on the amide group was the most effective, whilst the potency of ethylphenoxybenzamide was enhanced on addition of lipophilic groups to the 2'- and 4'-carbon atoms. Introduction of a 6-NO_2 group resulted in an approximately 3-fold decrease in inhibitory activity. These data confirm the results with both a thylakoid preparation of *Aphanocapsa* [14] and whole cells of *Scenedesmus* [15]. In this context, it is interesting to note that the 6-NO_2 derivative is still herbicidal *in vivo*, since it shows peroxidizing properties [16].

Of the 11 biphenyl compounds tested for inhibitory activity, 9 caused significant changes in the incorporation or radioactivity from [2-^{14}C]MVA into carotenes (Table III). The algal excretion product geranylacetone [2] was also a potent *in vitro*

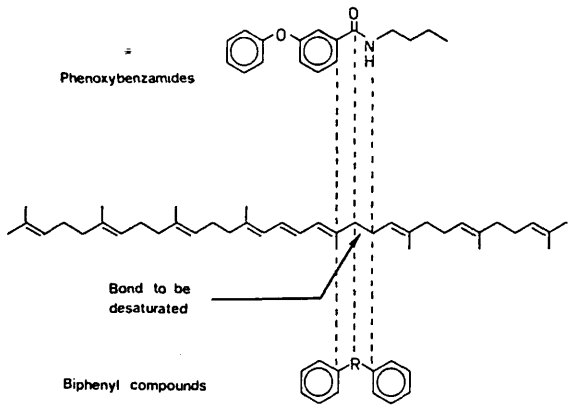


Fig. 2. Structural similarities between phytoene, alkylphenoxybenzamides and biphenyl compounds.

inhibitor of desaturation. A comparison of the structure-inhibitory activity relationships of these compounds with the C12–C8′ portion of the phytoene molecule (Fig. 2) provides support for the hypothesis that inhibitors of phytoene desaturation compete for the active site of the dehydrogenase [4, 17]. Diphenylamine has previously been shown to inhibit the activity, rather than the synthesis of phytoene desaturase [3]. Those compounds exhibiting maximum inhibitory activities fit most closely with this region of the phytoene molecule. They all contain at least one C=C bond, and in the case of the biphenyl compounds, have either 1 or 3 atoms between the rings (Fig. 1). Benzoin and deoxybenzoin, both of which have 2 inter-ring C atoms, and acetophenone which has a single aromatic ring, are poor inhibitors of phytoene metabolism. Apparently, the presence of either N-atoms or oxo groups has little effect on inhibitory activities, although a bulky, charged moiety such as a hydrazone presumable prevents efficient binding to the enzyme. The phenoxybenzamides can also be envisaged as competitive inhibitors of phytoene desaturase, due to the structural similarities between the C_{40} polyene chain and the side chain of the benzamide molecule (Fig. 2). The precise modes of action of these inhibitors must await a more detailed understanding of the enzymes involved in carotene formation.

Acknowledgements

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Phytotoxicity of m-Phenoxybenzamides: Inhibition of Cell-free Phytoene desaturation

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SUMMARY

Inhibition of carotenoid biosynthesis by herbicidal m-phenoxybenzamide derivatives has been investigated in a cell-free carotenogenic system from Aphanocapsa. Their target is the phytoene desaturase reaction. Double-reciprocal plots of β-carotene biosynthesis (from ^{14}C-labeled geranylgeranyl pyrophosphate) showed that 3-(2,5-dimethylphenoxy)-N-ethylbenzamide (S-3422) is a non-competitive inhibitor of the phytoene-desaturase complex. The K_i-value for cell-free inhibition of β-carotene formation was about identical to the I_{50}-value of intact cells.

Furthermore, the influence of certain substituents on herbicidal activity has been investigated. Inhibition increased with the length of the unbranched N-alkyl chains. In addition, substituents at the phenoxy group with higher lipophilicities showed greater inhibitory activities. The presence of a phenoxy or trifluoromethyl moiety at position 3 was essential.

INTRODUCTION

The biosynthetic pathway of carotenoids is one of the prominent targets for herbicides directed against the chloroplast [see (1) for review]. These inhibitors of carotene synthesis usually cause the accumulation of phytoene. Their interference with phytoene desaturation has been demonstrated directly with cell-free systems obtained from different organisms (2,3,4).

Recently, a certain group of diphenyl ethers, the N-alkyl-3-phenoxybenzamides has been found to inhibit carotene biosynthesis in microalgae (5,6). The inhibitory action of these compounds is strongly dependent on their substituent groups.

In this study, we have investigated the inhibitory properties of phenoxybenzamides in a cell-free carotenogenic system from Aphanocapsa (7). In addition, a range of benzamides substituted at various positions of the molecule, has been compared in vitro.

MATERIALS AND METHODS

The blue-green alga Aphanocapsa, strain 6714, was grown in a liquid mineral medium at 35 ^{o}C according to (7). Cell density was determined as packed cell volume (pcv) in calibrated microcentrifuge tubes of 80 µl capacity.

The chlorophyll content of membranes was measured by its absorbance in 80% acetone (8). Carotenoids were extracted from Aphanocapsa cells with hot methanol (65 ^{o}C) containing 6% KOH. From these extracts total colored carotenoids were removed by partition with 10% diethyl ether (v/v) in petrol (b.p. 60 to 80 ^{o}C), while β-carotene was isolated by partition with petrol

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alone. Values of K_i for cell-free β-carotene formation were determined as described by Dixon (9). The I_{50}-values for the formation of total colored carotenoids, in the presence of inhibitors, were obtained by a modified Dixon plot (10).

Cell-free carotene biosynthesis, with [3-^{14}C]geranylgeranyl pyrophosphate (specific activity 10 mCi/mmol) as the substrate, was carried our with membranes from Aphanocapsa. The details of this procedure have been described previously (7). ^{14}C-Geranylgeranyl pyrophosphate was synthesized by Dr. G. Schultz, Hannover, Germany, according to (11).

Compounds nos. 3 to 7 were prepared from phenoxybenzoic acid, following conversion to the acid chloride with thionyl chloride, by reaction with the appropriate alkylamines. The resulting products were easy to be crystallized and were recrystallized once or twice from chloroform by the addition of petrol. For the preparation of compounds no. 9, 12, 13, and 14, 3-(4-chlorophenoxy)benzoic acid, 2-phenoxybenzoic acid, and 4-phenylazobenzoic acid, were used, respectively. The purity of the synthesized phenoxybenzamides was verified by NMR-spectroscopy in $CDCl_3$. The compounds showed the expected displacement profiles and correlations of proton ratios.

Other inhibitors used in this study were kindly provided by the following companies: BASF AG, Ludwigshafen and Limburgerhof, Germany, nos. 11 and 15 to 18; Celamerck, Ingelheim, Germany, nos. 1, 8, and 3-(4-chlorophenoxy)benzoic acid; and Sumitomo Ltd., Takarazuka, Japan, nos. 2, 10. The compounds used for the syntheses of benzamides were purchased from Aldrich Company (German branch.).

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RESULTS

Increasing concentrations of S-3422 [3-(2,5-dimethylphenoxy)-N-ethylbenzamide] interfered with cell-free carotenogenesis performed by a membrane preparation from Aphanocapsa, as measured by the decreasing incorporation of radioactivity from ^{14}C-geranylgeranyl pyrophosphate (GGPP) into β-carotene (Fig.1). Simultaneously, an increase in radioactivity incorporated into phytoene was observed. Double-reciprocal plots of β-carotene biosynthesis from ^{14}C-GGPP, at 5 and 20 µM S-3422, resulted in straight lines, all of which intersected at the same position ($-1/K_m$) on the abscissa (Fig.2). This observation indicates that S-3422 inhibits carotene biosynthesis non-competitively. The apparent K_m-value is 52.6 µM. Dixon plots exhibited linearity between inhibitor concentration and reciprocal carotenoid formation, when either I_{50}-values (i.e. concentration of an inhibitor yielding 50% inhibition) for carotenoid synthesis in intact Aphanocapsa cells (Fig.3A), or the inhibition constant K_i (dissociation constant of the desaturase-inhibitor complex) of cell-free β-carotene formation (Fig.3B), were determined. The resulting I_{50}- and K_i-values are very similar, at 7.3 and 9.2 µM, respectively.

Table 1 shows the inhibition of cell-free carotene biosynthesis by m-phenoxybenzamides which contain different N-alkyl chains. All of the benzamides assayed decreased incorporation of ^{14}C from GGPP into β-carotene. Radioactivity accumulated in phytoene and phytofluene under these conditions. The ratio of radioactivity in phytoene versus β-carotene, which is an indicator of the inhibitory activity of the phenoxybenzamides (6), increased in parallel with the length of the N-alkyl chain. In

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the case of butyl derivatives, the n-butyl moiety (no.4) showed less inhibitory activity than the branched sec- (no.6) or even tert-butyl derivatives (no.7). The same was found for the n- (no. 3) and iso-propyl chains (no.5). Insertion of a double bond into the propyl chain (no.8) slightly decreased the degree of inhibition. These relative changes of in vitro inhibitory activities, after modification of the N-alkyl chain of the phenoxybenzamides, were also observed with intact Aphanocapsa cells, when assayed for their β-carotene content (Table 1, last column).

All the substituted m-phenoxybenzamides of Table 2 showed inhibition of the cell-free conversion of phytoene into β-carotene, regardless of the positions of the substituents. Accumulation of phytoene was highest with the 2-chloro, 4-trifluoromethyl compound (no.1), but decreased with the insertion of 2,5-dimethyl (no.10) groups, or a 4-chloro substituent (no.9).

Replacement of the 3-phenoxy moiety (no.4) by a 4-phenylazo (no.14) or a 2-iodo group (no.13) resulted in total inactivation of N-butylbenzamides as inhibitors of phytoene desaturation (Table 3). Accumulation of radioactivity in phytoene was no longer observed and the ^{14}C-incorporation ratio between phytoene and β-carotene was similar to that of the treated control. A 3-CF_3 moiety (no.15) was strongly inhibitory in vitro (10 µM), but Br, CH_3 or OCH_3 groups were not. When the same concentrations were used with intact cells, however, no effect was observed. Although the 2-phenoxy compound (no.12) showed substantial inhibition, it was much lower than that of the very active 3-phenoxybenzamide (no.4). A 4-phenoxybenzamide would have been useful for comparison, but it was not available.

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DISCUSSION

The decrease of β-carotene in whole cells of Aphanocapsa, after application of m-phenoxybenzamides, is similar to that reported previously for Scenedesmus cells (5). In addition, however, the use of a cell-free system from Aphanocapsa has made it possible to localize the site of action of these herbicides. The inhibition of *in vitro* β-carotene formation, from ^{14}C-GGPP, and the concurrent accumulation of radioactivity in phytoene (Fig.1) strongly indicates that these m-phenoxybenzamides specifically inhibit the desaturation of phytoene.

The possible nature of this inhibitory action as either direct competition with phytoene for the active site of the enzyme, or else non-competitive inhibition similar to that found for certain unsaturated carotenes (12), has been discussed recently (6). The double-reciprocal plots of *in vitro* β-carotene formation with increasing concentrations of ^{14}C-GGPP show identical K_m-values regardless of the presence of S-3422 (Fig.2). These data are indicative of non-competitive inhibition by this compound. A comparison of the I_{50}-value for the inhibition of *in vivo* β-carotene formation by S-3422 with the K_i-value in the cell-free system (Fig.3) shows them to be almost identical, within experimental error. This similarity also suggests non-competitive inhibition (13) provided that uptake by intact cells is non-limiting. This is the case for S-3422. However, the m-CF_3-benzamide (no.15), which is very active in the cell-free assay (Table 3), is probably not taken up by Aphanocapsa and Scenedesmus cells.

In cell extracts of Phycomyces, phytoene desaturation is under feed-back allosteric control by neurosporene, lycopene, β-zeacarotene and γ-carotene (12). From the data in Figures 2 and 3 it is possible that S-3422 inhibits in a similar way.

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It is noteworthy that S-3422 is a more potent inhibitor in the green alga Scenedesmus, where the I_{50}-value is about one tenth of that for Aphanocapsa (5). Since a cell-free system of Scenedesmus is not yet available, we cannot judge whether this difference in sensitivity is due to different uptake rates into whole cells, or to a more fundamental difference in the nature of the inhibitory action in green and blue-green algae.

The degree of inhibition of phytoene desaturation by phenoxybenzamides is dependent on the substituents of the molecule (5,6). The data in Table 1 give some indication as to the influence of certain groups on activity. An increase in the length of the N-alkyl chain results in more potent inhibitors. However, the presence of branched alkyl chains or the introduction of a double bond in the chain results in a decrease in inhibitory activity as compared to the straight chain moiety with the same number of carbon atoms. Clearly, unbranched N-alkyl chains are sterically favorable in this respect. Other comparative studies with inhibitors of phytoene desaturation have shown that the amide group of the phenoxybenzamides, including the alkyl chains, is essential for the inhibitory activity of these herbicides (4,6). The N-alkyl chain may mimic part of a carotene molecule with respect to binding to the enzyme.

All substituents at the phenoxy moiety increase herbicidal activity, regardless of their position. We tentatively assume that lipophilicity of this group alone determines the potency of these phenoxybenzamides.

As can be seen from the compounds assayed in Table 3, a phenoxy group gives the best inhibition when at position 3. Its replacement by other substituents, positioned at carbon 2 or 4,

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results in a total loss of inhibition. A benzamide with a CF_3-moiety at position 3, however, again shows herbicidal activity. This finding is a further indication that the amide group is the essential structural element (4,6). It has already been shown by in vitro (6) and in vivo (14) experiments that bulky substituents on the phenyl ring next to the amide group (e.g. the 4-NO_2 derivative) decrease the inhibitory action on carotene biosynthesis.

These results, together with a quantitative study on the structure-activity relationships of these compounds which is under way, may provide some help towards a rational approach for the synthesis of phenoxybenzamide herbicides of known phytotoxicity.

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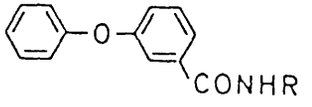
TABLE 1

Inhibition of carotene biosynthesis by N-alkyl-3-phenoxybenzamides assayed with intact cells or a cell-free system of Aphanocapsa*

 No. R	Incorporation of radioactivity (dpm) into Phytoene	Phytofluene	β-Carotene	[^{14}C] Incorporation (ratio phytoene: β-carotene)	β-Carotene content of Aphanocapsa cells (µg/ml pcv)
Control	82	52	1535	0.05	855
1 Methyl	172	95	1620	0.11	539
2 Ethyl	460	113	1150	0.40	505
3 Propyl	678	95	694	0.98	479
4 Butyl	1019	193	886	1.15	393
5 iso-Propyl	403	79	1093	0.37	530
6 sec-Butyl	838	185	1005	0.83	676
7 tert-Butyl	215	62	1300	0.16	710
8 Propenyl	941	115	1080	0.78	498

*) Inhibitor concentration in the cell-free system was 10 µM, with Aphanocapsa cells 30 µM. See Materials and Methods for experimental details.