THE SUBCELLULAR DISTRIBUTION OF CAROTENOIDS IN PHYCOMYCES BLAKESLEEANUS

A Thesis submitted by

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ABSTRACT

In order to study the subcellular distribution of carotenoids in Phycomyces blakesleeanus, homogenates of mycelia of the C115 carS42 mad-107(-) mutant were fractionated by both differential and density gradient centrifugation. Characterisation of the fractions was achieved by the distribution of marker enzyme activities.

Organelles prepared by differential centrifugation were shown to be associated with contaminating lipids, including β-carotene (β, β-carotene). These lipids could not be removed by either repeated washing, or by centrifugation procedures; so a separation technique based on density gradient centrifugation was devised.

Only two fractions isolated by the latter method contained carotenoid; the lipid particles, and a particulate fraction. The latter was tentatively identified, on the basis of its density (1.10g/ml), and its enzyme content, as a vacuole-containing fraction.

Both these pigmented fractions exhibited alkaline p-nitrophenylphosphatase activity, as well as containing sterols and phospholipids. The proteins from these organelles revealed close similarities on amino acid analysis.

The relative distribution of β-carotene between these fractions was investigated at various stages of growth.

The lipid droplets isolated from P.blakesleeanus resembled those prepared from other plants in both size and lipid content. They contained sufficient protein to form a monolayer around their surface.

Mycelia were grown in media containing [2-^{14}C]mevalonic acid (MVA), and the subcellular distribution of radioactive lipids was determined. The rates of incorporation of MVA in various organelles were compared.

Organelles were also incubated with [2-^{14}C]MVA *in vitro*. Cytosol enzymes were able to incorporate MVA into terpenols and squalene. No particulate fractions were capable of converting MVA into β-carotene or sterols in the absence of the cytosol.

These results are discussed in terms of the possible sites of biosynthesis of carotenoids in the mycelium, and the inter-relationships of the pigmented organelles.

CONTENTS

ABBREVIATIONS

The abbreviations used in this thesis are those listed in the Biochemical Journal (1976) 153, 1-21 (revised, 1977, 161, 1-2) with the following additions:

D.I.	Difference Index of Compositional Relatedness (Metzger *et al.*, 1968)
DMAPP	dimethylallyl pyrophosphate
E.R.	endoplasmic reticulum
FP	farnesyl phosphate
FPP	farnesyl pyrophosphate
GGP	geranylgeranyl phosphate
GGPP	geranylgeranyl pyrophosphate
GPP	geranyl pyrophosphate
HMG	3-hydroxy-3-methylglutaric acid
IP	isopentenyl phosphate
IPP	isopentenyl pyrophosphate
MVA	mevalonic acid
PPPP	prephytoene pyrophosphate
PSPP	presqualene pyrophosphate
SCP	sterol carrier protein

I N T R O D U C T I O N

A. THE BIOSYNTHESIS OF CAROTENES AND STEROLS

Biosynthesis of Isoprene Units

Both carotenes and sterols may be derived formally from five-carbon isoprene units, and are hence classified as terpenes (Ruzicka, 1953). The patterns of incorporation of [^{14}C]acetate into β-carotene (β,β-carotene; Grob and Bütler, 1955, 1956) and cholesterol(cholest-Δ^5-enol; Wiersch *et al.*, 1952; Ruzicka, 1953; Fig. 1.1) are both consistent with the involvement of a five-carbon unit derived from acetate via 3-hydroxy-3-methylglutaryl CoA (HMG-CoA).

Yokoyama *et al.* (1960) showed that the incorporation of [2-^{14}C] mevalonic acid (MVA) into β-carotene by a cell-free homogenate of *Phycomyces blakesleeanus* mycelia was greater than that of [^{14}C]HMG. MVA was incorporated into sterols with high efficiency by a rat liver homogenate (Tavormina *et al.*, 1956). Since Ferguson *et al.* (1958) demonstrated the reduction of HMG-CoA to MVA by a yeast extract, subsequent studies, involving enzymes purified from a variety of sources (reviewed by Beytía and Porter, 1976), have established that the major pathway of MVA biosynthesis is that shown in Fig. 1.2. Goodwin and Lijinsky (1951) found that the branched-chain amino acids leucine and valine stimulated carotenogenesis in *Phycomyces*. It has now been established that this is because they are efficient precursors of HMG via an alternative metabolic route (Goodwin, 1971a).

Ruzicka (1959) suggested that "terpenes are compounds formed by combination of isoprene units to aliphatic substances such as geraniol, farnesol, geranylgeraniol, squalene and others of a similar kind". The formation of squalene from MVA by a soluble yeast enzyme system requires ATP as a cofactor (Amdur *et al.*, 1957), suggesting that phosphorylated intermediates are involved. Bloch and coworkers (1959) isolated the enzymes catalysing the initial steps of this conversion from a yeast autolysate, and showed that 5-phosphomevalonic acid and MVA-5-pyrophosphate were produced sequentially, the latter being decarboxylated to isopentenyl pyrophosphate (IPP; Fig. 1.3).

Since each of these three reactions required ATP, the involvement of an unstable intermediate, 3-phospho-5-pyrophosphomevalonic acid, was suggested. Although the participation of this compound may be predicted theoretically, since phosphate is a better leaving group than

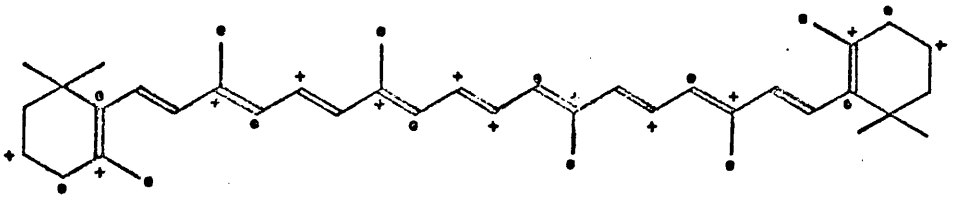


Fig. 1.1a Pattern of Labelling in ß-Carotene Synthesized from [^{14}C] Acetate

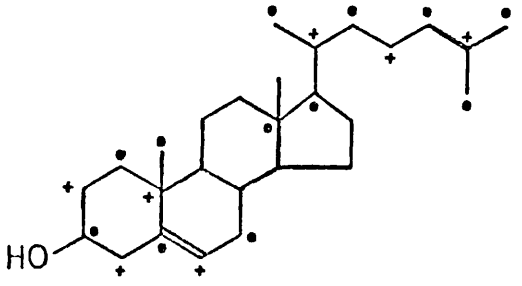


Fig. 1.1b Pattern of Labelling in Cholesterol Synthesized from [^{14}C] Acetate

♦ Carbon Atoms Labelled by [1-^{14}C] Acetate

● " " " " [2-^{14}C] "

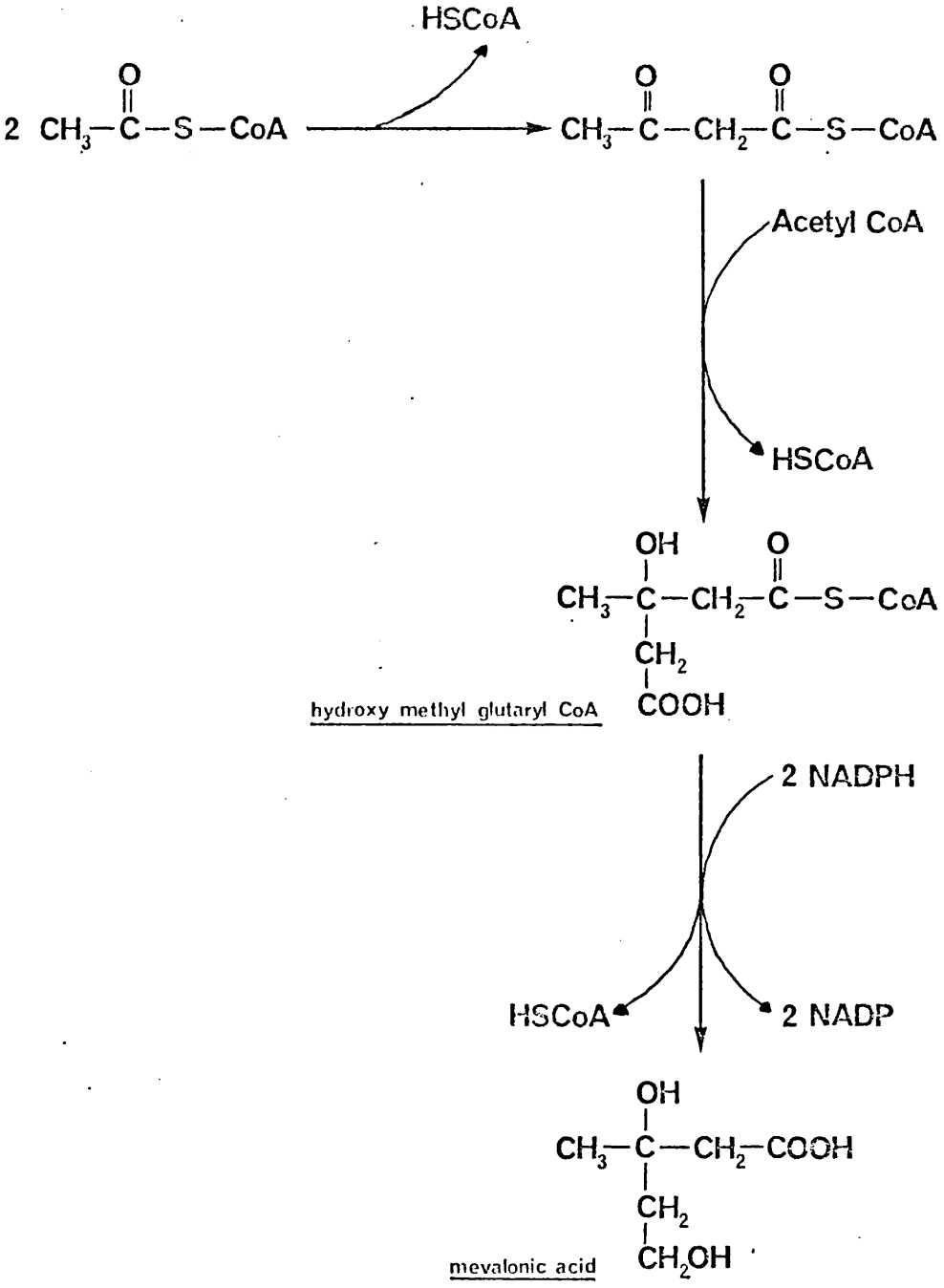


Fig. 1.2 Biosynthesis of Mevalonic Acid

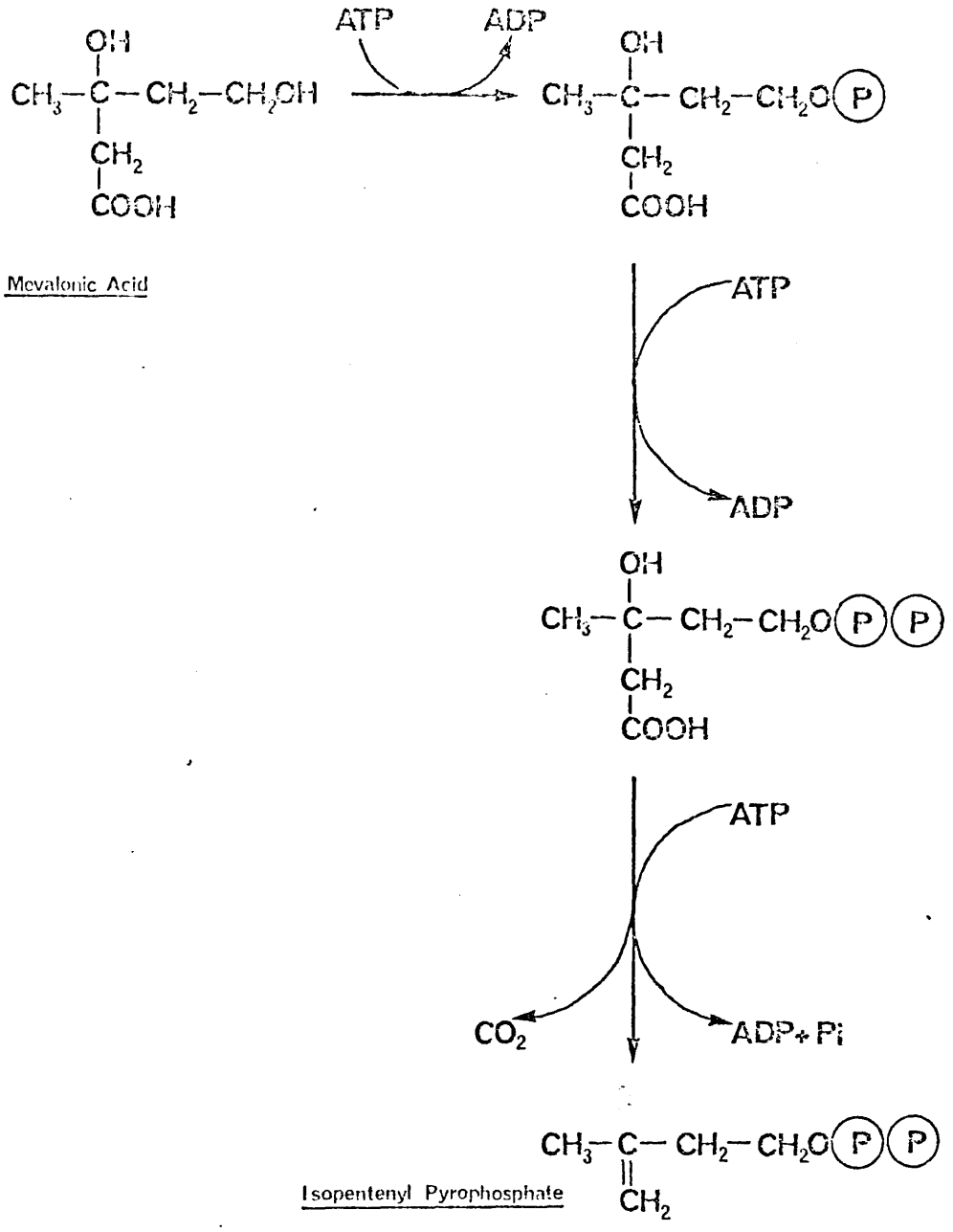


Fig. 1.3 Biosynthesis of Isopentenyl Pyrophosphate

the hydroxyl moiety, it has never been isolated from incubation mixtures (Beytía and Porter, 1976).

Biosynthesis of Polyisoprene Units

Bloch et al. (1959) showed that a yeast autolysate was capable of converting [^{14}C]IPP into squalene in the absence of ATP, while Varma and Chichester (1962) demonstrated the incorporation of [^{14}C]-IPP into lycopene (ψ,ψ-carotene) by a homogenate of tomato fruits similarly incubated without ATP. This indicates that IPP corresponds to the basic 5-carbon unit predicted from the early experiments on acetate labelling patterns (Willersch et al., 1952; Ruzicka, 1953; Grob and Bütler, 1955, 1956).

Agranoff et al. (1960) isolated from yeast an enzyme which catalysed the isomerisation of IPP to 3,3-dimethylallyl pyrophosphate (DMAPP). This reaction yields 87% DMAPP and 13% IPP at equilibrium (Porter, 1969). DMAPP then acts as an allylating agent in a condensation reaction with a second molecule of IPP to yield geranyl pyrophosphate (GPP). This molecule retains the allyl group, and so the condensation may be repeated to yield farnesyl pyrophosphate (FPP) and then geranylgeranyl pyrophosphate (GGPP; Fig. 1.4). These sequential allylations are catalysed by prenyltransferases (prenylpyrophosphate synthetases, E.C.2.5.1.1).

Prenyltransferases specific for the synthesis of FPP have been purified from yeast (Eberhardt and Rilling, 1975) and from chicken liver (Reed and Rilling, 1975). In each case the geranyl transferase and dimethylallyl transferase activities copurified, indicating that the formation of GPP and FPP is catalyzed by the same enzyme. Ogura et al. (1969) found that the geranyltransferase activity of prenyltransferase from pumpkin fruits was preferentially protected from heat denaturation by the presence of GPP, while dimethylallyl monophosphate inhibited the utilization of DMAPP, but not of GPP. The same group also found that 4-methylpent-4-enyl pyrophosphate, an analogue of IPP, was utilized as substrate with GPP but not DMAPP (Ogura et al., 1974). Since the purified enzyme consists of two subunits (Reed and Rilling, 1975), it is possible that these activities are located on separate proteins.

The biosynthesis of squalene involves the dimerisation of FPP

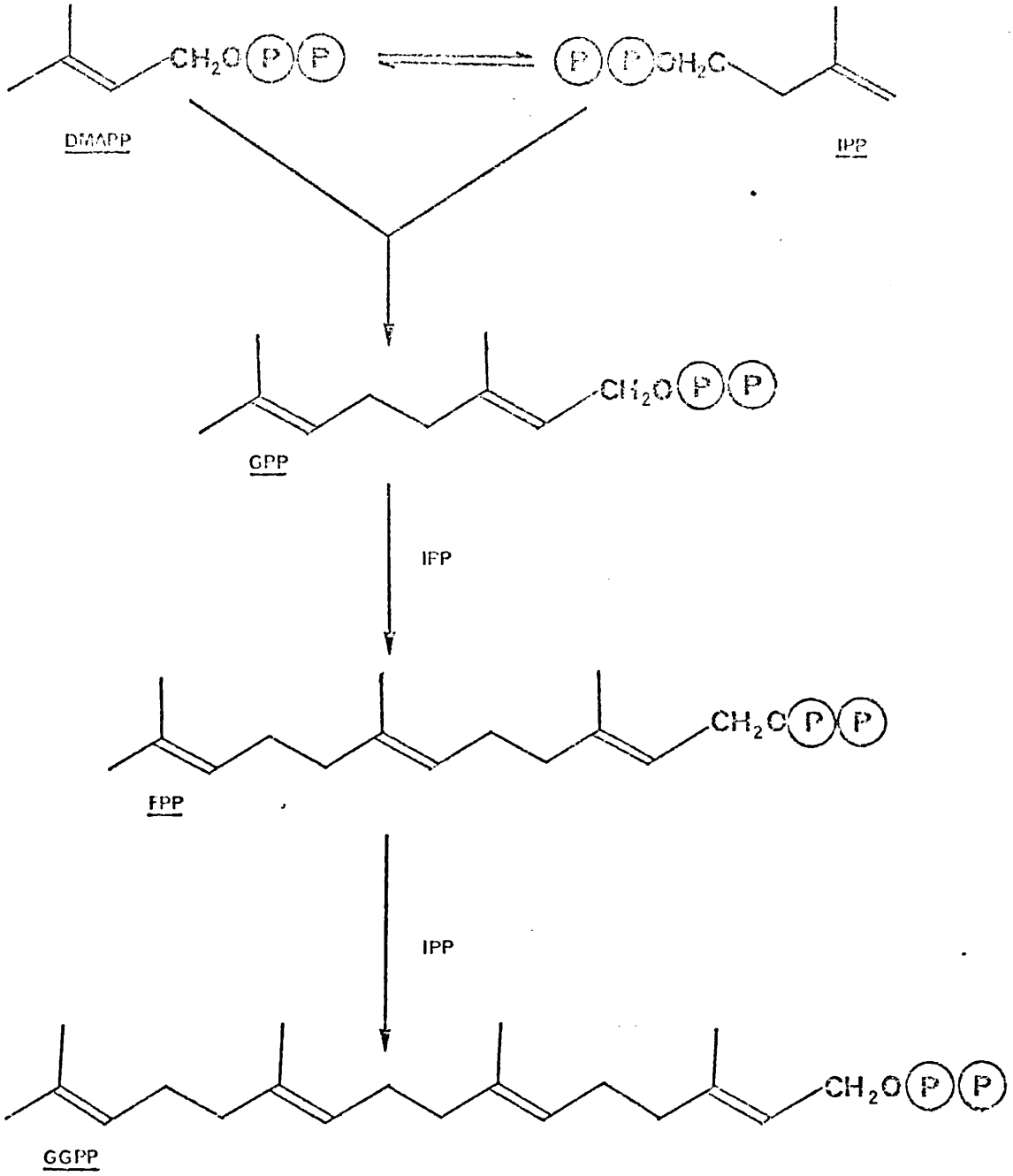


Fig.1.4 Conversion of IPP to FPP and GGPP

(Lynen et al., 1958), but the incorporation of FPP into carotenes by a cell-free homogenate of P. blakesleeanus mycelia was low except in the presence of MVA or IPP (Yamamoto et al., 1961). Since the labelling patterns of β-carotene and squalene derived from [^{14}C]-acetate are similar (Grob, 1959) these workers suggested that carotenes are formed by an analogous dimerisation of GGPP. However, the prenyltransferases purified from yeast and chicken liver are capable of synthesizing GGPP only when incubated with high concentrations of FPP and IPP (Eberhardt and Rilling, 1975; Reed and Rilling, 1975). This suggests that carotene-synthesizing organisms possess another prenyltransferase.

Since bacteria do not synthesize sterols, but some do produce carotenes, prenyltransferases in these organisms have been studied in relation to carotenogenesis. Johnson et al. (1974) examined the rate of incorporation of [^{14}C]IPP into other terpenyl pyrophosphates by cell-free supernatant fractions of a Mycobacterium species prepared before and after photoinduction of carotenogenesis. Photoinduction doubled the rate of incorporation of IPP when GPP or FPP were used as cosubstrates; however if either DMAPP or GGPP was employed there was no difference between the rates of incorporation of [^{14}C]IPP by induced and non-induced preparations. This indicates that illumination induced the production of a prenyltransferase specific for the biosynthesis of carotenogenic substrates.

The prenyltransferase of Micrococcus lysodeikticus, another carotenogenic bacterium, showed a greater affinity for FPP than for GPP or DMAPP, indicating that GGPP is formed preferentially (Kandutsch et al., 1964). Porter (1969) reported the partial purification of GGPP synthetase from an acetone powder of tomato fruit plastids. This formed both FPP and GGPP from IPP and DMAPP, but the rate of GGPP biosynthesis was three times greater than that of FPP production.

In addition to these enzymes, which are involved mainly in the formation of precursors of carotenes and sterols, other prenyltransferases have been described in several organisms, where they probably catalyse the biosynthesis of quinone side chains (Allen et al., 1967).

Squalene Biosynthesis

Lynen et al. (1958) found that yeast cell particles converted FPP into squalene when they were incubated in the presence of NADPH. The enzyme involved in this conversion, squalene synthetase (E.C.2.5.1.bb), is bound to the endoplasmic reticulum, unlike the enzymes converting IPP into FPP, which are cytosolic (Gaylor, 1974). This step thus represents a commitment to sterol biosynthesis.

Cornforth and Popják (1959) suggested that the condensation of two molecules of FPP to squalene occurred via the isomerisation of one to nerolidyl pyrophosphate, in an analogous reaction to the isomerisation of IPP to DMAPP. Condensation might then occur to produce a transient intermediate, which could be stabilised by the loss of two protons to form dehydrosqualene (an analogue of phytoene). The 12,12'-double bond could then be reduced by NADPH to yield squalene (Fig. 1.5).

This mechanism, however, was disproved by the results of Popják et al. (1961). They showed that although one of the four 12,13 hydrogen atoms was derived from NADPH, none of the others originated from water, as would be expected for a C=C bond-reduction mechanism. Since the remaining three all originated from the FPP molecules, an asymmetrical process must be involved in the final step of squalene biosynthesis, i.e. one of the two condensing FPP molecules must be subjected to different reactions from the other.

Rilling (1966) isolated an intermediate between FPP and squalene from yeast cells incubated with GPP and IPP, but without NADPH. On reincubation with NADPH this compound was converted to squalene. Epstein and Rilling (1970) established that this intermediate, presqualene pyrophosphate (PSPP), was a 30-carbon pyrophosphate ester which contained a cyclopropane ring. This suggests the overall reaction shown in Fig. 1.6. Although Cornforth (1973) suggested that PSPP could be an artefact produced by a reversible side reaction which was activated in the absence of NADPH, it has since been isolated from yeast microsomes incubated with NADPH (Muscio et al., 1974). Furthermore Qureshi et al. (1973a) showed that squalene synthetase from baker's yeast exists in two forms, protomeric and polymeric. The protomeric form catalyses the biosynthesis of only PSPP, while the polymeric form is able to convert it into squalene.

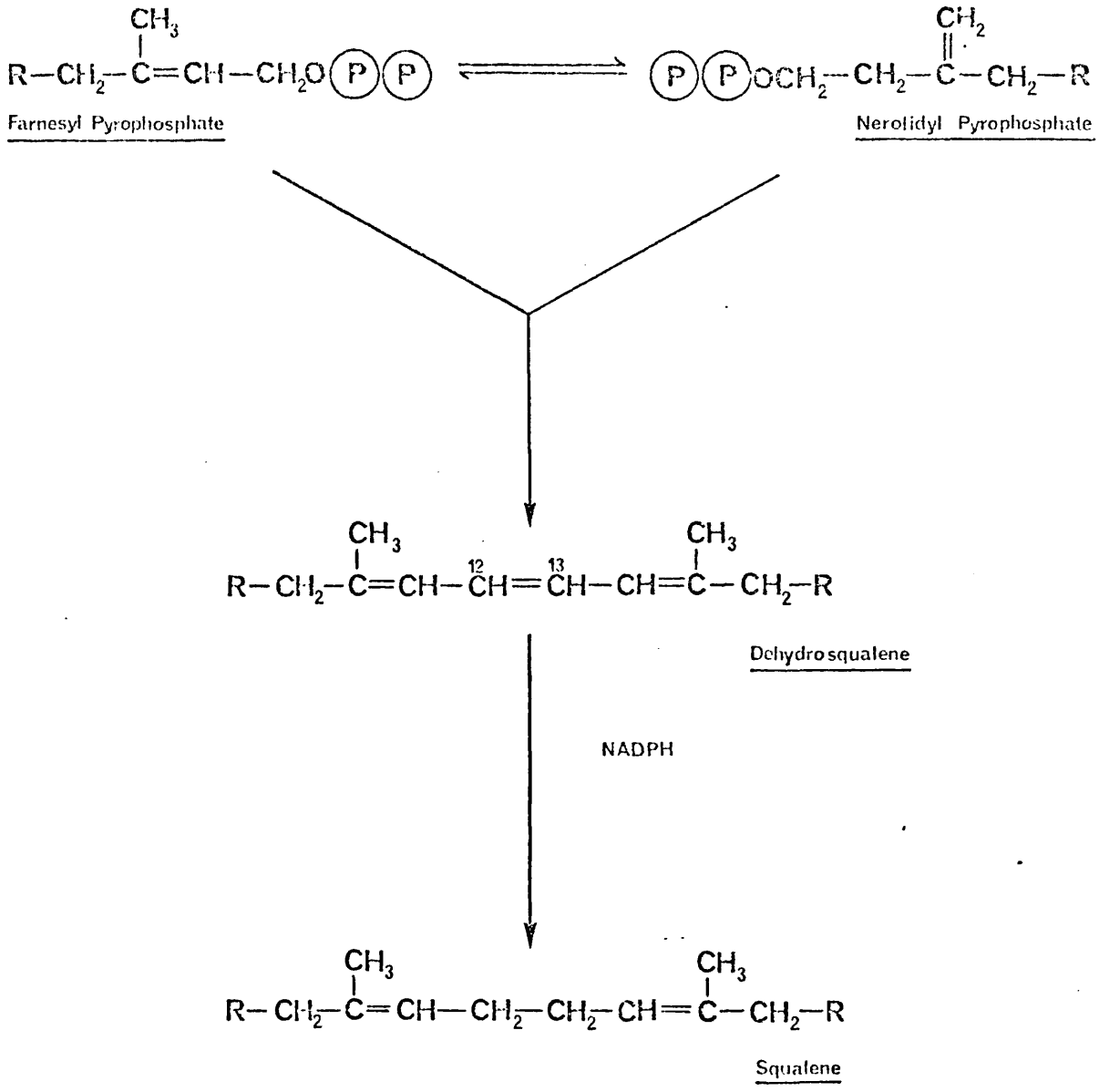


Fig. 1.5 Proposed Biosynthesis of Squalene via Dehydrosqualene

(Cornforth and Popják, 1959)

R = $CH_3-C(CH_3)=CH-CH_2-CH_2-C(CH_3)=CH-CH_2-$

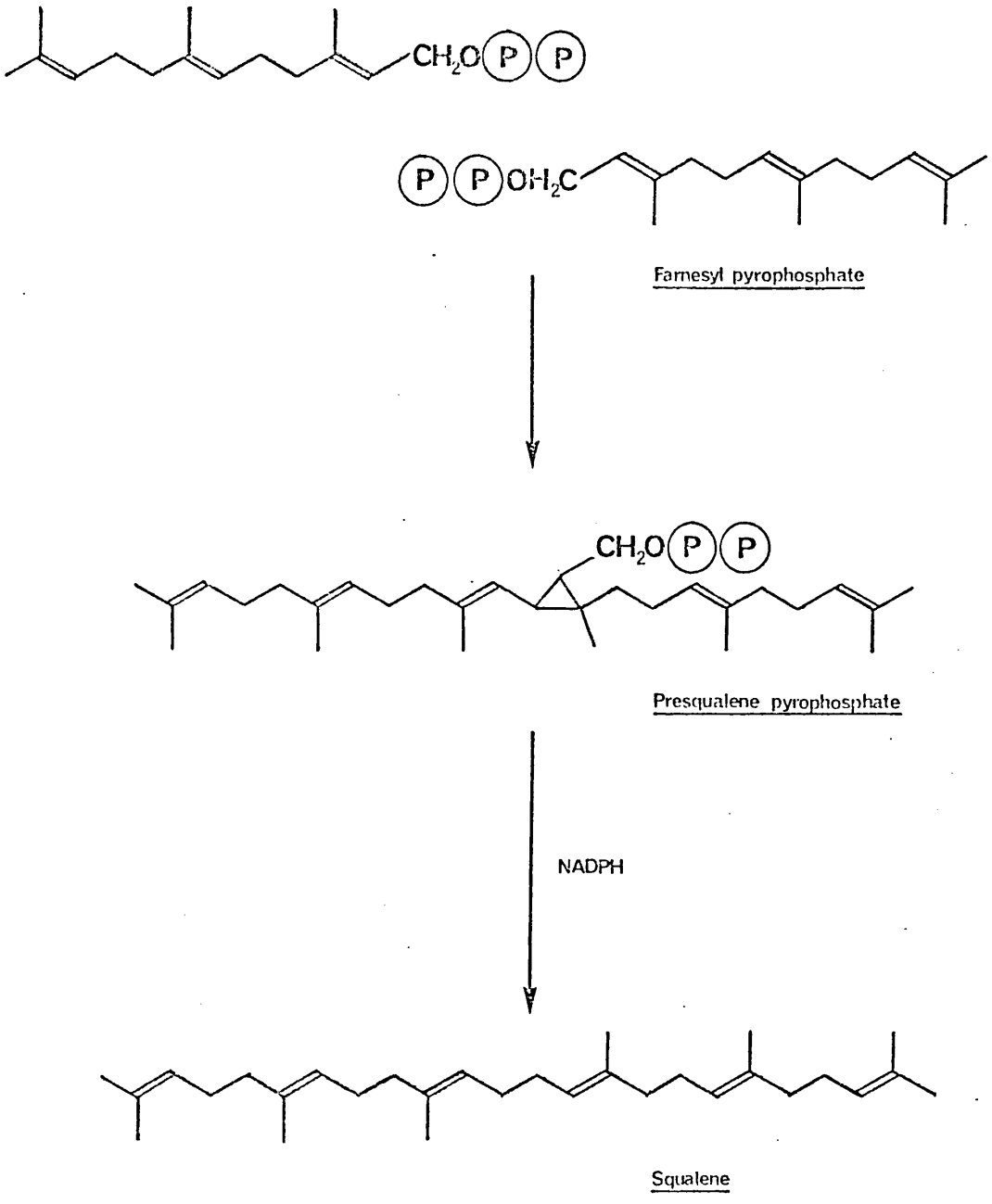


Fig. 1.6 Biosynthesis of Squalene

Beytía and coworkers (1973) studied the kinetics of purified yeast squalene synthetase. They concluded that the formation of PSPP occurs via a ping-pong type mechanism, involving nucleophilic addition of FPP to a farnesyl-enzyme intermediate. The reduction of PSPP to squalene involves an ordered sequential mechanism, whereby an enzyme-PSPP-NADPH complex rearranges to enzyme-squalene-NADP-PP_i. Based on these experiments the mechanism shown in Fig. 1.7a,b has been suggested (Edmond et al., 1971; Beytía et al., 1973; McCorkindale, 1976).

Biosynthesis of Lanosterol

Tchen and Bloch (1957) showed that squalene could be converted into lanosterol (lanosta - $\Delta^{8,24}$- dienol) and cholesterol by a rat liver homogenate. This was an aerobic reaction requiring NADPH. Corey et al. (1966) found that the incorporation of squalene 2,3-oxide into sterols by a rat liver homogenate was greater than the incorporation of squalene itself. They confirmed that the 2,3-oxide was an intermediate by trapping radioactivity from [^{14}C]squalene with non-radioactive squalene oxide. The same group have also shown that the second reaction occurs under anaerobic conditions (Dean et al., 1967).

The cyclization of squalene 2,3-oxide to lanosterol has also been demonstrated in cell-free systems from P. blakesleeanus (Mercer and Johnson, 1969) and Cephalosporium caerulens (Kawaguchi et al., 1973a), indicating that the sequence of reactions is similar in fungi. In higher plants, however, the first cyclic intermediate is probably cycloartenol (9,19-cyclolanosta-Δ^{24}-enol; Goodwin, 1971b).

The mechanism for the conversion of squalene 2,3-oxide to lanosterol is probably a proton-initiated cyclization (Mercer and Johnson, 1969), but it has not yet been investigated in detail (Gaylor, 1974). The overall sequence of reactions is shown in Fig. 1.8.

Biosynthesis of Ergosterol

The major sterol in most fungi is ergosterol (ergosta-$\Delta^{5,7,22}$-trienol; Weete, 1973), which Schwenk and Alexander (1958) showed was synthesized from lanosterol by yeast. This conversion involves the following reactions: (a) loss of the methyl groups at C_4 and C_{14};

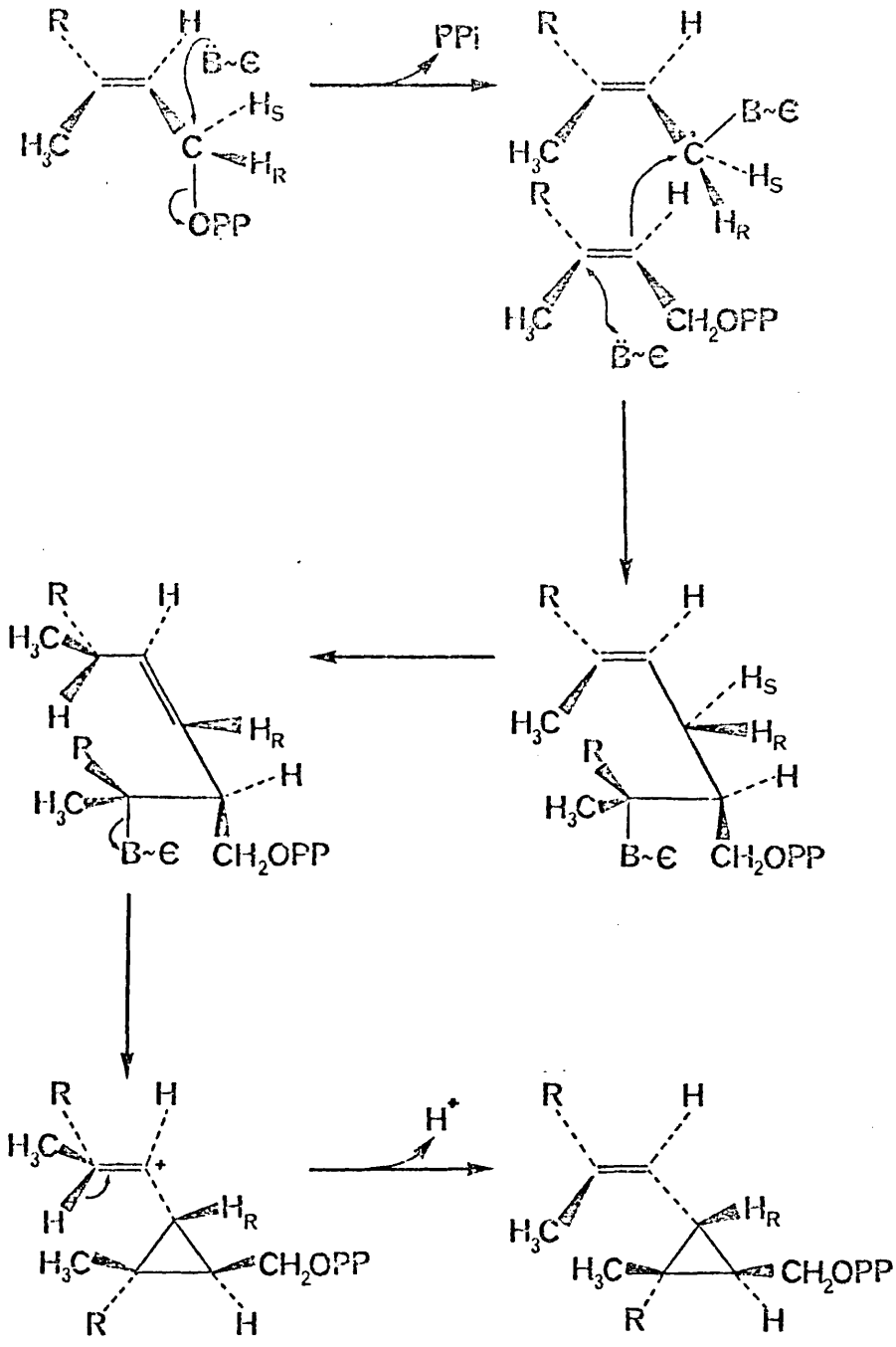


Fig.1.7a Dimerisation of FPP to PSPP

(Beytia et al., 1973)

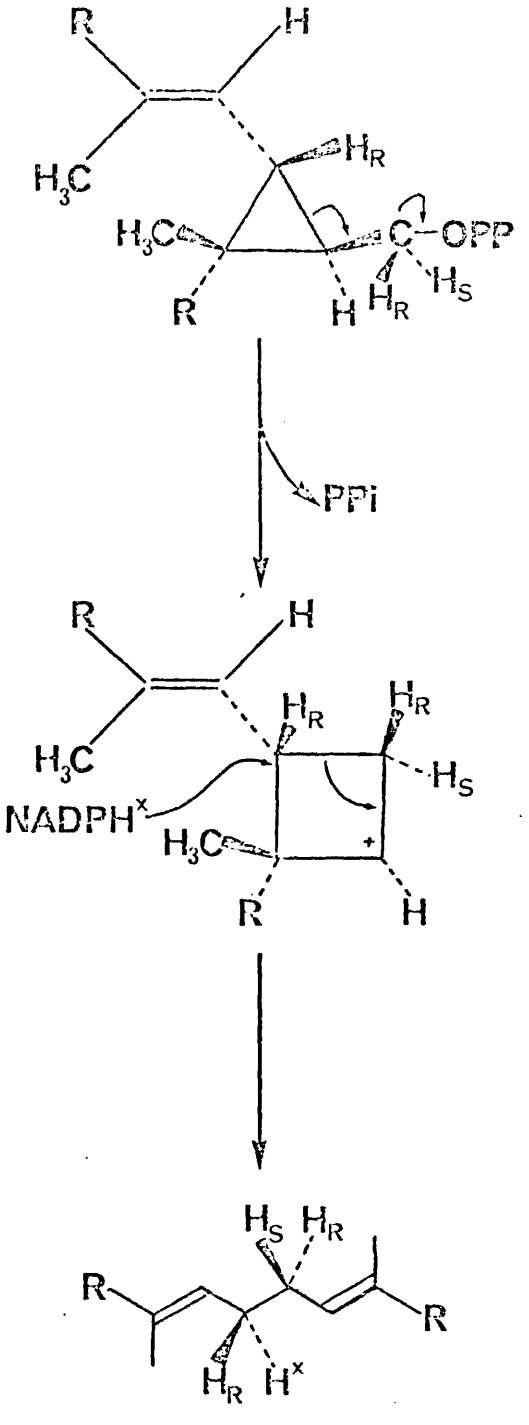


Fig.1.7b Conversion of PSPP to Squalene

(McCorkindale, 1976)

R = $CH_3-\underset{\underset{CH_3}{|}}{C}=CH-CH_2-CH_2-\underset{\underset{CH_3}{|}}{C}=CH-CH_2-CH_2-$

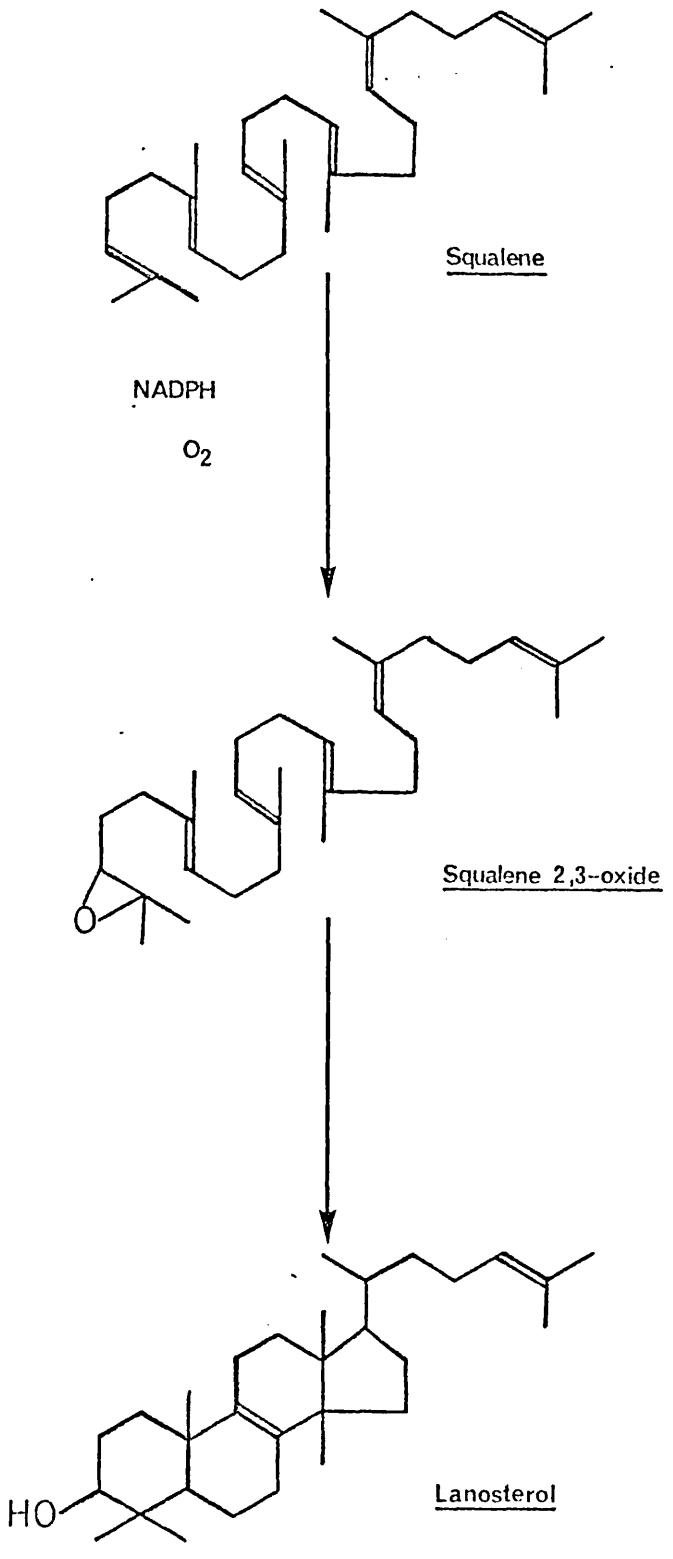


Fig. 1.8 Cyclisation of Squalene

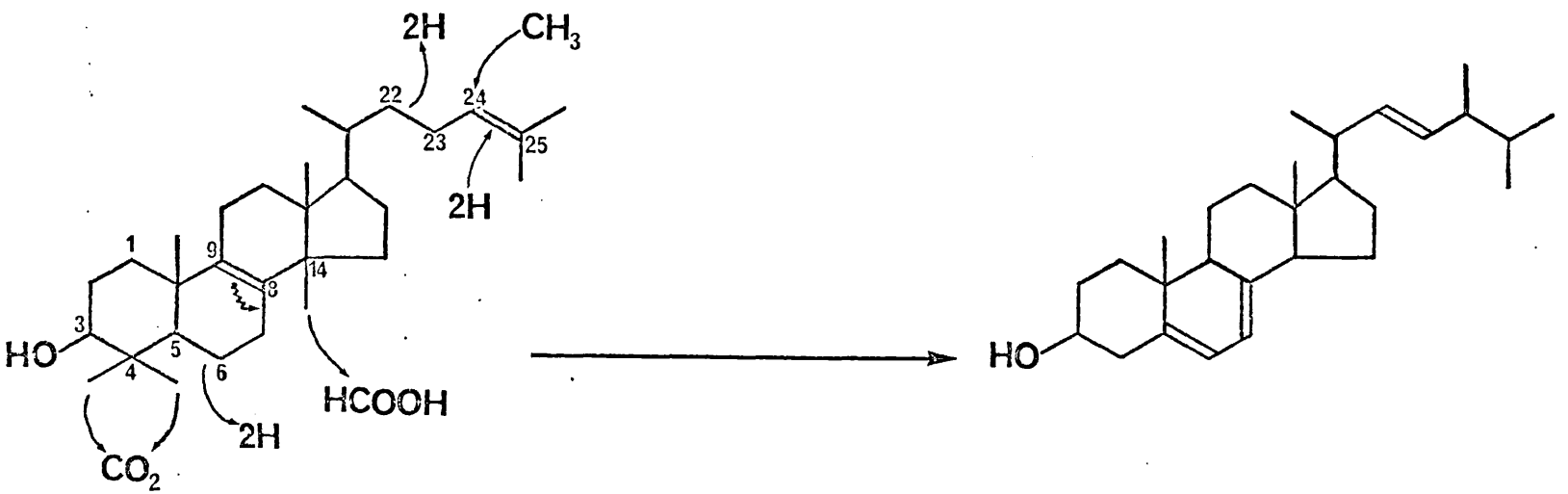
(b) reduction of the C_{24} double bond; (c) formation of a 5,6-double bond and isomerisation of the 8,9 to a 7,8-double bond; (d) methylation at C_{24}; (e) formation of a C_{22} double bond (Fig. 1.9).

In the conversion of squalene to the principal 4-desmethyl sterols the same general steps are present in most organisms, but the quantitatively important intermediates and the sequence in which certain reactions occur, differ not only between major taxonomic groups, but also between tissues and developmental stages of the same organism (Weete, 1973).

Interpretations of experiments in which proposed intermediates are shown to be incorporated into end products is complicated by the fact that such conversions may be due to a lack of specificity of the enzymes involved, so that the quantitative importance of these reactions *in vivo* cannot be demonstrated. Despite these difficulties Fryberg and coworkers (1973a) proposed that the major route of ergosterol production in yeast is that shown in Fig. 1.10. This scheme is based on both changes in the pool sizes of various intermediates when anaerobically grown yeast cells were transferred to aerobic conditions (since the absence of O_2 prevents cyclization of squalene), and on feeding/trapping experiments with postulated intermediates. They also showed a number of subsidiary conversions, indicating that a matrix of enzymes with low substrate specificity may be involved.

Nystatin is a polyene antibiotic which probably acts by complexing with membrane sterols (Kinsky, 1967). For this reason nystatin-resistant mutants of yeast normally show altered sterol patterns. Trocha *et al.* (1974) found that in a mutant in which demethylation at C_{14} was blocked (Fig. 1.10, step A), demethylation at C_4 (steps B and C) and methylation at C_{24} (step D) occurred normally, but $\Delta^{8,9}$ isomerisation, Δ^{22} desaturation and $\Delta^{24,28}$ reduction (steps E, F and G, respectively) did not occur. This indicates that the demethylation step must be performed early in the reaction sequence, since later enzymes are unable to accept 14-methyl sterols as substrates.

Barton and coworkers (1974) isolated several nystatin-resistant yeast mutants and showed that, in each case, only one mutation was involved. Blockages at the stages of C_{24}-methylation (Fig. 1.10; step D); $\Delta^{8,9}$-isomerisation (E); 22,23-dehydrogenation (F) and 5,6-



Lanosterol

Ergosterol

Fig. 1.9 Transformations involved in the Conversion of Lanosterol to Ergosterol

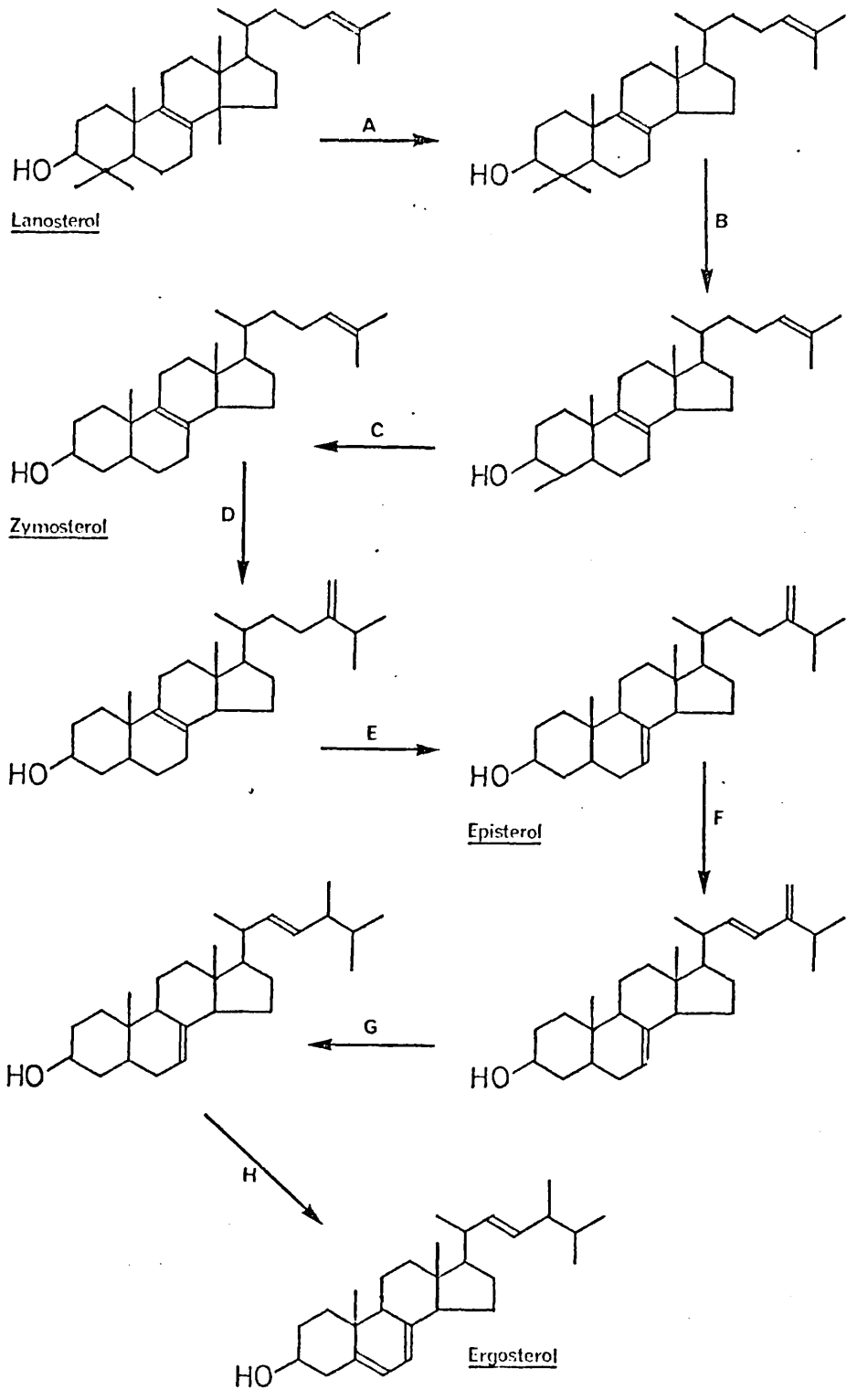


Fig. 1.10 Major Route of Ergosterol Synthesis in Yeast

(Fryberg et al., 1973a)

dehydrogenation (H) were found, and in each case all the other conversions occurred normally. This indicates that the later stages of ergosterol biosynthesis in yeast are simply a combination of five basic processes, which may occur in any order, although some routes are quantitatively more important than others.

In P. blakesleeanus the major sterols throughout growth are ergosterol (50-65% of total free sterol) and episterol (ergosta-$\Delta^{7,24(28)}$-dienol; 30-40%). In addition lanosterol, 24-methyllanosta-$\Delta^{8,24(28)}$-dienol, and ergosta-$\Delta^{5,7,24(28)}$-trienol have been isolated (Goulston and Mercer, 1969; Bartlett and Mercer, 1974). The presence of the methylene derivative of lanosterol indicates that, in Phycomyces, the methylene group is introduced before demethylation (i.e. step D precedes steps A to C of Fig. 1.10), while the tentative identification of other possible precursors of ergosterol in this fungus indicates that most of the changes in the ergosterol nucleus precede further modification of the side-chain (Goulston et al., 1975). The relatively high concentrations of episterol and ergosterol, and the presence of the intermediate ergosta-$\Delta^{5,7,24(28)}$-trienol further suggest that the 5,6-double bond is introduced before the side-chain is modified (i.e. step H precedes steps F and G of Fig. 1.10; Goulston and Mercer, 1969).

Mercer and Russell (1975) recently obtained confirmatory evidence for this sequence by growing Phycomyces in a medium containing [2-^{14}C, 3,4-^{3}H$_4$]MVA. They found an increase in the ^{14}C:^{3}H ratio of the isolated lipids from squalene (normalised to 6:6) to lanosterol and 24-methyllanosta-$\Delta^{8,24(28)}$-dienol (6:5), 4-methyl-5-ergosta-$\Delta^{8,24(28)}$-dienol and episterol (5:4), and ergosta-$\Delta^{5,7,24(28)}$-trienol and ergosterol (5:3). This was consistent with the expected loss of a tritium atom during cyclization, one ^{3}H and one ^{14}C atom during 4α-demethylation, and one ^{3}H atom during the formation of the 5,6-double bond.

The demethylation of lanosterol by a rat liver homogenate requires O_2 and NADPH, while three moles of CO_2 are released per mole of cholesterol formed (Olson et al., 1957). In addition, the conversion of lanosterol to zymosterol (cholesta-$\Delta^{8,24}$-dienol) by a yeast enzyme preparation is inhibited by carbon monoxide (Alexander et al., 1974), suggesting that the demethylation reactions involve a microsomal

mixed-function oxidase and cytochrome P_{450} (Gaylor, 1974).

The two C_4-methyl groups are oxidised to carboxylic acids, and thence to CO_2 (Sharpless et al., 1968), while the C_{14}-methyl group is oxidised to the aldehyde, and released as formic acid (Alexander et al., 1972). The latter reaction probably involves a $\Delta^{8,14}$-diene intermediate, since a proton is lost from C_{15} during the conversion of lanosterol to cholesterol (Gibbons et al., 1968).

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

The $\Delta^{7(8)}$-bond is formed by the reversible isomerisation of the $\Delta^{8(9)}$-bond (Fryberg et al., 1973a) while the introduction of the Δ^5-double bond probably involves a mixed-function oxidase, since it requires oxygen (Frantz et al., 1959) and is inhibited by cyanide (Dempsey, 1969). A hydroxylation-dehydration mechanism is probably involved (Fryberg et al., 1973b).

The introduction of the 22,23-trans double bond involves the elimination of the 22-pro-S- and 23-pro-S- hydrogen atoms in Aspergillus fumigatus (Bimpson et al., 1969), although the mechanism remains unknown (Goodwin, 1971b). Desaturation at C_{22} probably occurs after reduction of the $\Delta^{24(25)}$-bond, since sterols with the conjugated $\Delta^{22,24(25)}$-double bond system have not been identified in plants or fungi (Weete, 1973).

Esterification of Sterols

In addition to free sterols, steryl esters, steryl glycosides and acylated steryl glycosides are also present in plants (Grunwald, 1975). Bartlett and Mercer (1974) found that steryl esters represent about half of the total sterol content of P. blakesleeanus mycelia at most

stages of growth. The same range of fatty acids is found in the steryl esters, triacylglycerols and phospholipids of Phycomyces, but there are quantitative differences in the types of fatty acids esterified to sterols and to glycerol (Mercer and Bartlett, 1974). Furthermore, the proportion of each sterol in the free and esterified form varies widely with the stage of growth, indicating that esterification is not a random process (Bartlett and Mercer, 1974). Bartlett et al. (1974) demonstrated that the acyl group is derived from phosphatidylcholine in Phycomyces, as in animal tissues (Glomset, 1968).

When mycelia of P. blakesleeanus were transferred to a starvation medium no breakdown of steryl esters was observed, indicating that they do not act as reserves of energy (Bartlett and Mercer, 1974). Brady and Gaylor (1971) observed competition between esterification and demethylation of 4α-methyl sterols in cell extracts of rat liver, and showed that the esterified sterols were not demethylated, since the hydroxyl group was not available to form the 3-keto intermediate which is obligatory in C_4 demethylation (Rahman et al., 1970). This may indicate that esterification is a way of regulating lanosterol metabolism (Grunwald, 1975).

Sterol Carrier Proteins

All the early precursors of sterols up to FPP are soluble in water, but the subsequent intermediates, and the sterols themselves, are all lipophilic. Ritter and Dempsey (1970) found that washed rat liver microsomes exhibited very low activity in the enzymic reduction of cholesta-$\Delta^{5,7}$-dienol to cholesterol. This activity was enhanced by the addition of a heat-stable, non-dialyzable, trypsin-sensitive factor from the 100,000g supernatant. This protein was partially purified (Ritter and Dempsey, 1970; Scallen et al., 1971) and named "Sterol Carrier Protein (SCP)". These workers showed that SCP was able to bind to squalene in vitro, and to activate a number of specific reactions in the conversion of squalene to cholesterol. Bovine serum albumin, which also binds to sterols, did not activate these reactions.

Rilling (1972) found that SCP isolated from rat liver cells enhanced squalene biosynthesis in microsomal preparations from both rat liver and yeast. An analogous protein was isolated from a yeast 100,000g supernatant fraction, suggesting that a carrier protein may be

involved in fungal, as well as mammalian, sterol biosynthesis.

Scallen et al. (1972) purified SCP and obtained a molecular weight of 28,000, but Ritter and Dempsey (1973) found a form with a molecular weight of only 16,000, which aggregated to a higher molecular weight complex when bound to cholesterol precursors. This apparent anomaly was resolved by the separation of three distinct carrier proteins (designated SCP_1, SCP_2 and SCP_3), involved in the cyclisation of squalene, C_4-demethylation, and $\Delta^{7(8)}$-bond reduction, respectively (Scallen et al., 1975). Evidence for specific carrier proteins involved at other steps between squalene and cholesterol was also obtained.

SCP_1 has been purified 575 fold from rat liver (Srikantaiah et al., 1976). It is capable of binding squalene, desmosterol (cholesta-$\Delta^{5,24}$-dienol) and cholesterol, but not testosterone or oestradiol. Partially purified SCP_1 will also bind trolein, phosphatidylcholine, cholesteryl oleate, oleic acid and palmityl-CoA (Scallen et al., 1972), but it is capable of activating only the microsomal conversion of squalene to lanosterol. Although it has no catalytic activity the addition of excess SCP_1 to a mixture containing squalene-SCP_1 and microsomes increases the rate of the reaction, indicating that SCP_1 affects both binding and catalysis (Scallen et al., 1975). The apparent K_m for SCP_1 in the conversion of squalene to lanosterol (1.2×10^{-6} M) is of the same order as that for squalene (2.4×10^{-6} M), providing further evidence in favour of a carrier role for this protein (Srikantaiah et al., 1976).

Since many other reactions in lipid metabolism are stimulated by the presence of a 100,000g supernatant fraction Scallen et al. (1975) suggest that carrier proteins may be involved in all reactions involving water-insoluble substrates.

Biosynthesis of Phytoene

The formation of phytoene (7,8,11,12,7',8',11',12'-octahydro-ψ,ψ-carotene) from [^{14}C]FPP and [^{14}C]GGPP has been demonstrated using enzymes prepared from tomato plastids (Jungalwala and Porter, 1967; Shah et al., 1968), and in homogenates of P. blakesleeanus mycelia (Yamamoto et al., 1961; Lee and Chichester, 1969). The relative efficiencies with which these precursors are incorporated suggests that C_{40} carotenes are formed by dimerisation of GGPP.

By analogy with the formation of squalene by the dimerisation of FPP, the first C_{40} carotene should be lycopersene (7,8,11,12,15,7',8',11',12',15'-decahydro-ψ,ψ-carotene). Qureshi et al. (1973b) showed that this compound is formed when purified yeast squalene synthetase is incubated with GGPP in the presence of NADPH and Mg^{2+} ions. Omission of the reduced coenzyme caused the accumulation of prelycopersene pyrophosphate, a C_{40} analogue of presqualene pyrophosphate. However the rate of the reaction when GGPP was substrate was only 4% of the rate of dimerisation of FPP.

Considerable controversy surrounds the involvement of these intermediates during carotenoid biosynthesis in vivo. Grob et al. (1961) incubated [1-^{14}C]GGPP with a particulate fraction of mycelia from Neurospora crassa, and found that radioactive lycopersene was formed in the presence of NADPH. Grob and Boschetti (1962) also reported the isolation of this compound from whole cells of Neurospora grown in the presence of diphenylamine (an inhibitor of phytoene desaturation; Goodwin, 1952). However Davies et al (1963) could not detect lycopersene in cultures of Neurospora grown under similar conditions. The reason for this discrepancy may be the techniques used for the purification of lycopersene, since Grob and Boschetti employed paper chromatography, while Davies et al. separated their extracts more thoroughly by thin-layer chromatography. However, a recent observation by Barnes et al. (1973) that lycopersene is recovered only in very low yields after chromatography on silica gel, has reopened the question of the importance of this intermediate in vivo.

Altman et al. (1972) synthesized a forty-carbon analogue of presqualene pyrophosphate (Fig. 1.11), which they called prephytoene pyrophosphate (PPPP). They showed that it was produced by a cell-free extract of a Mycobacterium species incubated with [^{3}H]GGPP, and that this extract could also convert it into carotenoids.

Qureshi et al. (1972) found that a crude enzyme system isolated from tomato fruit plastids incorporated [^{14}C]GGPP into PPPP and lycopersene. In the absence of NADPH no lycopersene was formed, but when NADPH was added both PPPP and lycopersene were converted to more unsaturated carotenes. Barnes et al. (1973) used a similar enzyme preparation and found that although incorporation of [^{14}C]GGPP into

carotenes was unaffected by the substitution of NADP for NADPH, lycopersene production was drastically reduced under these conditions. Since the addition of NADPH did not increase the rate of biosynthesis of phytoene in a cell-free system from P. blakesleeanus (Bramley, 1973), it seems that lycopersene may not be an obligatory intermediate in phytoene synthesis.

The mechanism of squalene biosynthesis outlined previously (pp.17-20; Figs. 1.6 and 1.7) involves the formation of a cyclopropane intermediate and its reduction by NADPH. In contrast no protons derived from NADP^{3}H are incorporated into phytoene, both the remaining hydrogen atoms of the 15,15'-double bond being derived from GGPP (Gregonis and Rilling, 1974). Both pro-R-hydrogen atoms from C_1 of the condensing GGPP molecules are retained in 15-cis phytoene (Williams et al., 1967a), while the production of all-trans phytoene involves the loss of one pro-R and one pro-S-hydrogen atom (Gregonis and Rilling, 1974). The elimination of a hydrogen atom introduced from NADPH into lycopersene could only occur if cis-phytoene was produced by dehydrogenation of racemic lycopersene, or if it was derived from meso-lycopersene which is not free to rotate (Gregonis and Rilling, 1974). These authors argued that the configuration of lycopersene would be meso, and that the concentrations of lycopersene observed in the experiments of Qureshi et al., (1972) and Barnes et al., 1973) are inconsistent with a restriction on rotation, which could only occur if the intermediate remained bound to an enzyme throughout the reaction. A more plausible reaction scheme involves the synthesis of phytoene directly from prephytoene pyrophosphate (Fig. 1.11). The production of lycopersene would occur only if the cyclopropylcarbinyl cation derived from PPPP gained a hydride ion from NADPH, possibly because of the incomplete specificity of squalene synthetase (Qureshi et al., 1973b).

Desaturation of Phytoene

Coloured varieties of tomatoes are genetically dominant over colourless mutants. As a result of detailed analyses of the carotenoids of a number of varieties, Porter and Lincoln (1950) proposed that lycopene was formed from phytoene by a series of dehydrogenations. The series has since been extended to include 3,4-dehydrolycopene (3,4-didehydro-ψ,ψ-carotene; Davies, 1975; Fig. 1.12).

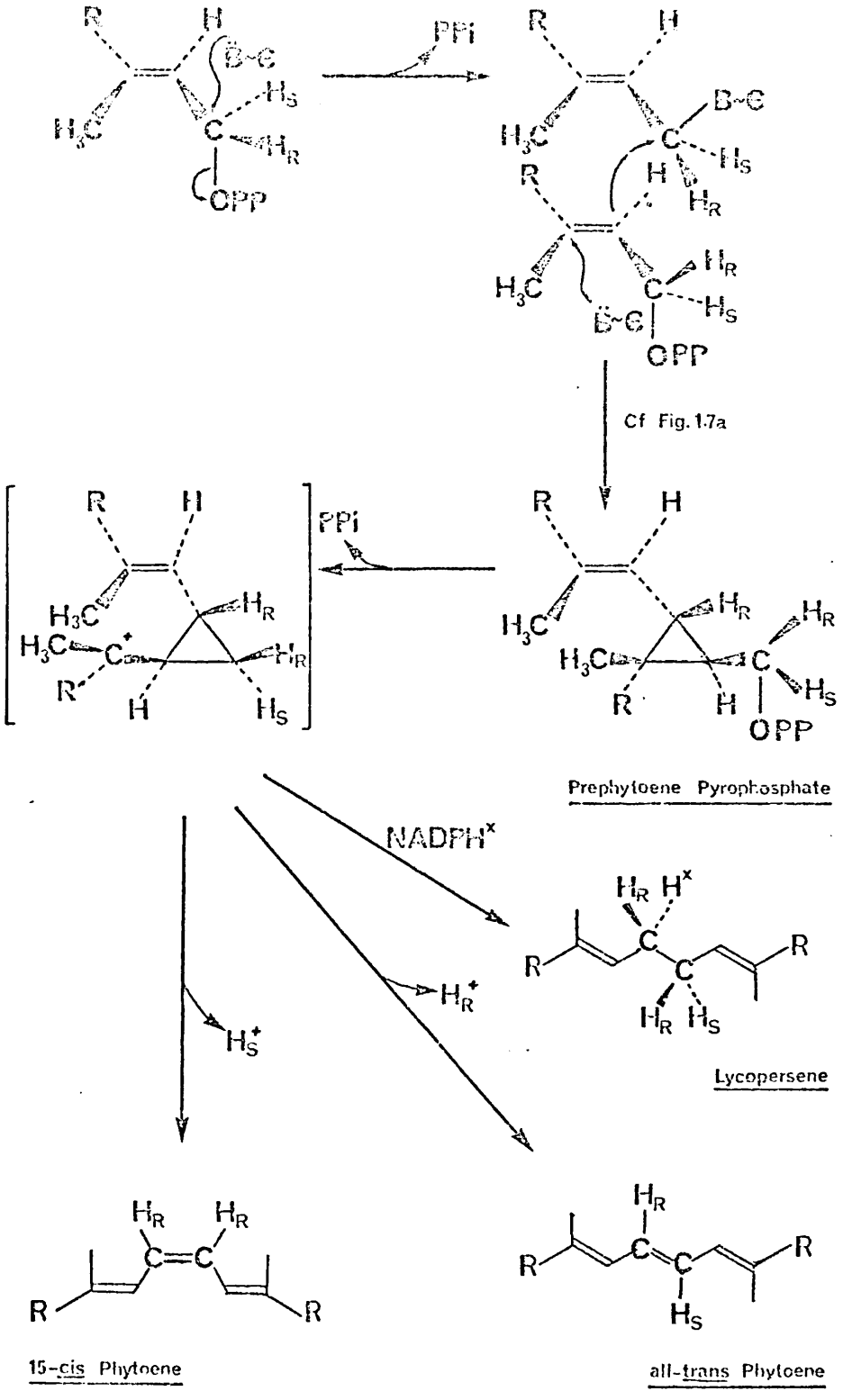
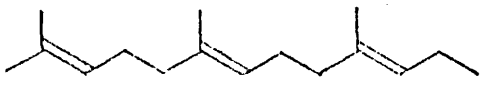


Fig.1.11 Biosynthesis of Phytoene and Lycopersene

R = 

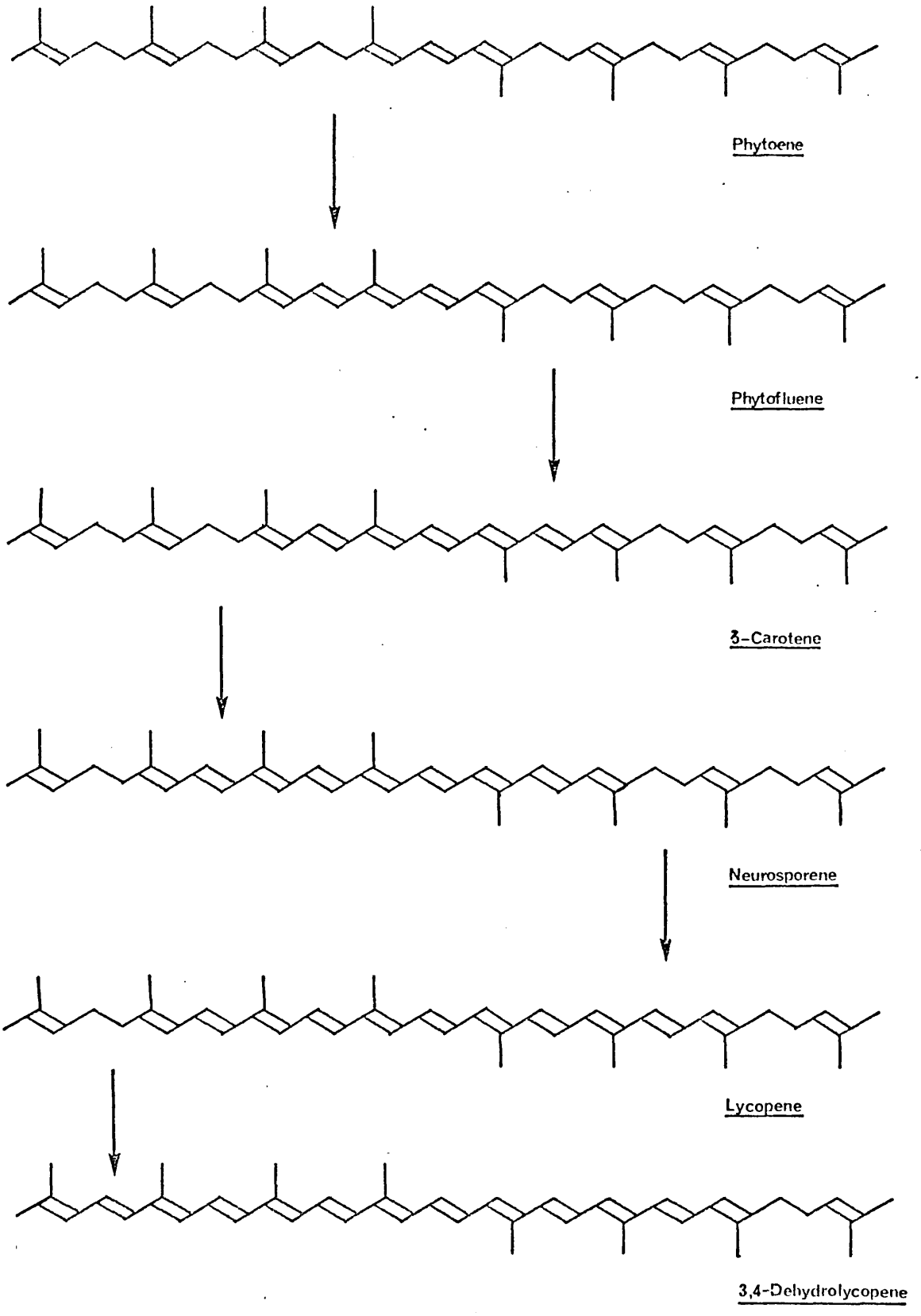


Fig. 1.12 Desaturation of Phytoene

When the pathway was first suggested the structures of the intermediates were unknown. However Zechmeister and Koe (1954) were able to show that each member of the proposed series could be converted to the next in high yield by dehydrogenation with N-bromosuccinimide, and final structural proof was provided by Davis et al. (1961). Further evidence has been provided by the studies of Porter's group on enzymes from tomato fruit plastids which are able to convert phytoene, phytofluene (7,8,11,12,7',8'-hexahydro-ψ,ψ-carotene) and 3-carotene (7,8,7',8'-tetrahydro-ψ,ψ-carotene) to more unsaturated members of the series (Kushwaha et al., 1970; Qureshi et al., 1974). The conversion of neurosporene (7,8-dihydro-ψ,ψ-carotene) to lycopene has been demonstrated in P. blakesleeanus (Bramley et al., 1977).

Davies et al. (1969) isolated an isomer of 3-carotene from Rhodospirillum rubrum grown in the presence of diphenylamine. This was shown to be 7,8,11,12-tetrahydrolycopene (7,8,11,12-tetrahydro-ψ,ψ-carotene), indicating that in Rhodospirillum dehydrogenation does not occur alternately on either side of the chromophore (Davies, 1970). Neurospora crassa and some mutants of Phycomyces blakesleeanus also contain the unsymmetrical isomer in addition to 3-carotene itself (Davies et al., 1974). This may indicate that desaturation proceeds via different routes in these organisms, or that the substrate specificity of the dehydrogenase enzymes is not absolute.

In higher plants and fungi the predominant stereoisomer of phytoene has a 13-trans, 15-cis, 13'-trans chromophore, although small amounts (<3%) of the all-trans isomer are also present (Aung Than et al., 1972; Herber et al., 1972). In higher plants phytofluene is present as the cis-isomer (Jungalwala and Porter, 1965), but 3-carotene and neurosporene have all-trans configurations (Davis et al., 1966).

Investigations on enzymes prepared from tomato fruit plastids showed that incorporation of [^{14}C]phytoene into cis-phytofluene was greater than into the trans isomer (Kushwaha et al., 1970). cis-Phytofluene was converted to trans-phytofluene, but the reverse reaction did not occur. trans-Phytofluene and trans-3-carotene were both converted to less saturated carotenes (Qureshi et al., 1974). This is evidence that cis-trans isomerisation of phytofluene occurs in higher plants.

In extracts of P. blakesleeanus total phytoene (which contains a small amount of the all-trans isomer) is a more effective diluent of [2-^{14}C]MVA incorporation into β-carotene than purified 15-cis-phytoene (Bramley and Davies, 1976), while cis-phytoene and trans-phytofluene are found in Verticillium agaricinum (Valadon et al., 1973). These data have been interpreted as suggesting that cis-trans isomerisation of phytoene occurs in fungi.

When the mycelia of a diphenylamine-inhibited culture of Phycomyces were washed and resuspended in buffer some of the accumulated phytoene (predominantly the cis-isomer) was desaturated. The changes in concentration of the all-trans isomer during this period of pigment production were consistent with the view that it is a precursor of phytofluene (Davies, 1973). It has been suggested on structural grounds that diphenylamine acts as an analogue of trans-phytoene by binding to the desaturase enzymes (Rilling, 1965). This mechanism has been discounted on the grounds that cis-, rather than trans-, phytoene is the intermediate involved (Goodwin, 1971a); but if isomerisation occurs at the phytoene stage in fungi, and at the phytofluene stage in higher plants, then the inhibitory effect of diphenylamine on microorganisms alone can be explained.

Many bacteria contain mainly trans-phytoene (Weeks, 1971; Gregonis and Rilling, 1973). In a cell-extract of Halobacterium cutirubrum both MVA and IPP were incorporated only into trans-carotenes, and no incorporation of [^{14}C]cis-phytoene or [^{14}C]cis-phytofluene into more unsaturated carotenes was observed (Kushwaha et al., 1976). This indicates that in some bacteria trans-phytoene is formed from GGPP without the involvement of the cis isomer (Gregonis and Rilling, 1974). However the possibility that the cis-phytoene observed in plants is merely a by-product of trans-phytoene is inconsistent with double-labelling experiments (Williams et al., 1967a; Gregonis and Rilling, 1974).

The stereochemistry of hydrogen elimination has been established by Williams et al. (1967a) and McDermott and coworkers (1973), working on higher plant tissue and a Flavobacterium species, respectively. They have shown that each double bond is formed by the trans elimination of the 2-pro-S- and 5-pro-R-hydrogen atoms originating from MVA. No evidence has yet been presented for the mechanism of dehydrogenation.

Formation of Cyclic Carotenes

In addition to acyclic carotenes many fungi also contain carotenoids cyclised at one or both ends of the chromophore. The major pigment in Phycomyces blakesleeanus is β-carotene (Goodwin, 1952), which consists of a chromophore of nine conjugated double bonds with a 5,6-cyclohexene (β-ionone) ring at each end of the molecule (Fig. 1.13). In addition to this pigment Phycomyces also contains two monocyclic carotenes, β-zeacarotene (7',8'-dihydro-β,ψ-carotene) and γ-carotene (β,ψ-carotene; Goodwin, 1972), both of which have a β-ionone ring at only one end of the chromophore. α-Carotene (β,ε-carotene) has a 5,6-cyclohexene ring at one end of the chromophore and a 4,5-cyclohexene (α-ionone) ring at the other end. This carotene is often found in higher plants (Weedon, 1971), but carotenes containing the α-ionone ring have not been unequivocally detected in fungi (Goodwin, 1976).

Porter and Lincoln (1950) found that there is only a single gene difference between tomato selections which accumulate lycopene and those which produce almost entirely β-carotene, and suggested that β-carotene is probably formed by cyclization of lycopene.

However β-zeacarotene, a monocyclic form of neurosporene, is present in diphenylamine-inhibited cultures of P. blakesleeanus (Williams et al., 1965) and in N. crassa (Davies, 1973), indicating that cyclization may occur at this stage of desaturation. Davies and Rees (1973) have also isolated a monocyclic form of unsymmetrical 3-carotene (7',8',11',12'-tetrahydro-β,ψ-carotene) from diphenylamine-inhibited cultures of P. blakesleeanus.

Evidence has also been accumulated to show that cyclization of lycopene can occur. Nicotine and CPTA [2-(p-chlorophenylthio) triethylamine HCl] both inhibit cyclization, and cause the accumulation of lycopene. When these inhibitors are removed by washing the accumulated lycopene is cyclised (Batra et al., 1973; McDermott et al., 1974; Hayman et al., 1974b). This takes place even in the presence of diphenylamine, which would be expected to cause the accumulation of β-zeacarotene if the lycopene were first reduced to neurosporene and then cyclized (Hayman et al., 1974b). The cyclization of lycopene to β-carotene has also been demonstrated in plastids isolated from higher plant tissues (Decker and Uehleke, 1961; Kushwaha et al., 1970;

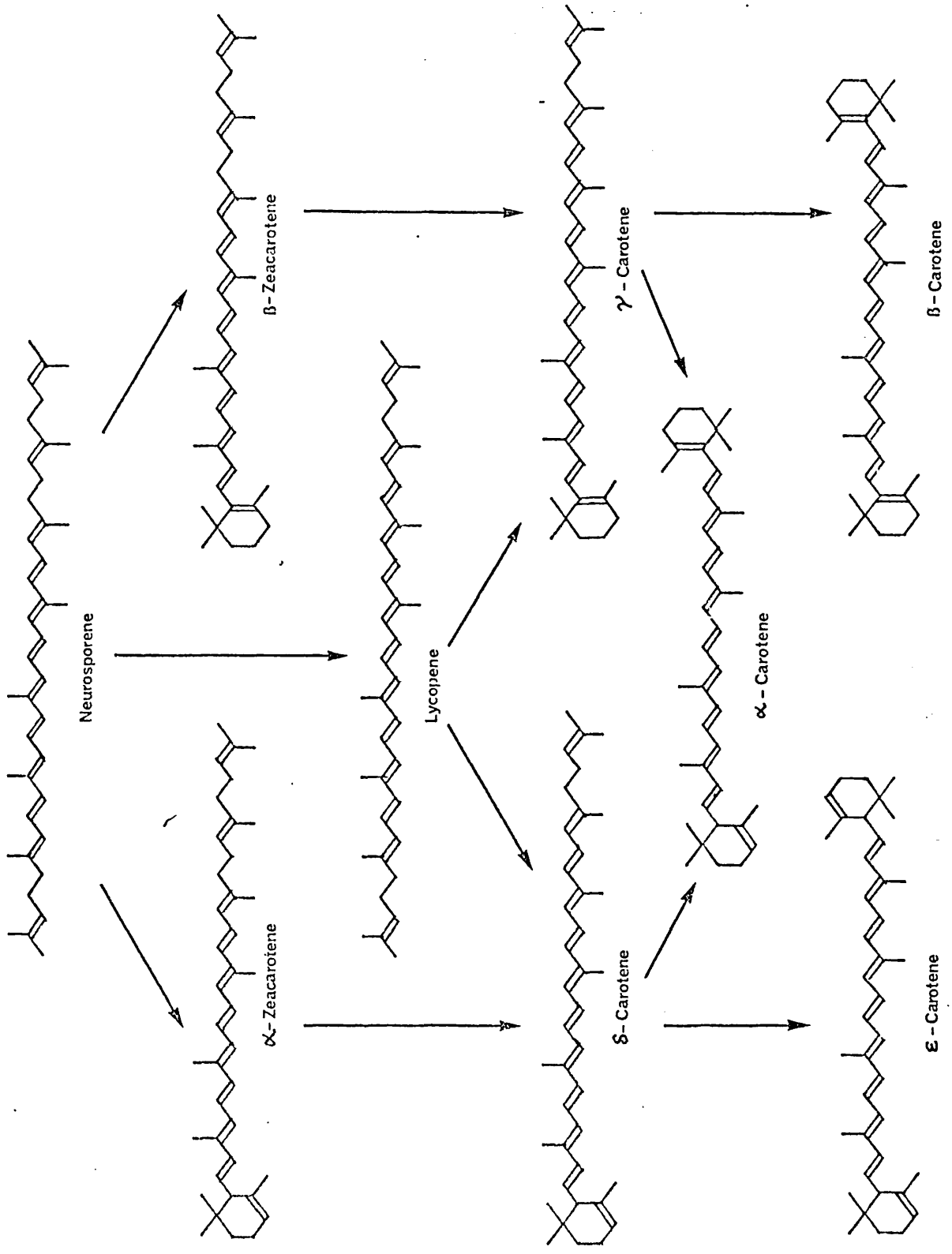
Hill et al., 1971), in acetone powders of tomato fruit plastids (Papastephanou et al., 1973), and in cell extracts of a carS mutant of P. blakesleeanus (Bramley et al., 1977) and of a Flavobacterium species (Brown et al., 1975).

However these experiments only show that both neurosporene and lycopene can be cyclized, and give no indication of the relative importance of each substrate in vivo. Bramley and Davies (1976) tested the relative efficiencies of a number of different carotenes in diluting out the incorporation of [2-^{14}C]MVA into β-carotene by a cell extract of a carS mutant of P. blakesleeanus. As expected γ-carotene and neurosporene were the most effective diluents, since both are intermediates in the conversion of phytoene to β-carotene, whichever pathway of cyclization is operative (Fig. 1.13). Lycopene and β-zeacarotene also reduced incorporation into β-carotene, however, indicating that cyclization can occur at either stage of desaturation. Dilution experiments involving either unlabelled β-zeacarotene or lycopene as diluent with [^{14}C]neurosporene as the substrate also showed that both pathways of cyclization are operative in this fungus (Bramley et al., 1977).

The mechanism of cyclization probably involves proton attack on the terminal double bond of the acyclic precursor to yield an activated intermediate. This could then lose a proton from C_4 or C_6 to form an α- or a β-ionone ring, respectively (Williams et al., 1967b; Fig. 1.14). Although the intermediate is normally depicted as a carbonium ion, the production of α- and β-carotene is stereospecific (Britton, 1976), and hence either a concerted reaction must occur, or the intermediate must be stabilized by interaction with an enzyme group.

A consequence of this proposed mechanism is that α- and β-ionone rings are produced separately, and not by isomerisation. This has been demonstrated by comparing ^{14}C:^{3}H ratios in α- and β-carotene and their precursors biosynthesized from doubly-labelled species of MVA in higher plant systems (Williams et al., 1967b). Goodwin and Williams (1965) showed that this mechanism also applies to the formation of β-carotene by P. blakesleeanus.

Fig. 1.13 Possible Pathways of Cyclisation of Neurosporene & Lycopene



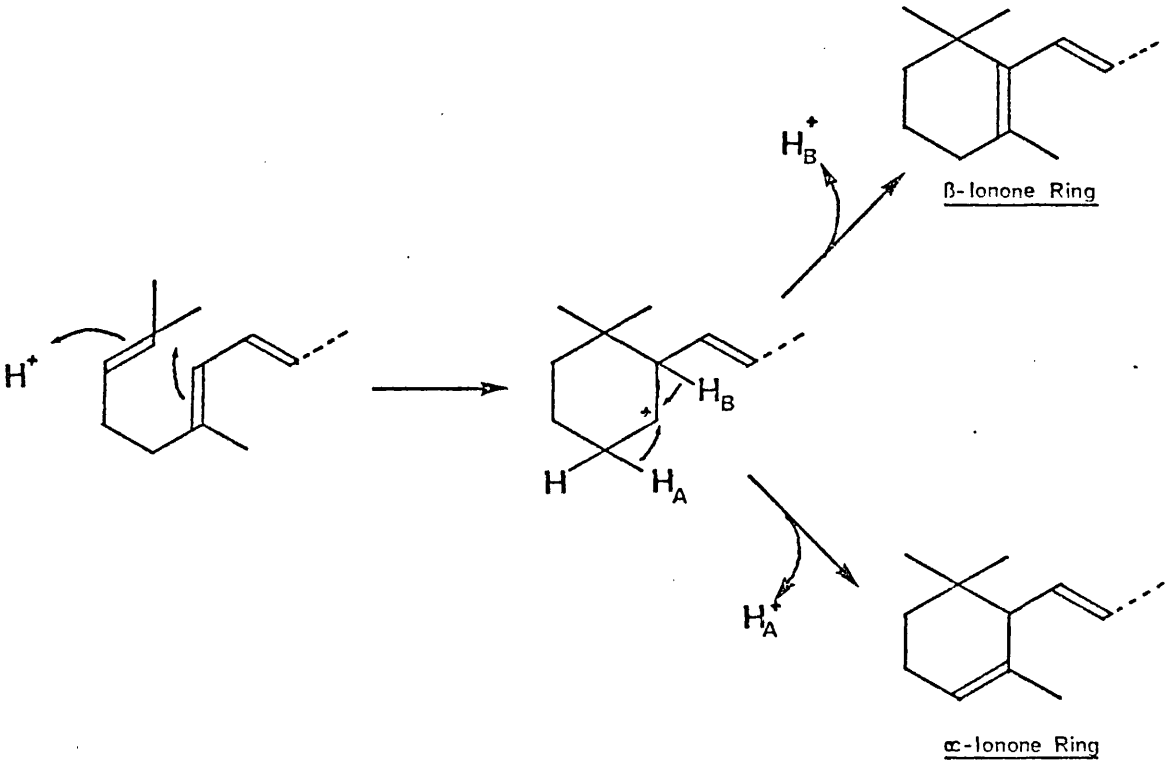


Fig.1.14 Probable Mechanism of Cyclisation

(Williams et al., 1967b)

Further Metabolism of Carotenes

Many fungi synthesize mainly β-carotene or γ-carotene, but others are able to modify these hydrocarbon chains, generally by the insertion of an oxygen function, to xanthophylls (Goodwin, 1972). The most common modifications (Fig. 1.15) are the introduction of a carbonyl group, e.g. canthaxanthin (β,β-caroten-4,4'-dione); oxidation of one of the terminal methyl groups to a carboxylic acid, e.g. torularhodin (3',4'-didehydro-β,ψ-caroten-16'-oic acid), and desaturation of the 3,4 bond, e.g. torulene (3',4'-didehydro-β,ψ-carotene) and torularhodin. A few fungi (e.g. N. crassa) also contain a 35-carbon apocarotenoic acid, neurosporaxanthin (4'-apo-β-caroten-4'-oic acid; Goodwin, 1972). In this case one isoprene unit has presumably been removed by oxidation.

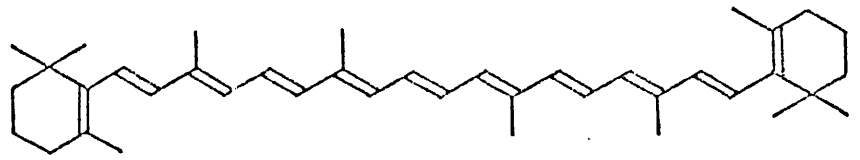
β-Carotene may be oxidatively cleaved to retinal, which is then metabolised to trisporic acids, fungal hormones which regulate gametogenesis (Austin et al., 1969, 1970; Fig. 1.16). It may also be oxidatively polymerized both in vitro and in vivo to yield sporopollenin, a component of the walls of pollen grains and fungal spores (Shaw, 1971; Gooday et al., 1973,1974).

Cofactor Requirements of Carotene Biosynthesis

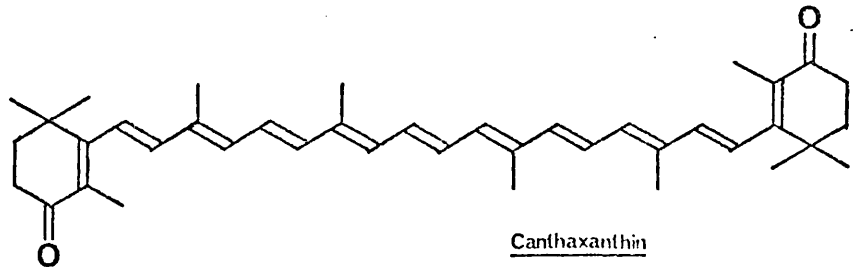
Although in vitro carotene biosynthesis by a number of crude enzyme preparations has been reported, not all of these extracts had been dialysed or similarly treated in order to remove endogenous cofactors before incubation, and in other cases evidence of cofactor requirements was not presented.

Anderson and Porter (1962) demonstrated the incorporation of [^{14}C]terpenyl pyrophosphates (biosynthesized from [2-^{14}C]MVA by a rat liver homogenate) into phytoene in extracts of carrots and also in tomato plastids. They showed that there was no requirement for oxygen in this system. Varma and Chichester (1962), however, using a crude homogenate of tomatoes, found that O_2 was required for the incorporation of either MVA or IPP into lycopene.

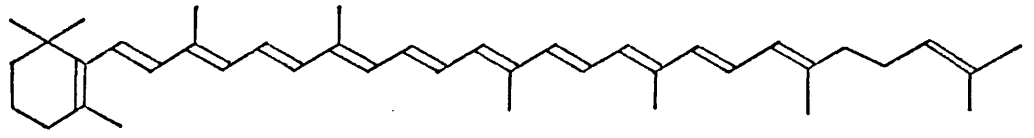
The cofactor requirements of carotene production have been rigorously examined by Porter and coworkers, using enzymes prepared from $(NH_4)_2SO_4$ fractionated acetone powders of tomato fruit plastids. Systems



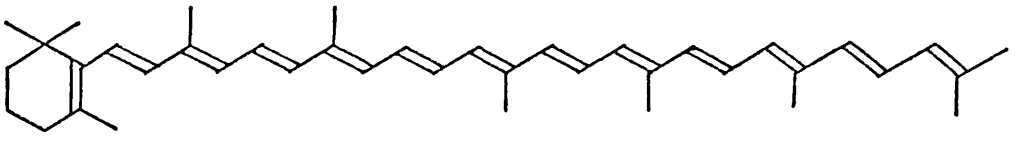
ß-Carotene



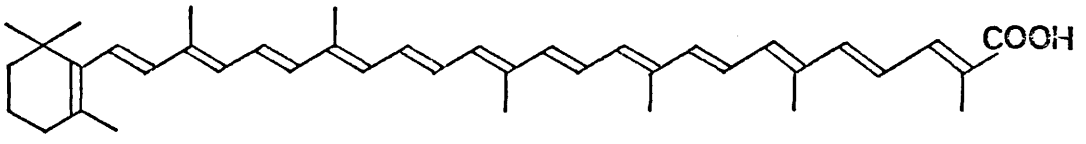
Canthaxanthin



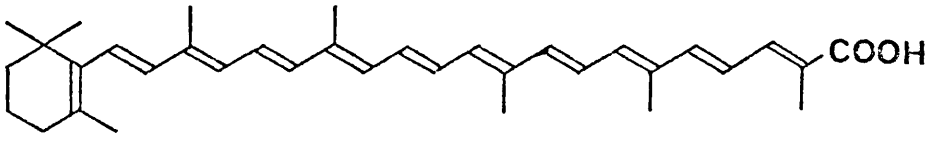
ɣ-Carotene



Torulene



Torularhodin



Neurosporaxanthin

Fig.1.15 Structures of Some Fungal Carotenoids

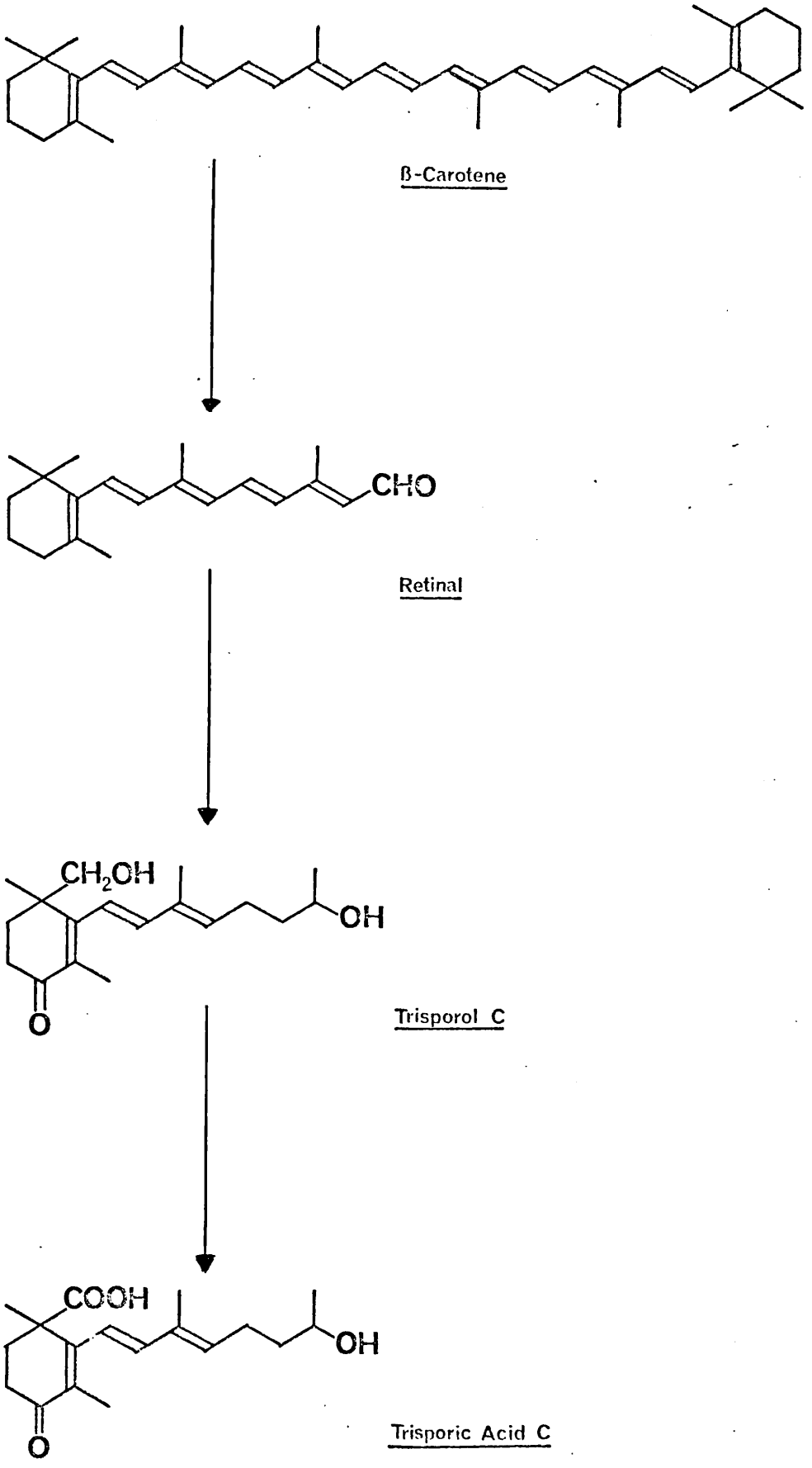


Fig. I.16 Biosynthesis of Trisporic Acid C

prepared in this way have been used to show that the condensation of IPP and FPP requires Mn^{2+} ions, while the conversion of GGPP into phytoene is stimulated by Mg^{2+} ions. No requirement for either pyridine nucleotides or O_2 was observed (Jungalwala and Porter, 1967; Shah et al., 1968). The conversion of phytoene to phytofluene by these enzymes requires NADP, while FAD is involved in the conversion of phytofluene to lycopene (Kushwaha et al., 1970). More recently Qureshi et al. (1974) have investigated the effects of cofactors on incorporation into each intermediate between phytofluene and lycopene when cis-phytofluene is incubated with an $(NH_4)_2 SO_4$ fraction of lyophilized tomato fruit plastids. Both FAD and Mn^{2+} ions were required for desaturation of phytofluene, while addition of Mg^{2+} or NADP was not necessary, although up to 2mM NADP was stimulatory. A phytoene synthetase complex purified by $(NH_4)_2SO_4$ fractionation and gel filtration was shown to require Mn^{2+} ions but not NADP or NADPH for the incorporation of [^{14}C]IPP into phytoene. Allosteric activation by ATP was also observed (Maudinas et al., 1975, 1977).

No requirement for pyridine nucleotides was observed for the conversion of [2-^{14}C]MVA into phytoene by an enzyme preparation from pea fruits (Graebe, 1968) or by isolated chloroplasts (Charlton et al., 1967; Buggy et al., 1969), but NADP was required by an $(NH_4)_2SO_4$ fraction of spinach chloroplast proteins (Subbarayan et al., 1970). In the latter case also FAD was necessary for the conversion of phytofluene to lycopene.

A number of investigations on cell-free extracts of P. blakesleeanus have been reported. The incorporation of [2-^{14}C]MVA into β-carotene by a dialysed homogenate required ATP, NADH, NADP, NADPH and Mn^{2+} or Mg^{2+} ions, whereas NAD reduced incorporation by increasing the formation of sterols (Yokoyama et al., 1962). Conversely, the conversion of [^{14}C]GGPP to phytoene by a similar system showed no pyridine nucleotide requirement (Lee and Chichester, 1969). The production of phytoene from [2-^{14}C]MVA by a dialysed extract of lyophilized mycelia of the carB10 mutant also showed no requirement for nucleotides or FAD (Bramley, 1973).

From these results it is possible to suggest the cofactor requirements for each step (Fig. 1.17).

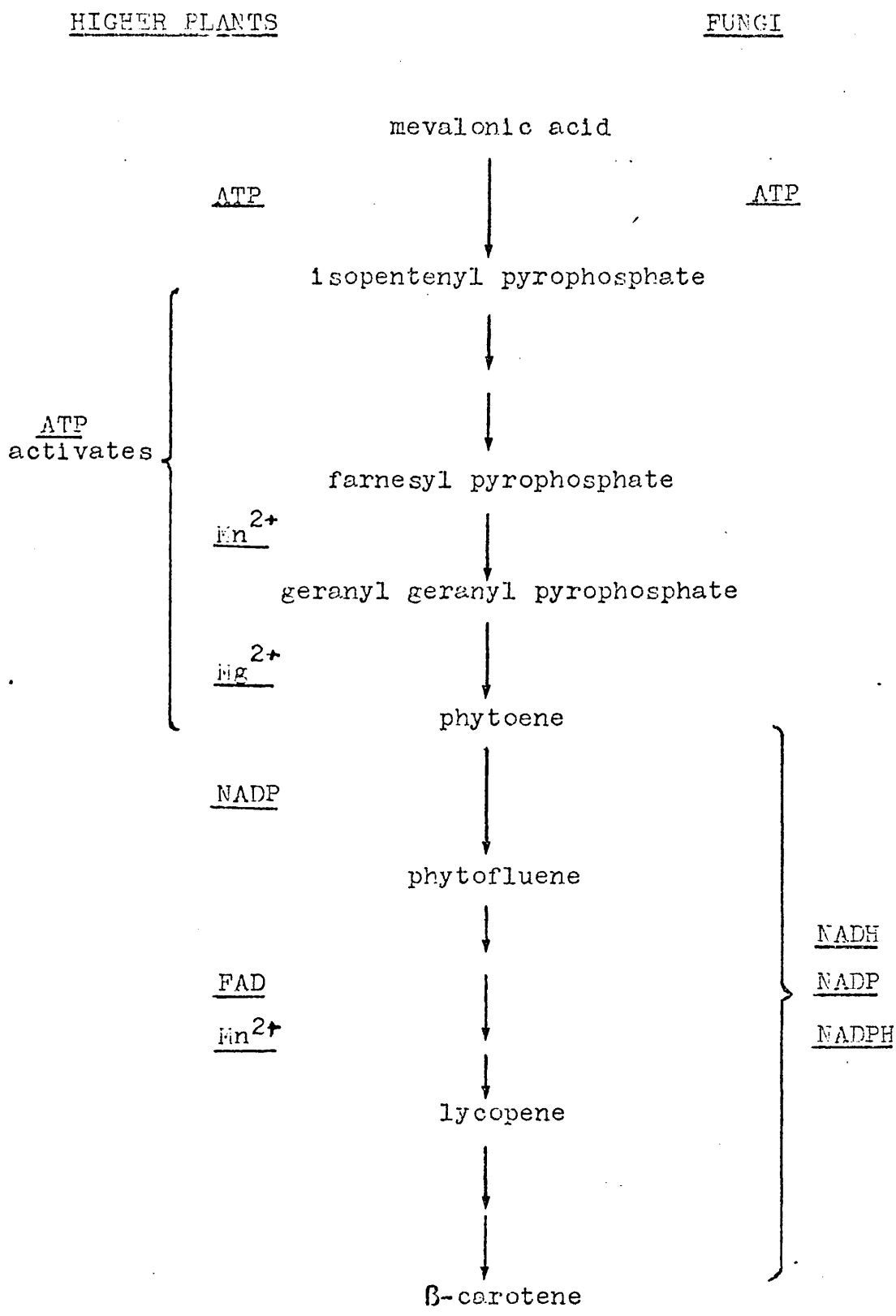


Fig. 1.17 Possible Cofactor Requirements for Carotene Biosynthesis in Higher Plants and Fungi

Most workers have reported a requirement for a thiol reagent (glutathione or dithiothreitol), although Qureshi et al. (1974) found no such requisite for the conversion of phytofluene to lycopene. The requirement is probably because the enzymes involved in the isomerisation of IPP to DMAPP and in the condensation of GGPP to phytoene contain important thiol groups, as implied by their sensitivity to inhibition by iodoacetamide (Popják and Cornforth, 1960; Lee et al., 1972).

Control of Carotenogenesis

The general pattern of carotenoid formation observed in fungi involves an initial period of active synthesis, leading to maximal concentration; an intermediate stage when these levels persist; and a final stage when the pigments disappear (Goodwin, 1976). The amount of pigment synthesized by P.blakesleeanus and Mortierella ramanniana (Mucor ramannianus) grown on solid medium depends on the availability of excess carbohydrate after growth has been completed (Goodwin and Willmer, 1952; Attwood, 1971). Carotenogenesis in many fungi may also be increased by the addition of trisporic acid. This fungal hormone is synthesized from β-carotene by the (+) strain of Blakeslea trispora (Austin et al., 1970; Sutter and Rafelson, 1968), in response to the presence of mycelia of the opposite mating type. Stimulation of carotenogenesis is restricted to the (-) strain (Thomas and Goodwin, 1967).

Carotene synthesis in many fungi is stimulated by exposure to light (Garton et al., 1951), while in other fungi, e.g. N. crassa, it is totally photoinduced; only phytoene being present in cultures grown anaerobically in the dark (Zalokar, 1954). The induction of carotenogenesis by low temperatures has also been reported (North, 1973).

Murillo and Cerdá-Olmedo (1976) have shown that end-product control of the carotenogenic pathway in Phycomyces is mediated by the product of one gene which is inactive or missing in carS mutants, which accumulate large concentrations of β-carotene. Sterol production is also end-product regulated, and exogenous ergosterol decreases carotene synthesis, indicating that a site of regulation lies on the common pathway between acetate and FPP (Lowry, 1968). On the basis of studies on a cell extract of a Phycomyces mutant Bramley and Davies

(1976) have proposed that neurosporene, lycopene, β-zeacarotene and γ-carotene inhibit desaturation of phytoene, while β-carotene inhibits cyclisation. Desaturation was not inhibited by β-carotene either in these cell extracts or in the growing mycelia (Bramley and Davies, 1975), but this is consistent with the results of Murillo and Cerdá-Olmedo (1976), since the strain employed in these experiments was a carS mutant.

Functions of Sterols and Carotenes

Sterols are present in the membranes of animal, plant and fungal cells, where sterol:phospholipid molar ratios of 0:10 to 0:35 are commonly found (Demel and Kruyff, 1976; Olsen, 1973). This suggests that sterols have an important function in biological membranes.

De Bernard (1958) first demonstrated an interaction between phospholipids and cholesterol by showing that the mean molecular area of a phosphatidylcholine monolayer was reduced in the presence of cholesterol. This condensation effect between sterols and phospholipids causes a decrease in the permeability of both artificial and natural membranes (Demel et al., 1968; Grunwald, 1974). In both of these experiments ergosterol was shown to be as effective as cholesterol.

Ladbrooke and coworkers (1968) showed, by differential scanning calorimetry, that the addition of cholesterol to dispersions of dipalmitoylphosphatidylcholine in water caused a reduction in the transition temperature between the crystalline and the liquid-crystalline phases, and decreased the heat absorbed during the transition. These alterations are because cholesterol controls the fluidity of the hydrocarbon chains of the phospholipid by disrupting the lattice structure in the crystalline phase, and also inhibits the flexing of these chains in the liquid-crystalline phase. A similar effect on transition temperatures has been observed in the intact membranes and the extracted lipids of Acholeplasma laidlawii and Mycoplasma mycoides cells, grown in the presence and absence of cholesterol (de Kruyff et al., 1972; Rottem et al., 1973). Arrhenius plots of the ATPase activities of Mycoplasma cells, grown in the absence of cholesterol, showed discontinuities at temperatures

corresponding to the phase transition of the membrane lipids. No discontinuities were observed for cells grown in the presence of cholesterol (Rottem et al., 1973). These data are in agreement with the suggestion of Overath et al. (1970) that the hydrocarbon chains of membrane lipids must be in a fluid state if transport and catalytic functions are to be performed normally, and suggest that a major function of sterols is to maintain this fluidity over a wide range of temperatures.

Not all the sterol present in fungi, however, can be located in membranes, since much of it is esterified to fatty acids. These steryl esters do not act as reserves of lipid for use under conditions of starvation (Bartlett and Mercer, 1974), but the sterol moiety may be used in the formation of new plasma membrane material during cell division, since large quantities of steryl esters have been observed in vesicles which resemble those visible around sites of membrane production in fungi (Rose, 1976).

In contrast to the information concerning the functions of sterols in cells, the functions of carotenoids in non-photosynthetic tissues are very poorly understood.

Carotenoids may be converted to sporopollenin and trisporic acids (p. 40), but since they are found in both mycelia and sporangiophores, and are produced by some fungi in large quantities even in the absence of hyphae of the opposite mating type, the production of these metabolites cannot be their sole function in fungi.

Phototropism and the photoinduction of carotenogenesis and sporangiophore initiation are examples of "blue-light effects" (Bergman et al., 1969). Curry and Gruen (1959) measured the action spectrum of these responses, and concluded that a carotenoid was involved. This was disputed by Delbrück and Shropshire (1960), who suggested that a flavin might be the photoreceptor. Flavoproteins with absorption spectra resembling the action spectrum of the photoresponse have since been isolated from Phycomyces (Bergman et al., 1969; Ootaki and Wolken, 1973). Recently the action spectra of these responses have been redetermined using more sophisticated techniques. The action spectrum for photoinduction of carotenogenesis in N. crassa shows a low response to u.v. light, correlating better with absorption by the cis peak of β-carotene than by flavins (De Fabo

et al., 1976). However, Delbrück and coworkers (1976) observed some phototropic response by sporangiophores of P. blakesleeanus to lasers of wavelength 575 to 630nm, and concluded that the magnitudesand positions of all the peaks in the action spectrum corresponded to observed transitions in riboflavin.

Since the balance of evidence is against β-carotene acting as a photoreceptor, and because even if it did only approximately $^1/_{3000}$th of the actual concentration in the sporangiophore would be required (Bergman et al., 1969), several other functions have been suggested.

Griffiths and coworkers (1955) suggested that carotenoids could protect cells from photodynamic destruction catalysed by chlorophyll. Evidence for this was obtained by Sistrom et al. (1956), who observed that carotenoid-less cells of Rhodopseudomonas spheroides were killed by light in the presence of oxygen only when chlorophyll was present to act as a photosensitizer; and by Kunisawa and Stanier (1958), who found that carotenoid-less mutants of Corynebacterium poinsettiae (a non-photosynthetic bacterium) were killed in the presence of toluidine blue, a photosensitizing dye. Cells containing carotenes, however, were not killed under these conditions.

Goldstrohm and Lilly (1965) found that cells of Dacryopinax spathularia were killed by a combination of light and O_2 unless they contained carotenes. Significantly, the insertion of solutions of β-carotene between the light source and the cells reduced the rate of cell death, suggesting that carotenes could filter out harmful radiation. However this cannot explain the photoprotective action of carotenoids in photosynthetic tissues, and even in non-photosynthetic organisms light-filtering would give no protection against photosensitizing compounds, e.g. porphyrins, which absorb light of longer wavelengths (Mathews , 1963; Burchard et al., 1966).

Fujimori and Livingston (1957) suggested that carotenes could quench the triplet energy state of chlorophyll, and thus prevent the generation of singlet oxygen ($^1O_2$), which could react with cell constituents (Schenck, 1954). Foote and Denny (1968) however, showed that the inhibitory effect of β-carotene on epoxide production was

due to quenching of $^1O_2$, rather than to any interaction with a triplet sensitizer. This quenching did not involve oxidation of the carotene, since each molecule must have quenched up to 250 molecules of $^1O_2$ in some experiments. Since $^1O_2$ causes the isomerisation of cis-β-carotene (Foote et al., 1970), and photochemical cis-trans isomerisation probably occurs via the triplet state (Fonken, 1967), the quenching reaction is probably:

$$^1O_2 + {}^1\text{Carotene} \longrightarrow {}^3\text{Carotene} + {}^3O_2$$

where $^{(1)}$, represents the singlet state and $^{(3)}$, the triplet state For such a reaction to occur the triplet energy level of the carotene must be below that of $^1O_2$. This is so for carotenes with nine or more conjugated double bonds, a number which corresponds to the minimum required for maximum quenching efficiencies (Land, 1975).

If this proposed function is importat in vivo then carotenes should be present in fungal membranes, especially those membranes which are particularly important, or which might be exposed to $^1O_2$. Neupert and Ludwig (1971) found that the outer membranes of mitochondria of N. crassa contained neurosporaxanthin, which they suggested could have a photoprotective function. However, the molar ratio of carotenoid: phospholipid is approximately 1:300 (Hallermayer and Neupert, 1974), a concentration which had very little protective effect on phospholipid liposomes (Anderson and Krinsky, 1973).

Several other functions for carotenoids in non-photosynthetic organisms have been proposed. Lucy and Dingle (1962) suggested that vitamin A can stabilise erythrocyte membranes by interacting with both lipid and protein, since the projecting methyl groups are suitably positioned for binding to cholesterol by van der Waal's forces. Carotenoids could act in an analogous manner, since the methyl groups are similarly positioned.

Smith (1963, 1969) suggested that carotenol-glucosides and esters act as carriers of glucose and acetate across the bacterial membrane, while Huang and Haug (1974) showed that carotenoids were able to substitute for sterols in the regulation of membrane fluidity in

A. laidlawii. However both these suggestions have been seriously criticized (Krinsky, 1971), and in addition they are unlikely to apply to fungal cells, which do possess sterols, but have not been reported as containing carotenol-glucosides.

B. FUNGAL GROWTH AND MORPHOLOGY

Taxonomy

Fungi are heterotrophic, eukaryotic organisms, living either parasitically or saprophytically. The yeasts are unicellular, but the majority of fungi possess tubular filaments, or hyphae, which develop to form a three-dimensional network throughout the substrate. The hyphae are usually microscopic, but the mycelium may develop thicker portions (rhizomorphs), resting bodies (sclerotia), and fruiting bodies, which may reach considerable dimensions. Growing hyphae may be in the form of single multinucleate cells (coenocytes), or divided into segments by transverse walls (septa), which are often perforated.

The features by which fungi are classified include mycelial development, the types of spores and sporangia, and the nature of the life cycle. Table 1.1 shows the major sub-divisions of fungi as defined by Burnett (1968) and Ainsworth (1971).

Phycomyces blakesleeanus

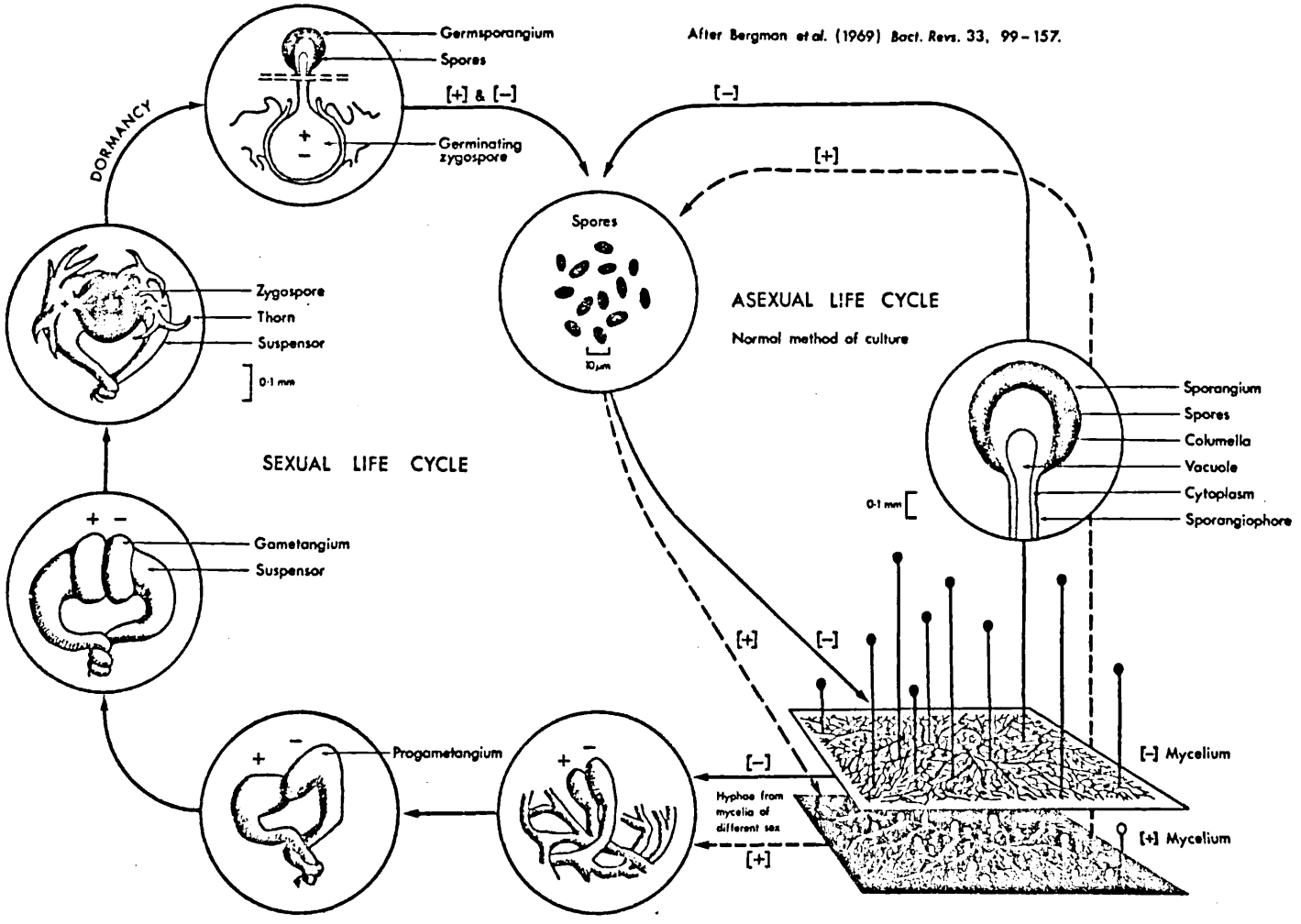
Most of the work described in this thesis has been carried out on a mutant strain of the fungus Phycomyces blakesleeanus(-). This is a member of the class Zygomycetes (sub-division Zygomycotina), and of the order Mucorales, members of which are characterized by the development of spores in a specialised sporangium. Phycomyces nitens was first described by Agardh (1817), and reclassified by Kunze (1823). Burgeff (1925) divided the species into P. nitens and P. blakesleeanus on the basis of a fertility barrier.

Phycomyces is a saprophyte which normally grows on dung or on dead wood (Bergman et al., 1969). The standard strain used in scientific research is of unknown origin (Cerdá-Olmedo, 1974). The life cycle shows asexual and sexual phases (Fig. 1.18). In the asexual phase the spores germinate to produce a branched coenocytic mycelium. This forms aerial sporangiophores which

TABLE 1.1 TAXONOMY OF FUNGI

Division	Sub Division	Characteristics and Representative Genera
Myxomycota		Slime fungi - life cycle involves free-living amoebae which later unite to form a plasmodium. Representative: Dictyostelium
Eumycota	Mastigomycotina	Lower fungi - No dikaryotic phase in the life cycle. Motile zoospores produced asexually. Representatives: Pythium, Phytophthora, Blastocladiella, Achyla
	Zygomycotina	Lower fungi - No dikaryotic phase in the life cycle. Only non-motile spores produced Representatives: Phycomyces, Mucor, Blakeslea, Pilobolus, Mortierella, Rhizopus
	Ascomycotina	Mycelium usually septate. Asexual reproduction often by conidia (in the Hemiascomycetes by budding). Sexual reproduction involves ascospores produced within asci. Representatives: Yeasts (Saccharomyces, Lipomyces, Rhodotorula, Candida*), Neurospora, Aspergillus*, Cephalasporium*, Verticillium*, Fusarium*, Cochliobolus, Ciliaria, Ophiobolus, Whetzelinia, Sclerotinia
	Basidiomycotina	Mycelium usually septate. Sexual reproduction by basidiospores borne externally in basidia, which may be grouped together as highly organised fruiting bodies. Representatives: Ustilago, Coprinus, Dacryopinax, Sclerotium*, Clathrus
	Deuteromycotina	Fungi Imperfecti: Sexual reproduction absent. Mostly Ascomycotina which have lost the sexual phase or where it has not been recognised. Representatives: Those Genera marked '*' are correctly classified as Fungi Imperfecti, but they have been placed elsewhere by analogy with similar fungi showing a sexual phase.

Fig. 1.18 Sexual and Asexual Life-Cycles in *Phycomyces blakesleeanus*



differentiate at the tip into spore-producing sporangia. The sexual cycle, involving the contact of mycelia of different mating types ((+) and (-)), results in the production of a zygospore. Unlike the higher fungi (Ascomycotina and Basidiomycotina) there is no dikaryotic phase in the sexual cycle, i.e. the nuclei contributed by each strain fuse before the zygospore develops. After a period of dormancy the zygospore produces a germsporangium, and the germspores liberated from this develop into new mycelia (Bergman et al., 1969).

Mutants of Phycomyces

A number of mutants have been prepared by treatment of heat-shocked spores with N-methyl-N'-nitro-N-nitrosoguanidine. A nomenclature for these mutants has been proposed whereby mutations are designated by three-letter symbols representing the morphological or biochemical alterations (Delbrück, 1973). Different loci governing the same phenotypic alteration are indicated by a single letter, which is followed by an isolation number. When the affected locus is not known a hyphen is used. These designations are preceded by a number which characterizes the strain and the laboratory where the mutant was produced. The genetic symbol 'mad' is used to describe a mutant showing normal morphology but altered phototropism, while the symbol 'car' represents mutants showing alterations in carotene production. Mutants of this latter kind have been used extensively in the study of carotenogenesis.

Genetic Control of Carotenogenesis

Some of the enzymology of carotenogenesis has been deduced from genetic studies on mutants of Phycomyces and some other fungi.

If two mutants are affected at the same gene (function) then a heterokaryon will show an intermediate phenotype, but if different genes are involved then a wild phenotype may be produced, provided that each mutation is recessive to its wild-type allele. Using these criteria Ootaki et al. (1973) were able to show the presence of three types of car mutant, designated A, B and R. The carA mutants are white, and produce less than 1% of the wild-type carotene concentrations, while carB mutants are also white, but accumulate phytoene. Mutants of the carR type produce large amounts of lycopene.

Evidence was obtained for the existence of only three genes, carA, carB and carR indicating that only one enzyme is involved in desaturation, and only one other in cyclisation. Recently Murillo and Cerdá-Olmedo (1976) have shown the existence of another gene, carS, which is probably involved in the production of a diffusible, cytoplasmic protein involved in the feedback inhibition of carotenogenesis by β-carotene. As expected carS mutants are characterized by large β-carotene concentrations.

De la Guardia et al. (1971) produced heterokaryons from carA and carR mutants, and found that the relative amounts of lycopene, γ-carotene and β-carotene in the progeny depended in a predictable way on the ratio between the number of nuclei containing the allele for normal cyclization and the number of nuclei containing the mutated cyclization gene. They suggested that a multienzyme complex was involved, and that this required two copies of the normal cyclase to form β-carotene. If the mutant gene produces a "blocking" enzyme, which can enter the enzyme complex in place of the active cyclase, then lycopene or γ-carotene would be released depending on the position of the defective enzyme (Fig. 1.19). However, this theory has been criticized by Hsu et al. (1974), since the proportions of lycopene, γ-carotene and β-carotene are not constant throughout growth; lycopene is produced at earlier stages of development than β-carotene.

Esleva and Cerdá-Olmedo (1974) analysed the carotenoid content of a "leaky" carB mutant, and found that although it accumulated phytoene it also produced decreasing amounts of phytofluene, ζ-carotene, neurosporene and lycopene. The relative proportions of these intermediates were consistent with a model in which the gene carB produces only one type of dehydrogenase, four identical copies of which then associate to perform the dehydrogenation of phytoene to lycopene (Fig. 1.19). Further evidence for this theory was obtained from quantitative complementation experiments on heterokaryons (Aragon et al., 1976).

Studies on other fungi have indicated different genetic controls. In N. crassa the product of the al-1 locus appears to be involved solely in the dehydrogenation of phytoene to phytofluene (Subden and

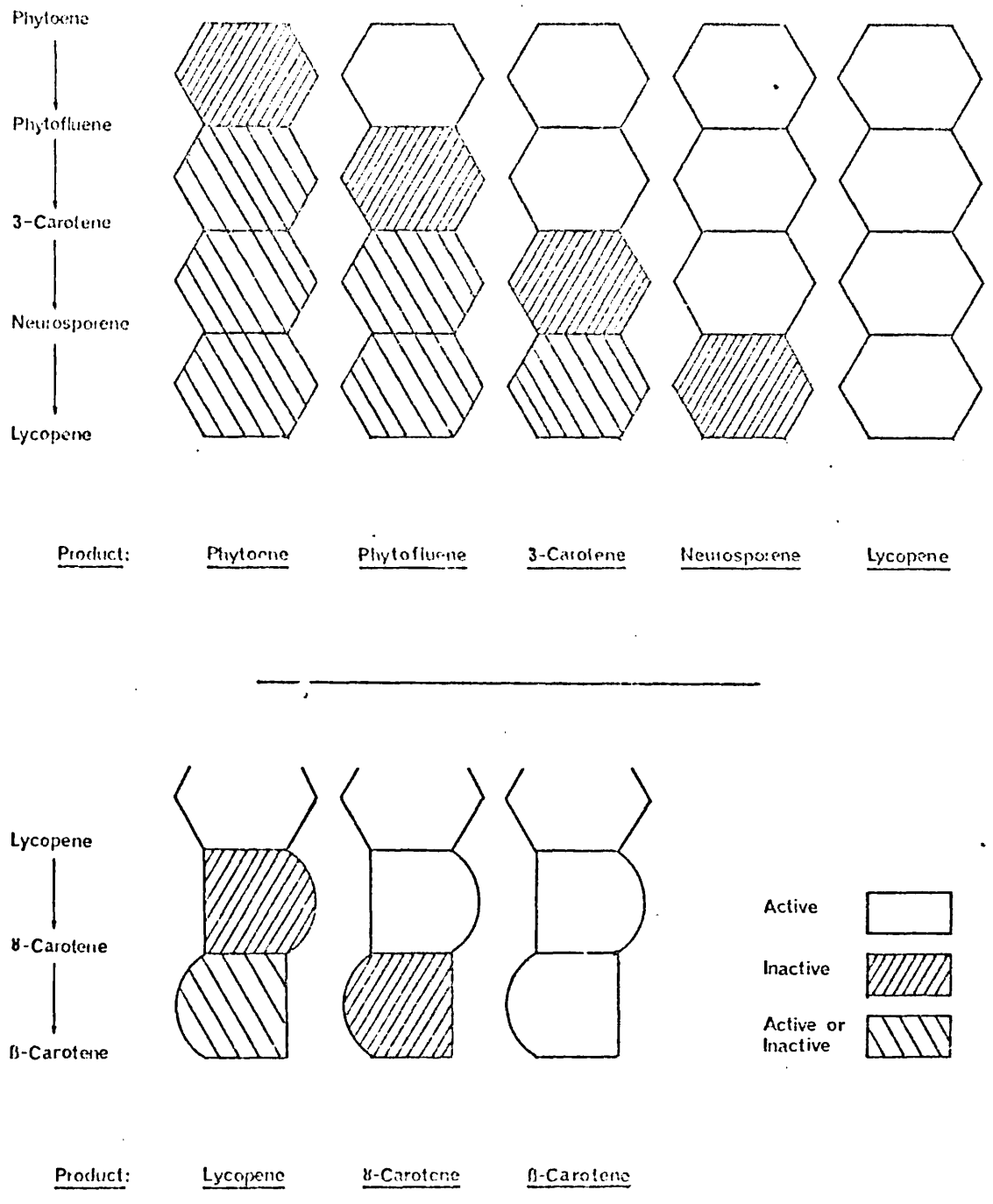


Fig.1.19 Possible Structure of a Carotenogenic Enzyme Complex

(de la Guardia et al., 1971; Aragon et al., 1976)

Goldie, 1973), while in Ustilago violacea complementation studies have indicated that at least two identical dehydrogenases and two identical cyclases are involved in carotenogenesis (Garber et al., 1975). However, since both organisms are members of the "higher fungi" (Table 1.1), it is possible that the enzymology of carotenogenesis in these cases could be different from that of Phycomyces.

Fungal Growth

Most fungi may be grown either statically on solid medium or on the surface of liquid medium, or in "shake culture", where they develop within an aerated liquid medium. When Phycomyces is grown on solid media sporangiophores develop after two to three days, but only mycelia are produced if the fungus is grown in liquid culture with agitation (Bergman et al., 1969).

Reinhardt (1892) was the first to claim that hyphal extension was confined to the apex, and this has since been confirmed for all classes of fungi (Henderson-Smith, 1923). The rate of growth may vary from 0.1 to 6mm/h (Burnett, 1968), and in one of the faster-growing species, N. crassa, Zalokar (1959a) calculated that if this wall growth was matched by growth of the protoplast then the whole of a growing zone approximately 100μm long would have to duplicate itself every minute. Since this would require protein synthesis at more than 100 times the observed rate, he suggested that the protoplast is pushed forward by vacuolation of the older areas of hyphae.

Because of this mode of growth fungal hyphae show a well-defined zonation. Cytochemical studies on hyphae of N. crassa have shown that the enzymes involved in glucose metabolism are most active in the younger, growing zone, while hydrolases are located in older areas. Both RNA and protein are also found predominantly in the first 100μm of the hypha, while glycogen is almost completely absent from this zone (Zalokar, 1959a). The distribution of organelles within hyphae of Pythium ultimum has been studied by Grove et al. (1970; Fig. 1.20). The growing zone (3-5μm) contains mainly small vesicles, presumably involved in wall synthesis. Immediately behind this area is a zone rich in mitochondria, while nuclei are first observed about 40μm behind the hyphal tip. Approximately 150μm

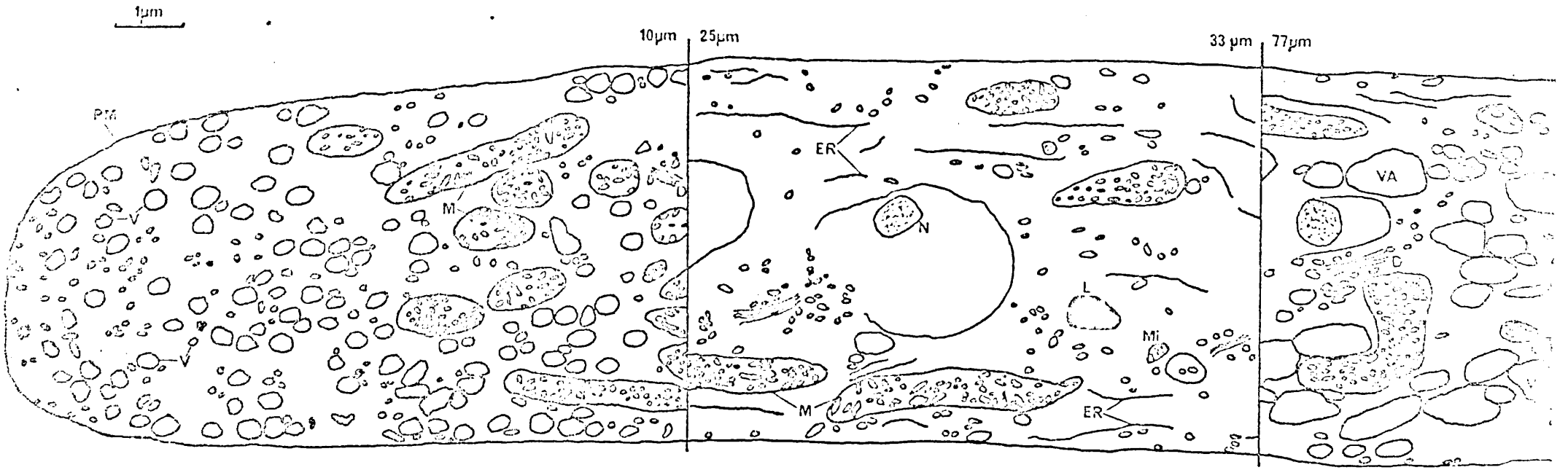


Fig. 1.20 Longitudinal Section Through Hypha of Pythium (after Grove et al., 1970)

(ER: Endoplasmic Reticulum M: Mitochondria Mi: Microbody N: Nucleus PM: Plasma Membrane V: Vesicle VA: Vacuole D: Dictyosome)

behind the apex the hyphae are almost completely filled with vacuoles.

A consequence of this mode of growth is that each area of the mycelium undergoes a series of well-defined changes. Park and Robinson (1967) described cell ageing as "the progressive maturation and senescence of an individual part of a hypha, from its formation at the apex to its death". As the cells of a static culture grow older they become more highly vacuolated, and lateral hyphae may be produced. Eventually hyphae of narrower diameter, lacking definite growth orientation, are produced; reproductive structures are formed; and the cells begin to autolyse.

When a fungal spore germinates in shake-culture it usually develops apically-extending hyphae, from which lateral branches grow in various planes. Eventually a spherical colony, the outermost region of which contains young, actively-growing hyphae, is developed (Burnett, 1968). The kinetics of such growth have been investigated by Koch (1975). He found that the state of "balanced growth", in which every extensive property of the culture increases exponentially with the same growth-rate constant as every other, only persists for a very short period following the initial lag phase. This exponential growth phase is eventually limited by depletion of nutrients or space, and is succeeded by the "idiophase", during which the growth rate declines, and secondary metabolites accumulate (Bu'Lock et al., 1965).

In statically-grown cultures hyphae are only able to develop into a new area while making contact with the nutritive surface. After this initial invasion the area can be occupied, and hence static cultures grow linearly in radius but exponentially in weight. In shake cultures, however, the new domain is simultaneously invaded from the entire surface of the culture, and full occupancy is almost immediate. Consequently, although these cultures also grow linearly with respect to radius, the increase in weight is proportional to the cube of the time of growth (Koch, 1975). Because the invasion of new areas occurs throughout the surface of mycelia growing in this way, the supply of nutrients to hyphae in the inner layers is quickly reduced, and consequently ageing proceeds faster in shaken than in statically-grown cultures. This difference means that the

comparison of results obtained with fungi grown in these two different ways is not always justifiable.

C. FUNGAL ULTRASTRUCTURE AND ORGANELLES

The Cell Wall

Fungal cell walls are often less than 0.2μm in thickness (Burnett, 1968), but under certain circumstances, e.g. in aerial sporangiophores, they have to withstand considerable stresses. This rigidity is supplied by a structure composed of a network of microfibrils embedded in an amorphous, polymer-containing matrix (Rosenberger, 1976).

Three classes of polysaccharides; cellulose, chitin and insoluble β(1→3)-glucan, may be found in these microfibrils. Cellulose is present in the walls of only a limited number of fungi, mainly species of Oomycetes (Mastigomycotina), but chitin and insoluble glucan are widely distributed (Bartnicki-Garcia, 1968). Kreger (1954) showed, by X-ray diffraction, that the hyphae and sporangiophores of P. blakesleeanus contain both chitin and chitosan (a deacylated analogue of chitin), while in the cell walls of Ascomycetes and Basidiomycetes insoluble glucans are probably the predominant structural material, although chitin is often present in appreciable amounts, except among the yeasts (Bartnicki-Garcia, 1968).

In addition to these complex, insoluble polymers, fungal cell walls contain other polysaccharides, together with 10-15% by weight of proteins and 5-10% of lipids (Rosenberger, 1976). These, together with some β(1→3)-glucan, compose the matrix phase.

Electron-microscopic examinations of ultra-thin sections of hyphae have suggested that the cell walls are composed of several layers (Hawker, 1965). On the basis of ultrastructural examination of cell walls which had been partially digested enzymically Hunsley and Burnett (1970) concluded that the walls of both Ascomycetes and Basidiomycetes consist of an outer layer of amorphous glucan, a layer of protein in which coarse strands of glycoprotein are embedded, and an inner layer of chitin microfibrils, possibly intermixed with protein. In addition Basidiomycete cell walls are covered by an amorphous layer of α(1→3)-glucan.

In older cultures of some fungi melanins formed by the polymerization of indoles or catechols, are produced in large amounts, and are deposited on the exterior surface of the hyphae, where they increase the resistance of the wall to attack by hydrolytic enzymes (Rowley and Pirt, 1972).

Nuclei

Fungal nuclei are usually smaller than their counterparts in higher plant and animal cells, being 2-3μm in diameter (Burnett, 1968). Like bacteria they do not contain histones (Leighton et al., 1971), but they do possess a central, basophilic body resembling the nucleolus of other eukaryotic nuclei. Since this is not dyed by Unna's RNA stain it may not be functionally equivalent to the nucleoli of higher organisms (Zalokar, 1959a).

The nuclei are enclosed by a porous envelope consisting of a double membrane which is frequently continuous with the endoplasmic reticulum (E.R.; Bracker, 1967). In Phycomyces the hyphae at all stages of the asexual cycle are multinucleate, and nuclei may occupy 20 to 25% of the total cell volume (Burnett, 1968).

Mitochondria

Fungal mitochondria are normally 1 to 2μm long and about 1μm wide, although they may elongate to 15μm or more in some fungi (Beckett et al., 1975). Their ultrastructure resembles that of mitochondria from higher plants and animals (Hawker, 1965). Extensive investigations of mitochondria isolated from Aspergillus spp., N. crassa and yeasts have shown that they are also bichemically similar to those of green plants and animals (Watson, 1976).

Studies on the separated membranes of mitochondria from N. crassa and Saccharomyces cerevisiae (Cassady and Wagner, 1971; Bandlow, 1972) have shown that the internal distribution of enzymes is the same as that in mammalian mitochondria (Ashwell and Work, 1970). The cristae of the inner membrane contain the enzymes of electron transport, together with the enzymes of the tricarboxylic acid cycle, although some of these (e.g. malate dehydrogenase, E.C.1.1.1.37) are in solution in the matrix. The outer membrane resembles the E.R., and possesses antimycin A-insensitive electron transport chain enzymes. Adenylate kinase (E.C.2.7.4.3) is located in the intermembrane space

of yeast mitochondria (Bandlow, 1972).

Luck (1963) showed that when mycelia of a choline-requiring auxotroph of N. crassa were labelled with [^{14}C]choline and then allowed to undergo several divisions in unlabelled media, the distribution of radioactivity among individual mitochondria followed a Poisson curve. This suggests that newly formed mitochondria arise by the growth and division of pre-existing ones.

Mitochondria isolated from N. crassa and yeast are able to synthesize proteins which are incorporated exclusively into the inner membrane (Neupert and Ludwig, 1971; Bandlow, 1972), and it is now well established that 10-15% of the proteins of the inner membrane are synthesized by the mitochondrion (Watson, 1976). The remainder are presumably synthesized on the E.R. and transported to the mitochondrion, a process which has been demonstrated in rat liver cells (Kadenbach, 1966). The proteins of the outer mitochondrial membrane are synthesized cytoplasmically (Neupert and Ludwig, 1971). Cobon et al. (1976) have shown that phosphatidylcholine is also synthesized extra-mitochondrially in yeast, and is transported to the mitochondrion by an exchange process involving supernatant proteins.

Endoplasmic Reticulum

The endoplasmic reticulum of fungi appears, under the electron microscope, to be looser and more irregular than that found in the cells of green plants or animals (Burnett, 1968). Although this could be an artefact of fixation, Klein et al. (1967) also concluded, from results obtained by cell fractionation, that the E.R. of yeast cells was less developed than that of higher plants.

Endoplasmic reticulum is generally most prevalent in young, actively growing cells (Bracker, 1967), and it is now established that ribosomes may be attached to it (Beckett et al., 1975), although the presence of smooth E.R. has yet to be proved conclusively. Specific associations of the E.R. with other cell components, especially the nuclear envelope, have also been observed by electron microscopy (Bracker, 1967).

In many fungi, especially in apical regions, the E.R. is highly vesicular. Three types of vesicle are associated with the E.R. of P. blakesleeanus sporangiophores (Peat and Banbury, 1967), and one of

these, a "multivesicular body", resembles a vesicle observed in the developing conidia of Fusarium spp., where it may develop into a lipid body (Schneider and Seaman, 1977; see p. 72).

Cytoplasmic Membranes

The cell contents are enclosed within a cytoplasmic membrane called the plasmalemma or plasma membrane. This is generally closely attached to the cell wall, and has a characteristic tripartite structure (Burnett, 1968).

A number of small vesicles, present near the plasma membrane at the hyphal apex, are probably derived from the Golgi apparatus (see p. 74), and may be involved in the secretion of enzymes and cell wall precursors across the plasmalemma (Heath and Greenwood, 1970). In so doing the vesicles probably fuse with the plasma membrane, thus increasing its area at the same time as the area of the cell wall is increased (Heath et al., 1971).

Vesicular invaginations of the plasma membrane, termed lomasomes, have been observed in a number of fungi (Moore and McAlear, 1961). These may be artefacts of fixation (Bracker, 1967), but Heath et al. (1971) have suggested that they could be produced when growth of the cell wall proceeds at a slower rate than the extension of the plasmalemma by the addition of secretory vesicle membranes. Under such conditions invaginations of the plasma membrane (plasmalemmasomes) could become sequestered within the cell wall to form lomasomes.

The preparation of purified plasma membrane fragments from a number of yeasts and fungi (reviewed by Rose, 1976), has shown that they possess both $(Na^+ + K^+)$- and Mg^{2+}- stimulated ATPases (E.C.3.6.1.4). These enzymes are probably involved in the generation of a membrane potential which drives the uptake of various compounds across the plasma membrane (Slayman and Gradman, 1975). A sugar carrier, with a high affinity for glucose and a broad specificity, is present in yeast cells (Kotyk, 1967). Kotyk and Matile (1969) showed that isolated yeast plasma membrane fragments bind both glucose and other sugars with the same affinity constants as intact cells, establishing that these transport systems are located on the plasmalemma.

Microbodies

Fungal microbodies are small vesicles, 0.25 to 2.5μm in diameter, bounded by a single membrane (Maxwell et al., 1975). Ultrastructurally they resemble the microbodies of higher plants and animals (Mollenhauer and Morré, 1966; De Duve, 1969). The matrix is of moderate electron density, and normally appears granular, except in the Ascomycotina, where inclusions resembling Woronin bodies (see p. 75) are often found (Wergin et al., 1973). A close association with the E.R. is often observed (Maxwell et al., 1975), and they may be derived from this membrane (Vigil, 1973; Bowden and Lord, 1976).

In higher plants two major types of microbodies are found (Huang and Beevers, 1971; Schopfer et al., 1976). Peroxisomes are involved in glycollate metabolism in green leaves, and also produce and degrade H_2O_2 (de Duve, 1969; Tolbert, 1971), while glyoxysomes contain the enzymes of the glyoxylate cycle, and are involved in the conversion of fats to carbohydrates (Briedenbach and Beevers, 1967; Tolbert, 1971).

In higher plants glyoxysomes are involved especially in the metabolism of the lipid reserves of fatty seeds during germination (Briedenbach and Beevers, 1967). An analogous process in fungi is the metabolism of lipids during spore germination (Casselton, 1976). Throughout this process a close association between microbodies and lipid globules has been observed (Mendgen, 1973; Wergin et al., 1973; Murray and Maxwell, 1974; Mills and Cantino, 1975a), suggesting that, in fungi, microbodies may act in the same way as plant glyoxysomes. Further evidence for this assumption has been provided by Maxwell and coworkers (1975) who observed that more microbodies are present in fungi growing on substrates which may be metabolised via the glyoxylate cycle than in fungi growing on glucose-containing media.

Hoffman et al. (1970) demonstrated by ultracytochemical staining that catalase (E.C.1.11.1.6) is present in the microbodies of yeast cells, and organelles containing both this enzyme and others involved in the glyoxylate cycle have been isolated from N. crassa (Theimer, 1973) and S. cerevisiae (Parish, 1975a). Similar organelles, possessing some of the enzymes of the glyoxylate cycle, have been isolated from

several other species (Casselton et al., 1969; Kobr et al., 1969; Kobr and Vanderhaeghe, 1973; O'Sullivan and Casselton, 1973; Mills and Cantino, 1975b). In none of these cases, however, have all the enzymes of the glyoxylate cycle been demonstrated, the presence of both citrate synthetase (E.C.4.1.3.7) and malate dehydrogenase being particularly dubious (Kobr and Vanderhaeghe, 1973). This may indicate that although fungal microbodies are functionally similar to the glyoxysomes of higher plants, the inter-relationships of the microbodies, the mitochondria and the cytosol are different (Fig. 1.21; Casselton, 1976). Such an interdependence might explain the frequently observed association of fungal microbodies with mitochondria (Maxwell et al., 1975).

Yeast microbodies contain the enzymes glycollate oxidase (E.C.1.1.3.1) and NADPH-glyoxylate reductase (E.C.1.1.1.79; Szabo and Avers, 1969), and it seems likely that these enzymes, together with catalase, are involved in the oxidation of NADPH by a glyoxylate reductase-glycollate oxidase cycle (Szabo and Avers, 1969; Fig. 1.21). This role is likely to be of minor importance under most conditions, however, since mitochondria are also able to carry out this cycle (Avers, 1971).

Vacuoles

In filamentous fungi vacuoles cannot be seen in the apices of the hyphae, but they are present further away from the growing tip (Burnett, 1968). They are surrounded by a single unit-membrane, the tonoplast (Hawker and Abbott, 1963).

Unlike the vacuoles of yeast cells, which follow a cyclic pattern of development through growth and division of the cell (Matile et al., 1969), the vacuoles of filamentous fungi and higher plants develop unidirectionally (Matile, 1975). The young, tip regions of fungal hyphae contain very small vacuoles, apparently interconnected by narrow canals (Park and Robinson, 1967). As the hypha grows the protoplast is pushed forward by the enlargement of these provacuoles, until the older regions are completely vacuolated (see p. 56). The enlargement of the vacuoles occurs as a result of the movement of water from the cytoplasm across the tonoplast, by a "vacuolation factor" secreted into the medium by ageing mycelia (Park and Robinson, 1967).

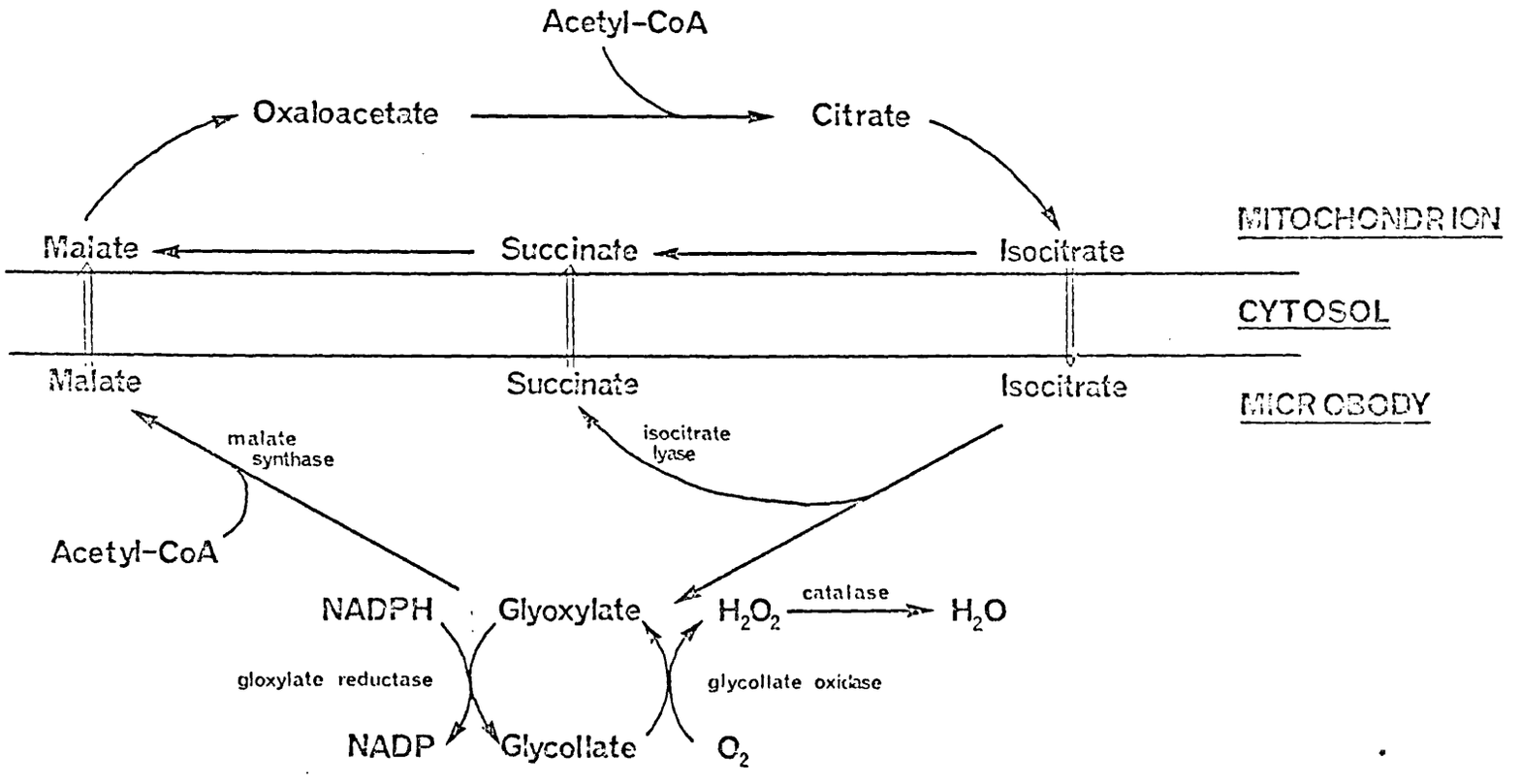


Fig.1.21 Possible Metabolic Pathways in Fungal Microbodies

Matile and Wiemken (1967) isolated vacuoles from protoplasts of S. cerevisiae, and showed that they contained several hydrolytic enzymes. The existence of proteinases (E.C.3.4), ribonuclease (E.C.2.7.7.16), phosphatases (E.C.3.1.3.1/2) and glycosidases (E.C.3.2.1) in these organelles has since been demonstrated (Wiemken, 1975). The presence of these hydrolases suggests that vacuoles act as the lysosomes of yeast cells (Matile and Wiemken, 1967).

Evidence that the vacuoles of filamentous fungi play a lysosomal role was obtained by Iten and Matile (1970) and Matile (1971), who isolated vacuoles from mycelia of Coprinus lagopus and from hyphae and conidia of N. crassa. In each case the isolated organelles contained proteinase, glycosidase, ribonuclease and phosphatase, and some of these enzymes were partly latent.

Gahan (1967) defined lysosomes as organelles which possessed a single limiting membrane, reacted cytologically with vital dyes, and contained one or more acid hydrolases possessing the property of latency.

Ultracytochemical studies on a number of fungi have indicated that they contain organelles which correspond to these criteria (Pitt and Walker, 1967; Pitt, 1968). The distribution of these particles within the hyphae has since confirmed that they are vacuoles (Hänssler et al., 1975; Armentrout et al., 1976).

Yeast vacuoles are able to accumulate large amounts of purines, S-adenosylmethionine and amino acids (Matile et al., 1969; Nakamura and Schlenk, 1974; Boller et al., 1975), and this has led Wiemken and Nurse (1973) to suggest that vacuoles of higher plant and fungal cells are able to act as a buffer against changes in the external environment of the cell, and thus maintain a homeostatic equilibrium within the cell.

Spherosomes and Lipid Droplets

Highly refractile bodies of "dense substance" in plant cells were first described by Hanstein (1880), and were later named spherosomes (Dangeard, 1922). They may be stained with lipid dyes such as Sudan black, Sudan III and Nile blue, and also fluorescesce with cationic dyes such as berberine sulphate (Frey-Wyssling et al., 1963). Although this staining by fluorochromes has been taken as

evidence for the presence of anionic proteins (Drawert, 1953), it may be only because these dyes are lipophilic in their unionised states (Frey-Wyssling et al., 1963).

Dangeard (1922) concluded that plant spherosomes accumulated lipids, and developed into "oleosomes"; but Guillermond (1921) suggested that spherosomes were merely a stage in the development of fat deposits (lipid granules) in the cytoplasm, and that they were not organelles but simply products of metabolism.

Sorokin and Sorokin (1966) and Sorokin (1967) examined a number of plant cells with varying lipid contents, and concluded that spherosomes were present in every case, and that they were fairly constant in size (normally 0.8 to 1.0μm diameter). In contrast, the reserve oils present in some seeds were contained in globules of widely varying sizes, which did not appear to be surrounded by a limiting membrane (Sorokin and Sorokin, 1966). Histochemical staining reactions showed that spherosomes contained predominantly polar lipids, while the reserve oil droplets contained neutral lipids (Sorokin and Sorokin, 1966). Because of these differences Sorokin (1967) concluded that spherosomes were not precursors of fat droplets.

Cytochemical staining has indicated that plant spherosomes contain a number of acid hydrolases, suggesting that they may be functionally equivalent to lysosomes (Matile, 1969). However some of these results may be artefactual, since some of these enzymes cannot be detected in cell homogenates (Matile, 1969), while in other cases there may have been confusion between spherosomes and small vacuoles (Matile, 1975).

Fungal hyphae also contain fat droplets, particularly in mature or aged cells and in spores (Bracker, 1967). Thus older hyphae of N. crassa contain osmiophilic globules approximately 0.3μm in diameter, together with much larger vacuoles (Zalokar, 1961). Submerged hyphae from shake cultures are almost completely filled with vacuoles and fat globules (Zalokar, 1959b). Two classes of lipid globules are present in yeast cells; one contains amorphous, unsaturated fats and the other myelin-like lamellae and polar lipids (Matile et al., 1969; Bauer et al., 1975).

There have been several reports of acid phosphatase (E.C.3.1.3.2) activity in small spherosomes of filamentous fungi (Armentrout et al.,

1968; Wilson et al., 1970; Palczewska and Jagodzka, 1972; Hislop et al., 1974), but recently Hänssler et al. (1975) and Armentrout et al. (1976) have concluded that this enzyme is not located in the lipid bodies of either Sclerotium rolfsii or Whetzelinia sclerotiorum, since the intrahyphal distributions of phosphatase activity and lipid droplets are considerably different. This interpretation is acceptable in most cases, but Palczewska and Jagodzka (1972) did observe activity of both alkaline and acid phosphatases (E.C.3.1.3.1/2) inside larger lipid bodies of Achyla flagellata mycelia; while Bauer and Sigarlakie (1975) found alkaline phosphatase activity on the membranes of vacuoles and spherosomes (here almost certainly small vacuoles), and also along the membranes of those lipid globules which were inside vacuoles.

Studies have also been carried out on the chemical composition of, and the presence of enzymic activities in suspected spherosomes, isolated by centrifugation procedures. Matile et al. (1965) separated two hydrolase-containing organelles from seedlings by density gradient centrifugation, and identified them by electron microscopy as spherosomes and "prospherosomes". They also obtained a fraction of lower density than the homogenisation medium which they assumed corresponded to lipid bodies and which possessed esterase activity. Prospherosomes and spherosomes differed mainly in their lipid contents, and this was reflected in the densities at which they equilibrated. Semadeni (1967) showed that prospherosomes, spherosomes and fat globules from maize seedlings were able to incorporate [^{3}H]acetate into lipids, and concluded that spherosomes were precursors of lipid droplets. Fatty acid and triglyceride biosynthesis has also been detected in fat droplets isolated from seeds of castor bean and sea kale (Harwood et al., 1971; Harwood and Stumpf, 1972; Gurr et al., 1974).

Although Matile's group have published several papers on the hydrolase content of isolated plant spherosomes (Matile et al., 1965; Balz, 1966; Semadeni, 1967; Matile and Spichiger, 1968), Matile (1975) has conceded that some of these results may have been due to contamination of the spherosomes with other organelles, while Jelsema et al. (1975) have shown that the acid phosphatase activity associated with spherosomes isolated from wheat seeds is located on contaminating membrane fragments. The only hydrolytic enzyme which has been

consistently localised in floating fat fractions from higher plants is lipase (E.C.3.1.1.3; Ory et al., 1968; Muto and Beevers, 1974). This enzyme is probably derived from the cytoplasm, and is involved in the mobilization of endogenous materials, rather than in any lysosomal function (Ching, 1968).

Thus the combined results of microscopic examinations and cell fractionation experiments indicate that lipid droplets are derived from smaller organelles, which probably do not have any lysosomal function in most organisms, being primarily involved in lipid biosynthesis, storage, and mobilization. However the smaller amounts of protein present in mature droplets as compared to spherosomes (Table 1.2) may reflect the loss of hydrolases from these organelles as they become specialized for lipid synthesis.

Most of the analyses of the lipid contents of isolated oil droplets (Table 1.2) have been performed on material which floated to the top of the homogenate during centrifugation, although spherosomes from maize seedlings were isolated by sedimentation (Semadeni, 1967). This difference is immediately obvious, since in these cases the protein:lipid ratios are higher than for those fractions isolated by flotation. With these exceptions the chemical compositions of fat fractions isolated from higher plants are very similar to those of fungal lipid droplets.

The presence of small amounts of protein and phospholipid in all these fractions suggests that the droplets are membrane-bound. Jacks et al. (1967) showed that lipid droplets from peanuts contained sufficient protein to form a bilayer membrane and also sufficient phospholipid for a monolayer. Yatsu and Jacks (1972) provided electron microscopic evidence that lipid droplets are surrounded by a "half-unit" membrane, and that this was similar biochemically to structural protein isolated from other organelle membranes. Conclusive evidence for the presence of a membrane around lipid globules has now been obtained by scanning electron microscopy of droplets isolated from Lipomyces starkeyi (Uzaka et al., 1975).

Frey-Wyssling et al. (1963) suggested that in some higher plant seeds spherosomes developed from terminal sacs on the E.R. Subsequent deposition of lipids within some of these organelles produced lipid globules (Fig. 1.22a). Electron microscopy of developing conidia of

TABLE 1.2. CHEMICAL COMPOSITION OF LIPID GLOBULES ISOLATED FROM HIGHER PLANTS AND FUNGI

Reference	Source	Diameter (μm)	Composition (%w/w)			
			Neutral Lipids	Sterols/ Steryl Esters	Phospho lipids	Protein
1	Maize seedlings a)*	-		62.3		37.7
	b)*	-		68.0		32.0
2	Douglas fir seeds	0.2 - 1.8	91	4	3	2.0
3, 4	Peanut	1.0 - 2.0	99.5		0.1	0.2
4	Cotton seed	4.0	98.4		0.4	0.6
	Cabbage leaves	0.5	80.8		0.2	4.0
5	Bush beans	0.5 - 3.0	92.4	-	2.3	5.3
6	Kale seeds	0.25- 1.7	65.6		9.6	19.1
	Sunflower seeds	-		89.3		4.3
	Safflower seeds	-		85.5		10.9
7	Saccharomyces cerevisiae	0.3 - 1.0	44	41	0.5	0.6
8	Mucor hiemalis	0.2 - 1.8	85	10	1.5	4
9	Lipomyces starkeyi	1 - 4	86+	1+	4+	-
10	Blastocladiella emersonii zoospores	0.4 - 0.5	84.5	7.4	2.5	4.4

*see text for explanation a = spherosomes b = prospherosomes

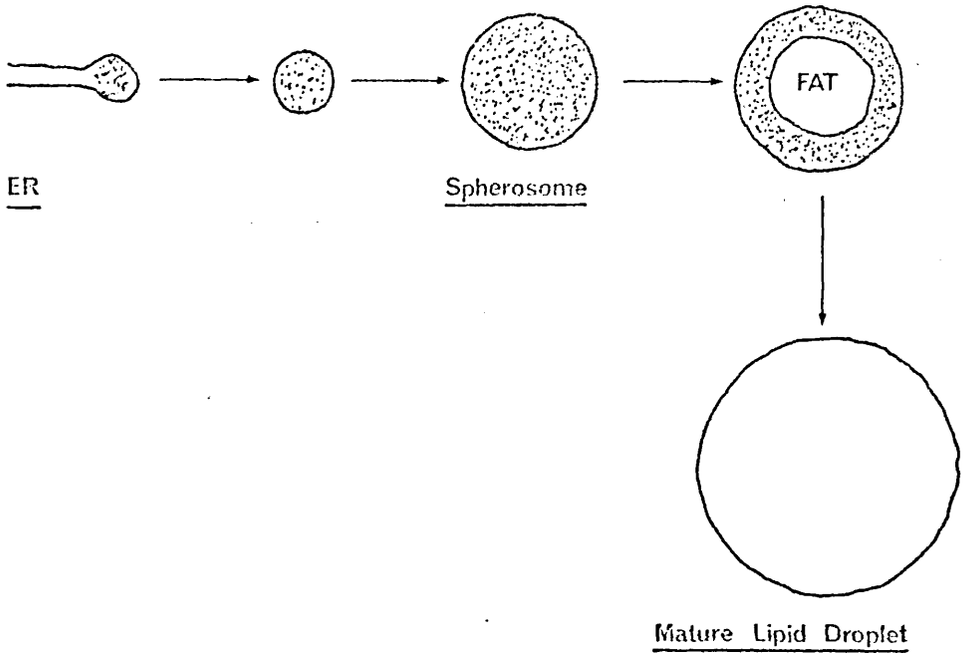
+based on distribution of radioactivity incorporated from [U-^{14}C]glucose.

References:

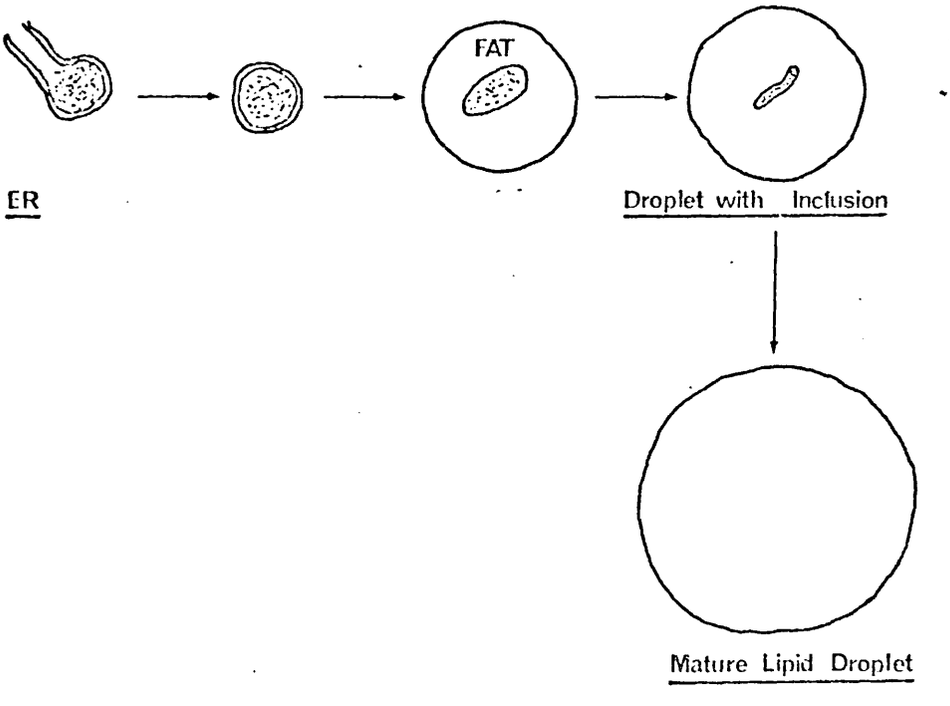
1. Semadeni (1967)
2. Ching (1968)
3. Jacks et al. (1967)
4. Yatsu et al. (1971)
5. Allen et al. (1971)
6. Gurr et al. (1974)
7. Clausen et al. (1974)
8. Herber (1974)
9. Uzaka et al. (1975)
10. Mills and Cantino (1977)

Fig.1.22 Development of Lipid Droplets

(a) Maize (Frey-Wyssling et al, 1963)



(b) Castor Bean (Schwarzenbach, 1971b)



Fusarium sulphureum has indicated that in this organism oil droplets are formed by a similar route (Schneider and Seaman, 1977). However since the oil droplets of F. sulphureum are surrounded by a distinct tripartite membrane their ontogeny is probably different from that of globules which do not appear to be surrounded by a unit membrane.

Schwarzenbach (1971a,b) has produced an alternative explanation for the development of oil droplets in castor bean seeds. This involves the deposition of lipids not in the cisternae of the E.R., but in the middle layer of the membrane (Fig. 1.22b). Lipid droplets with a central vacuolar inclusion which could have been produced in this way have also been observed by Harwood et al. (1971).

Other workers (Harwood et al., 1971; Rest and Vaughan, 1972; Smith, 1974) have suggested that oil bodies in castor beans, mustard and kale seeds originate around a cluster of enzymes in the cytoplasm, and hence are not surrounded by a genuine membrane. These conflicting results show that, at present, the origin and development of lipid globules is imperfectly understood, and also suggest that they may be produced in alternative ways by different plants.

Respiratory quotients for fungi deprived of exogenous substrates indicate that they are able to utilize stored lipids as food reserves (Throneberry, 1970). The major site of lipid deposition in most cells is the fat globules, and during starvation a reduction in both the lipid content and the number of lipid globules has been observed (Suberkropp and Cantino, 1973). This mobilisation of lipid reserves is particularly important during the germination of seeds and spores. Under these circumstances there is a close association of lipid droplets and microbodies (see p. 63) and the levels of some enzymes of the glyoxylate cycle may be increased (Mills and Cantino, 1975b).

If fat droplets are surrounded by a half-unit membrane, as suggested by Yatsu and Jacks (1972) then they cannot fuse with structures which possess normal membranes. Hence mobilization of the accumulated lipids either takes place within the globule itself, or possibly the oil bodies may enter vacuoles (Matile, 1975). The enzymes involved in lipid mobilization, i.e. lipase and fatty acyl-CoA synthetase (E.C.6.2.1.2/3), are not present in fat droplets isolated from peanut cotyledons (Jacks et al., 1967), suggesting that intra-vacuolar digestion of lipid globules probably occurs in some organisms.

When stationary phase cells of S.cerevisiae are transferred to a fresh growth medium there is a drastic decrease in the number of lipid droplets. These organelles attach themselves to the vacuolar membrane, are absorbed phagocytically, and are then quickly dissolved (Matile et al., 1969; Bauer and Sigarlakie, 1975). The presence of lipids within vacuoles has also been observed in a number of higher plants (Matile, 1975), and in the yeast Rhodotorula gracilis (Ruinen et al., 1968), suggesting that this may be a common site of lipid degradation. The transport of exogenous hydrocarbons into the vacuoles of yeast cells has also been demonstrated (Meissel et al., 1973).

Golgi Complex

The Golgi apparatus is present in both plant and animal cells, and is a complex, membranous system of stacks of flattened cisternae bounded by smooth unit membranes, together with associated vesicles and tubules, which are usually at the edges of the stacks. At one side (the proximal pole) it is usually closely appressed to the E.R. This basic form is present in both higher plant and animal cells (Mollenhauer and Morré, 1966; Cook, 1973), and similar structures have been observed in some Oomycetes (e.g. Pythium ultimum; Grove et al., 1968), but they have not been identified in the Zygomycotina, Ascomycotina or Basidiomycotina. In the latter cases ring-like, Golgi cisternae surrounded by vesicles are present, but the stacking characteristic of other organisms has not been observed (Beckett et al., 1975).

In mammalian cells the Golgi apparatus is involved in membrane assembly and transport, in oligosaccharide biosynthesis and protein glycosylation, and in the secretion of polysaccharides and lysosomal hydrolases (Dauwalder et al., 1972; Cook, 1973). Similar functions have been proposed for this organelle in higher plants and fungi.

In plant cells oligosaccharides are synthesized within the Golgi apparatus, and then transported across the cytoplasm to the cell wall (Northcote and Pickett-Heaps, 1965). These materials are transported in Golgi-derived vesicles, which fuse with the plasmalemma (Whaley et al., 1960; Mollenhauer et al., 1961). Similar vesicles, containing polysaccharides and enzymes, have been observed in fungal hyphae

(Grove and Bracker, 1970; Meyer et al., 1976), where they fuse with the plasmalemma, thus also providing a source of new plasma membrane material (Heath et al., 1971; p. 62). The modification of membranes from E.R.-like to plasmalemma-like has been observed in the Golgi apparatus of Pythium ultimum (Grove et al., 1968).

A current theory concerning the mammalian lysosomal system involves the production of hydrolase-containing vesicles (primary lysosomes) from the smooth E.R. and the Golgi apparatus. These vesicles may be incorporated into autophagosomes to produce secondary lysosomes, or they may fuse with the plasma membrane and secrete their enzyme contents extracellularly (Dean and Barrett, 1976). In this respect a major function of the Golgi apparatus is the provision of glycoproteins for the lysosomal system (Rambourg et al., 1969).

The secretion of extracellular hydrolytic enzymes has been observed in a number of fungi (Matile, 1969), and those which have been purified have been shown to be glycoproteins (Matile, 1975). Vesicles which could be involved in the transport of these enzymes to the plasmalemma have been implicated in the secretion of invertase (E.C.3.2.1.26) in S. cerevisiae (Holley and Kidby, 1973), and similar vesicles, containing proteinases, have also been observed in hyphae of N. crassa (Matile, 1965), where they are involved in intracellular secretion (Heiniger and Matile, 1974). By analogy with mammalian systems these vesicles correspond to primary lysosomes, and they are probably derived from the Golgi apparatus.

Cytological and biochemical evidence indicates that higher plant and fungal vacuoles, which correspond to the secondary lysosomes of mammalian cells (Matile and Moor, 1968), are produced by vesiculation of the E.R. (Berjak , 1972; Bracker, 1974; Parish, 1975b), but dictyosome vesicles may be incorporated into them during their development (Matile and Moor, 1968; Berjak, 1972; Matile, 1975). Although it has not yet been proved that these vesicles are a source of hydrolytic enzymes, this seems likely, and thus the role of the Golgi body in the lysosomal systems of higher plant and fungal cells is probably similar to its function in the mammalian lysosomal system.

Other Components of Fungal Cells

In addition to the major cell organelles discussed previously micrographs of fungal hyphae have revealed a number of minor components.

The hyphal tips of septate fungi (Ascomycotina, Basidiomycotina and Deuteromycotina) contain a small, densely staining or refractive body called the SpitzenkÜrper (Brunswik, 1924; McClure et al., 1968). However electron microscopy has now shown that this is a specialised region within the cluster of apical vesicles, which contains micro-vesicles, tubules, and a few ribosomes (Grove and Bracker, 1970).

Highly refractile, spherical bodies (Woronin bodies) are also found in the vegetative hyphae of Ascomycotina (Bracker, 1967). These are formed at the hyphal apex within single-membrane bound sacs (possibly microbodies; Wergin et al., 1973), and develop to a size of 0.6 to 0.8μm. They are usually associated with septa, and coalesce to plug the pores in the cross walls if a section of the hypha is injured (Brenner and Carroll, 1968).

The hyphae of N. crassa contain electron-opaque, hexagonal crystalloids about 1μm in length (Shatkin and Tatum, 1959). Similar crystals have since been observed in the hyphae of many other genera, and the existence of a membrane around them has been claimed, suggesting that they may develop within microbodies (Maxwell et al., 1970; Hoch and Maxwell, 1974). Tsuda and Tatum (1961) isolated crystals from hyphae of N. crassa and showed that they contained about 20% by weight of ergosterol, while Hoch and Maxwell (1974) showed that the crystalline structure was due to the presence of protein. The function, if any, of these crystals remains unknown, but similar crystals have been observed in the sporangiophores of P. blakesleeanus, where they are regularly aligned along the central vacuole (Wolken, 1972). These crystals contain proteins and lipids, and have an absorption spectrum which resembles that of the flavoprotein implicated in responses to blue light (p. 47; Ootaki and Wolken, 1973).

D. THE SUBCELLULAR DISTRIBUTION OF CAROTENOIDS AND STEROLS

Subcellular Locations of Terpenoid Biosynthesis

HMG-CoA is an intermediate in the formation of both terpenoids and acetoacetate, etc., and hence the first step which represents a commitment to terpenoid formation is the essentially irreversible reduction of HMG-CoA to MVA (Gaylor, 1974).

In mammalian liver cells the enzyme which catalyses this reaction, HMG-CoA reductase (E.C.1.1.1.34), is bound to the E.R. (Bucher et al., 1960), but differential centrifugation of homogenates of S. cerevisiae indicates that in this organism the conversion of acetyl-CoA to MVA occurs in the mitochondrion (Shimizu et al., 1971; Trocha and Sprinson, 1976). Density gradient centrifugation of mitochondria isolated from S. cerevisiae demonstrated a coincidence of HMG-CoA reductase activity with mitochondrial enzymes (Shimizu et al., 1973), but similar purification of mitochondria from N. crassa resulted in the removal of most of the HMG-CoA reductase activity, indicating that the enzyme may be bound to the E.R. in this organism (Imblum and Rodwell, 1974).

Shimizu et al. (1973) also showed that a 105,000g supernatant from yeast cells was able to convert [2-^{14}C]MVA into non-saponifiable lipids, while the incorporation of [1-^{14}C]IPP into FPP has been demonstrated in a 105,000g supernatant fraction isolated from Cochliobolus heterostrophus (Kawaguchi et al., 1973b). Mevalonate kinase (E.C.2.7.1.36) was present in the 70,000g supernatant of a homogenate of N. crassa (Imblum and Rodwell, 1974).

In mammalian cells the conversion of FPP into lanosterol is catalysed by microsomal enzymes, but the production of the intermediate, squalene 2,3-oxide, requires the additional presence of a supernatant factor (Tchen and Bloch, 1957; Anderson et al., 1960; Tai and Bloch, 1972). Similar results have been obtained with Cochliobolus heterostrophus, where the particulate fraction alone is able to catalyse the conversions of [^{14}C]FPP into squalene and [^{14}C]squalene 2,3-oxide into lanosterol, but the addition of the supernatant fraction is required for the epoxidation of squalene (Kawaguchi et al., 1973b). Schechter et al. (1970) found that squalene 2,3-oxide: lanosterol cyclase (E.C.1.99.1.13) was distributed between the supernatant and particulate fractions of yeast homogenates, but they concluded that it was probably a particulate enzyme which had been solubilized during extraction.

The conversion of lanosterol to cholesterol occurs on the E.R. of mammalian cells (Chesterton, 1968), but no comparable investigations of the subcellular location of fungal ergosterol biosynthesis have been reported, with the exception of one study on yeast S-adenosylmethionine:

Δ^{24}-sterol methyltransferase (E.C.2.1.1.41), which concluded that this enzyme is located on the E.R. (Moore and Gaylor, 1969). However, Thompson et al. (1974) have claimed that the presumed microsomal fraction contained promitochondria, and that this methyltransferase is actually located in the mitochondrial matrix or on the inner mitochondrial membrane. However, since a close connection exists between sterol biosynthesis and respiratory competence in yeast (Thompson et al., 1974), it is possible that the enzymes of ergosterol formation may not be mitochondrial in those fungi which are obligate aerobes.

The only reported investigation on carotene biosynthesis in subcellular fractions of fungi involved a particulate fraction from N. crassa, which was able to convert [1-^{14}C]GGPP into lycopersene (Grob et al., 1961). However, it now seems likely that this conversion was due to a lack of specificity of the squalene synthetase present in the extract (Qureshi et al., 1973b), and lycopersene is probably not involved in carotene biosynthesis (see pp. 30-31).

Subcellular Location of Carotenoids

Unlike sterols, which are present in most cell membranes (see p. 46), carotenoids have a very restricted distribution within cells. Zopf (1892) observed that the fat globules of Pilobolus kleinii contain carotenes in solution in lipids, and Schopfer (1928) used histochemical stains to demonstrate that the lipid droplets of M. hiemalis contain both carotenes and sterols. Guillermond et al. (1933) studied the distribution of carotenoids in a number of Pezizales (Ascomycotina) and carotenogenic yeasts, and concluded that the carotenes were present exclusively in the lipid globules of these fungi.

Heim (1946a), however, observed that in the Phallales (Basidiomycotina) most of the carotenoids were present in crystals, with only small amounts of pigment in the lipid globules. Similar crystals were observed in other Basidiomycetes (R. Heim, 1949), in the paraphyses of several Pezizales (Heim, 1946b), and in the sporangiophores of Pilobolus spp. (members of the Mucorales; P.Heim, 1949). These crystals were apparently found only in structures associated with the reproductive processes, but they were absent from basidia, asci or sporangia (P. Heim, 1949).

Heim (1946a, 1947) observed that in some Pezizales and Phallales carotenoids were synthesized in long filaments, which developed in a similar way to higher plant chromoplasts. Recently these observations have been repeated using the electron microscope. Schrantz (1965) concluded that, in Ciliaria hirta (a member of the Pezizales), carotenoid crystals developed in the E.R. and subsequently entered the lipid globules of older cells; while Eymé and Parriaud (1970) suggested that, in Clathrus cancellatus (a member of the Phallales), the crystals were deposited within membranes derived from the plasma membrane, although they were probably synthesized inside the lipid droplets.

In Dacryopinax spathularia the carotenoids may be located in the cell wall (Vail and Lilly, 1968).

There have been relatively few studies on the distribution of carotenoids in isolated cell fractions. Zalokar (1960; 1969) showed, by intracellular centrifugation, that most of the carotenoids present in hyphae of N. crassa and sporangiophores of P. blakesleeanus are confined to the fat globules, although several membranous fractions of Phycomyces sporangiophores were stained with carotenes. The presence of carotenes in lipid droplets isolated from mycelia of B. trispora and M. hiemalis, and from zoospores of Blastocladiella emersonii has also been demonstrated (Cederberg and Neujahr, 1970; Herber, 1974; Mills and Cantino, 1977).

Ninety percent of the β-carotene in mycelia of B. trispora is present in the lipid globule fraction, and the remainder is mainly in a fraction sedimented at 4,900g for 30 min (Cederberg and Neujahr, 1970). Since this fraction probably contains mitochondria, it is in agreement with the reports of Neupert and Ludwig (1971) and Keyhani et al. (1972), who detected carotenoids in the mitochondria of N. crassa and P. blakesleeanus, respectively. However, Herber (1974) concluded that the association of carotenoids with mitochondria isolated from M. hiemalis was due to contamination with lipid globules, and stated that the lipid globules contained 'the majority, if not the totality' of the carotenes present in the hyphae. Thus, although cell fractionation has confirmed that carotenoids are located in lipid globules, and has also provided some evidence for an additional particulate location, the identities of the latter, sedimentable fractions remain uncertain.

E. AIMS OF THE PRESENT INVESTIGATION

The pathways of carotenoid biosynthesis in fungi are now well understood, but the few reported investigations on the intracellular distribution of fungal carotenoids have yielded conflicting results (see pp.77-78). This controversy may be due in part to the analysis of fractions of uncertain purity, and so the present investigation was undertaken with the object of isolating and purifying organelles which contain or are able to synthesize β-carotene.

The fungus used in these studies, Phycomyces blakesleeanus, has been the subject of a number of previous investigations on carotene biosynthesis, and in most experiments a mutant (C115 carS42 mad-107(-)), which synthesizes only β-carotene (Meissner and Delbrück, 1968), was used.

The isolation of an organelle(s) able to synthesize carotenoids in vitro would be advantageous in subsequent investigations concerning the enzymology and regulation of this process; while knowledge gained from intracellular studies of carotenoid distribution may help to clarify the functions of fungal carotenoids.

MATERIALS AND METHODS

A. MATERIALS

Solvents: These were obtained from various suppliers. Analytical grade solvents were used for chromatographic and spectroscopic procedures. Light petroleums and diethyl ether were dried over sodium-lead alloy and then redistilled. Ether was distilled over reduced iron powder to remove peroxides.

Chemicals: General laboratory chemicals were obtained from B.D.H. Ltd., Poole, Dorset, and were of "AnalaR" grade whenever possible. Kieselgel G was supplied by Merck Laboratory Chemicals, Darmstadt, Germany; while Woelm neutral alumina, 4-methylumbelliferyl-N-acetyl glucosaminide, geraniol, Triton X-100, dodecenylsuccinic anhydride and N-benzyl dimethylamine were purchased from Koch-Light Ltd., Colnbrook, Bucks. Enzymes, coenzymes, bovine serum albumin (BSA), Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), and standards for chromatography were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey. The Boehringer Corporation (London) Ltd., Lewes, Sussex, provided some coenzymes. Glutaraldehyde, methyl nadic anhydride and Epikote 812 resin were purchased from George T. Gurr Ltd., London, N.W.9, and osmium tetroxide was supplied by Johnson Matthey Chemicals Ltd., London, E.C.1. Saboraud dextrose agar and yeast extract were provided by Oxoid Ltd., London, S.E.1; 2,5-diphenyloxazole (PPO) by International Enzymes Ltd., Windsor, Berks, and farnesol by Ralph N. Emmanuel Ltd., Wembley. A standard solution of amino acids was obtained from Calbiochem, Bishop's Stortford, Herts. Metrizamide was a sample from Nyegaard and Co. A/S, Oslo, Norway. DL-[2-^{14}C]Mevalonic acid lactone was supplied by the Radiochemical Centre, Amersham, Bucks. X-Ray film and developers were from Kodak Ltd., Lancs., and plates for electron microscopy were Type EM-5 from Ilford Ltd., Essex.

Microorganisms: *Phycomyces blakesleeanus* (-) Burgeff, strain CBS (284.35) was obtained from the Commonwealth Mycological Institute, Kew, Surrey. A superyellow mutant and an albino mutant, designated C115 *carS42 mad-107* (-) and C5 *carB10* (-) respectively (Delbrück, 1973; Murillo and Cerdá-Olmedo, 1976), were gifts from Prof. M. Delbrück, Division of Biology, California Institute of Technology, Pasadena, California, U.S.A.

B. GENERAL EXPERIMENTAL METHODS

Culture Conditions: The fungus was maintained on agar slopes stored at 4°C, which were subcultured every 6 to 8 weeks. Originally these slopes

were prepared from Saboraud dextrose agar, but it was observed that cultures maintained in this way eventually became sterile, and so an alternative growth medium was employed. This contained, per litre:

New Zealand agar	10g
D-Glucose	30g
L-Asparagine	2g
$MgSO_4.7H_2O$	0.5g
KH_2PO_4	1.5g
Yeast extract	1g
Thiamine hydrochloride	0.25mg

The agar, glucose and salts solutions were sterilised separately by autoclaving (120°C, 15 min), and then mixed. Aliquots (10ml) were dispensed into sterile boiling tubes, which were allowed to cool at an angle of approximately 20° to the horizontal. These slopes were inoculated with sporangia from a mature culture, and they were then grown under illumination for 6 to 7 days at 24°C.

Stock cultures were maintained as spore suspensions in deionised water stored at 4°C, or as small blocks of agar containing young hyphae, which were stored at room temperature in sterile, deionised water (Boesewinkel, 1976). The latter method was preferred since spore suspensions are not stable for longer than about six months (M. Delbrück, personal communication).

For experimental purposes mycelia were grown in liquid medium which contained, per litre:

D-Glucose	25g
L-Asparagine	1.25g
L-Leucine	1.25g
$MgSO_4.7H_2O$	0.5 g
KH_2PO_4	1.5 g
Yeast extract	0.5 g
Thiamine hydrochloride	0.25mg

In most cases aliquots (500ml) of medium were dispensed into 2 litre conical flasks and autoclaved, but occasionally 100ml portions in 250ml flasks were used. These flasks were inoculated with 0.005 volumes of a spore suspension prepared by irrigating a mature slope with sterile medium (7.5ml) and "heat shocking" this suspension at 45°C for 10 min. Liquid cultures were shaken at 150 rev/min at 24°C

in an orbital incubator. During growth they were illuminated by overhead fluorescent lighting which provided approximately 5 400 lx (Bramley, 1973).

Mycelia were harvested by filtering the medium through 2 layers of prewashed muslin. They were thoroughly washed with deionised water before use, then squeezed dry and weighed. Dry weights were determined by freeze-drying.

Preparation of Cell Extracts: Washed mycelia were cut into small pieces, suspended in ice-cold homogenisation buffer (8:1 v/w) and homogenised with a teflon-glass homogeniser (clearance 0.4mm, Pierce et al., 1953). The pestle was driven at approximately 1500 rev/min either by a Tri-R stirrer motor (Camlab, Cambridge) or by an electric drill. In some cases a more concentrated homogenate was prepared by finely chopping mycelia before homogenisation in 5 vol. of buffer.

After homogenisation the extracts were strained through two layers of prewashed cheesecloth, and then centrifuged at 800g (3 min) to remove cell debris and intact hyphae.

Centrifugation Procedures: Low speed centrifugations (< 2 000g) were performed in an MSE Minor centrifuge operated in a cold room (6-8°C). Higher speeds were obtained using angle rotors in MSE Highspeed and Superspeed refrigerated centrifuges, operating at 4°C. Density-gradient centrifugations were performed using 6 x 16.5ml, 3 x 40ml and 3 x 70ml swing-out rotors, operating in MSE Superspeed centrifuges.

Linear density gradients were established using a gradient former similar to that described by Britten and Roberts (1960), but consisting of two glass tubes joined by a teflon tap (Fig. 2.1). The denser solution was placed in the front compartment. After starting the mixer both taps were opened, and the gradient allowed to run down the side of the centrifuge tube in order to minimize mixing. Each gradient took 2 to 3 min to prepare, and could be stored at 4°C for up to 3h before use. Discontinuous gradients, prepared by carefully layering solutions in a centrifuge tube by means of a 10ml pipette, were used immediately.

After centrifugation the gradients were analysed by pumping the solution from the bottom of the tube via a hollow needle (16 gauge), using an LKB "varioperpex" peristaltic pump. The samples passed through the flow cell (path length 3mm) of an LKB Uvicord I operating at 258nm, and fractions were then collected manually or automatically. The

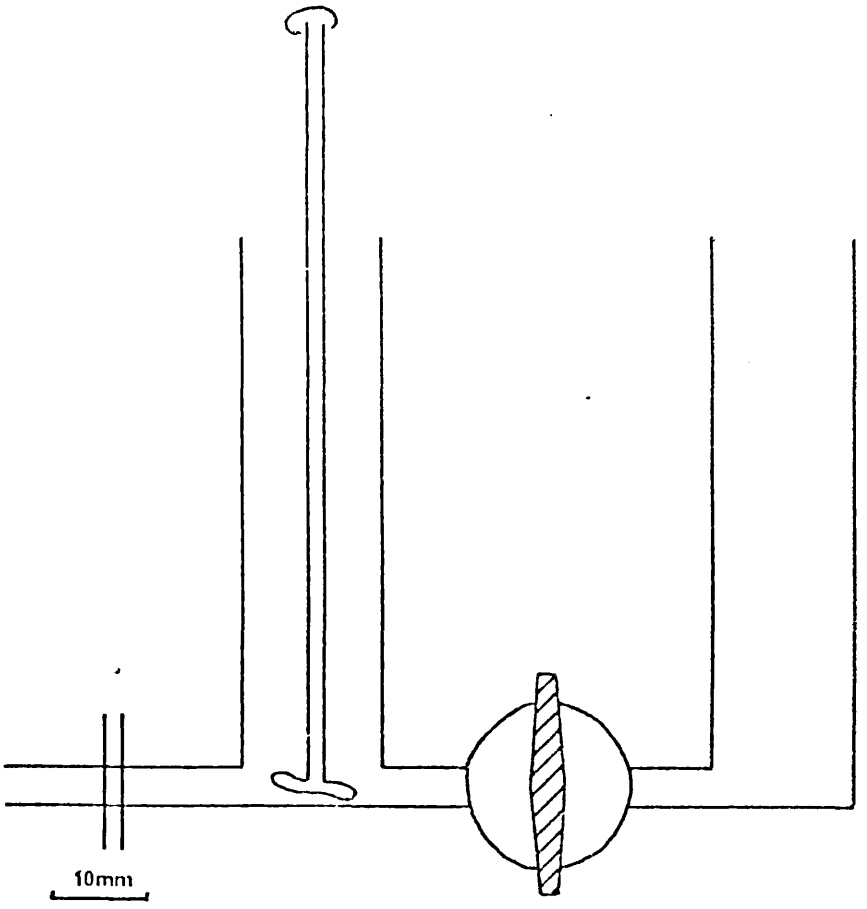


Fig.2.1 Density Gradient Apparatus

concentration of sucrose or sorbitol in each fraction was determined using a hand refractometer (Atago, Japan), which was calibrated directly as % (w/w) sucrose at 20°C. This value was converted to the density of sorbitol at 4°C using the data of Dobrota (1972) and Suerth (1974; Fig. 2.2).

Marker Enzyme Assays: Table 2.1 shows the enzymes assayed during these investigations, together with the assay methods employed. All enzymes were assayed at room temperature (19-22°C) except for proteinase, arylsulphatase, hexosaminidase and α-glycosidases, which were assayed at 37°C. The rate of a reaction was monitored continuously whenever possible, using a Pye-Unicam SP 1800 recording spectrophotometer. In the other cases the absorption was measured with an SP 500 spectrophotometer after standard intervals of time. The fluorescence due to the action of hexosaminidase on 4-methylumbelliferyl-N-acetylglucosaminide was determined using a Locarte fluorimeter fitted with a blue band pass filter (LF_2) as the primary filter and a yellow (LF_5) cut-off secondary filter.

Most of these assays were conducted using documented procedures, although the assays for α-mannosidase, α-galactosidase and proteinase were modified so that the activities of these enzymes were estimated at their pH optima.

Preliminary experiments were performed with crude homogenates to ensure that the rates of enzyme reactions were proportional to the amounts of protein in the assays. When assaying fractions, suitable volumes were used to ensure that the measured reaction rates were within the ranges determined for the crude homogenate.

NADPH: Cytochrome c reductase was employed as a marker for microsomal membranes in these studies because it has previously been located in the microsomes of higher plant and yeast cells (Donaldson et al., 1972; Cobon et al., 1974). Since interference in this assay from nitrate reductase (E.C.1.6.6.3), which is able to use mammalian cytochrome c as an electron acceptor (Garrett and Nason, 1969), is a possibility, the crude homogenate was assayed for NADPH: nitrate reductase by the method of Hageman and Flesher (1969). It was shown that the activity of this enzyme in a cell homogenate from the carS mutant was less than 0.1% of the activity of NADPH: cytochrome c reductase.

Other Determinations: Protein was measured by the method of Lowry et al. (1951), using BSA as a standard. Cell fractions were treated

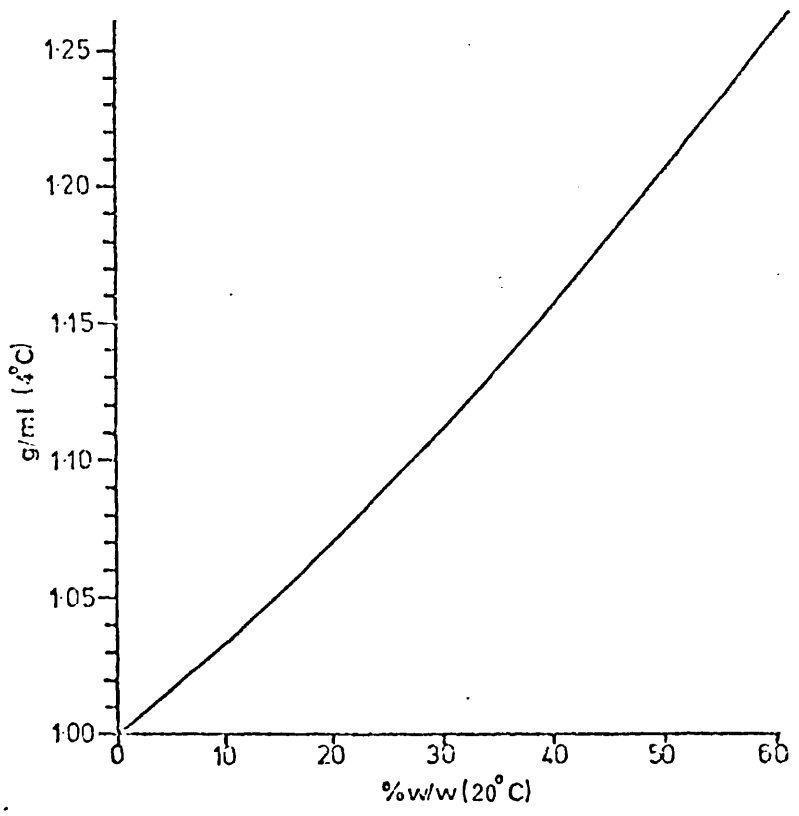


Fig. 2.2 Graph for Determination of the Density of Sorbitol Solutions

TABLE 2.1 ENZYME ASSAY METHODS

Samples were treated with 0.05% Triton X-100 prior to assays for catalase, protease, arylsulphatase, alkaline phosphatase and hexosaminidase

Enzyme	Subcellular location	Substrate(s) (concn. in reaction mixture)	Other additions (concn. in reaction mixture)	λ (nm)	ϵ ($mM^{-1}cm^{-1}$)	Reference
Succinate:DCPIP* reductase (E.C.1.3.99.1)	Mitochondrion (inner membrane)	0.02mM Succinate; 0.053mM DCPIP	0.1M Potassium phosphate buffer, pH 7.4; 0.3mM EDTA, pH 7.4; 1mM KCN	600	21	King (1967)
Succinate: Cyt c oxido-reductase (E.C.1.3.99.1)	Mitochondrion (inner membrane)	0.01mM Succinate; 0.05mM cyt c	5mM Potassium phosphate buffer, pH 7.2; 1mM KCN; 0.3M sorbitol	550	19.2	Douce et al., (1972)
NADH: Cyt c oxidoreductase (E.C.1.6.2.1)	Mitochondrion (inner membrane	0.3mM NADH; 0.05mM cyt c	5mM Potassium phosphate buffer, pH 7.2; 1mM KCN, 0.3M sorbitol	550	19.2	Douce et al., (1972)
Malate dehydrogenase (E.C.1.1.1.37)	Mitochondrion (matrix)	1mM Oxaloacetate; 0.15mM NADH	50mM Potassium phosphate buffer, pH 7.2	340	6.22	Watson et al., (1975)
Kynurenine 3-hydroxylase (E.C.1.14.1.2)	Mitochondrion (outer membrane)	0.05mM L-Kynurenine sulphate; 0.1mM NADPH	0.3M Tris-acetate buffer, pH 7.4; 0.45mM KCN; 20mM KCl	340	7.3	Bandlow (1972)
Antimycin A-insensitive NADH: Cyt c reductase (E.C.1.6.2.1)	Mitochondrion (outer membrane)	0.15mM NADH	25mM Tris-maleate buffer, pH 6.5; 5mM KH_2PO; 10mM KCl; 1mM EDTA; 0.3M sorbitol; 1µg/ml antimycin A	340	6.22	Bandlow (1972)
Catalase (E.C.1.11.1.6)	Microbodies	10mM H_2O_2	0.2M Potassium phosphate buffer, pH 7.0	240	40	Aebi (1974)
Glucose 6-phosphate dehydrogenase (E.C.1.1.1.49)	Cytosol	5mM Glucose 6-phosphate; 40µM NADP	0.2M Tris-HCl buffer, pH 7.5; 0.05M $MgCl_2$	340	6.22	Langdon (1966)

* DCPIP 2,6-dichlorophenol indophenol

TABLE 2.1 (continued) ENZYME ASSAY METHODS

Enzyme	Subcellular location	Substrate(s) (concn. in reaction mixture)	Other additions (concn. in reaction mixture)	λ (nm)	ϵ ($mM^{-1}cm^{-1}$)	Reference
NADPH: Cyt c reductase (E.C.1.6.99.1)	Microsomes	0.1mM NADPH; 0.11mM DCPIP	40mM Potassium phosphate buffer, pH 7.4	600	21	Williams & Kamin (1962)
Alkaline phosphatase (E.C.3.1.3.1)		0.67mM Ditris p-nitrophenyl phosphate	0.67M Tris-HCl buffer, pH 8.5	405	18.5	Torriani (1960)
Neutral proteinases (E.C.3.4)		0.1% (w/v) Azocasein	66mM Potassium phosphate buffer, pH 6.5. Reaction stopped with an equal vol. 10% trichloroacetic acid. Centrifuged (2000g; 10 min). 0.5 vol. 4M NaOH added to supernatant	440	$A^{1\%}_{1cm}$=35	Hazen (1974)
Arylsulphatase (E.C.3.1.6.1)	Lysosomal compartments	8mM 4-Nitrocatechol	200mM Sodium acetate buffer, pH 6.0. Reaction stopped with an equal vol. of 0.2M NaOH	515	18.9	Agogbua & Wynn (1976)
N-Acetyl-β-D-hexosaminidase (E.C.3.2.1.29)	Lysosomal compartments	0.39mM 4-Methylumbelliferyl-N-acetylglucosaminide	54mM Citrate in 92mM phosphate buffer, pH 4.5; 0.1 mg/ml bovine serum albumin. Reaction stopped with 16 vol. 0.2M glycine-NaOH buffer, pH 10.3	365 (excitation) 460 (emission)	*	Price & Dance (1972)
α-Mannosidase (E.C.3.2.1.24)	Tonoplast	0.66mM p-Nitrophenyl-α-D-mannoside	13mM Citrate in 17mM phosphate buffer, pH 5.5. Reaction stopped with equal vol. 1M Na_2CO_3	405	18.5	van der Wilden et al.,(1973)
α-Galactosidase (E.C.3.2.1.22)		0.66mM p-Nitrophenyl-α-D-galactoside	As above	405	18.5	

* Concn. of 4-methylumbelliferone liberated was determined using a standard curve

with an equal volume of cold 10% (w/v) trichloroacetic acid, and the precipitated proteins were collected by centrifugation (2 000g; 10 min) and resuspended in deionised water before addition of the appropriate reagents. Since this precipitation procedure was not possible for the determination of protein concentrations in isolated lipid globules, these were removed by filtration through a Millipore membrane after colour development was complete.

In preliminary experiments nucleic acids were extracted by the method of Burton (1968). Isolated fractions were extracted with 0.1M tris-HCl, pH 9.0, containing SDS (sodium dodecyl sulphate; 1% w/v; Miura, 1967), and nucleic acids were then precipitated with 10% (w/v) trichloroacetic acid (2.5 vol). The precipitates were collected by centrifugation (2 000g; 10 min), washed with 10% trichloroacetic acid, and digested with 0.5M $HClO_4$ for 20 min at 70°C. DNA and RNA were estimated by the diphenylamine and orcinol methods respectively (Schneider, 1957).

Unfortunately the orcinol test in particular is sensitive to interference from sucrose, sorbitol and mannitol, so that extensive washing of the pellets is necessary before this test can be applied. Since this washing procedure results in a considerable loss of material from small fractions, an alternative method was sought. Blobel and Potter (1968) selectively extracted RNA by alkaline digestion with 0.3M KOH for 1 h at 37°C. RNA can then be estimated spectrophotometrically at 260nm, and DNA by dissolving the unhydrolysed precipitate in 0.6M KOH, and adding $HClO_4$ and diphenylamine reagent. However, when a sample of BSA was subjected to this procedure for RNA extraction, a measurable absorbance at 260nm was obtained, indicating that some of the protein had been dissolved. Furthermore, since DNA can no longer be quantitatively extracted from the pellet remaining after alkaline digestion (Burton, 1968) it can only be estimated by a colour reaction, although direct spectrophotometric estimation is more sensitive.

Consequently, the method of Ogur and Rosen (1950) was used in later experiments. Ice-cold 0.2M $HClO_4$ (2 vol) was added to fractions suspended in deionised water or buffer. After 10 to 15 min in an ice bath the precipitates were collected by centrifugation (2 000g; 10 min) and resuspended in a measured volume of ice-cold 1M $HClO_4$. The mixtures were then left at 6-8°C for approximately 18h, during

which the RNA was extracted. The tissue was then removed by centrifugation, and the absorbance of the supernatant measured at 260nm. The concentration of RNA was determined using an $A^{1\%}_{1cm}$ of 312.5 (Munro and Fleck, 1966). After the removal of RNA, the DNA was extracted by digestion with 1 M $HC1O_4$ for 30 min at 80°C.

Although this method is unable to completely separate RNA from DNA (Munro and Fleck, 1966), it was shown that no loss of protein occurred when a 10 000g pellet, prepared from a homogenate of Phycomyces mycelia, was extracted by this procedure. It was concluded, therefore, that this method was suitable for the spectrophotometric determination of both RNA and DNA in cell fractions. The $A^{1\%}_{1cm}$ of a solution of commercial DNA in 1M $HC1O_4$ was found to be about 315, so the same value was used for the calculation of DNA and RNA concentrations.

Biosynthesis of Terpenoids from [2-^{14}C]MVA: Mevalonic acid lactone was hydrolysed by the addition of the requisite amount of 0.2M NaOH solution to the dry substrate. For in vivo incorporation experiments it was added to cultures, either at inoculation or during growth, by filtration through a Millipore membrane.

In vitro incorporation experiments were carried out essentially as described by Bramley and Davies (1975). The incubation mixtures (1ml) contained:

[2-^{14}C]DL-Na mevalonate	0.1 or 0.5µCi(7.7 or 38.5 nmoles)
ATP	10µmoles
Glutathione (reduced)	20µmoles
$MnC1_2$	6µmoles
$MgC1_2$	4µmoles
NAD	1µmole
NADH	1µmole
NADP	1µmole
NADPH	1µmole
FAD	1µmole
Buffer	
Enzyme	

Anaerobic incubations were carried out in 15ml Thunberg tubes which were evacuated with an oil vacuum pump and flushed with oxygen-free nitrogen several times.

Incubations were carried out at 24°C in a shaking water bath (100 rev./min) in the dark. Reactions were terminated by the addition of 3 vol of ethanol:diethyl ether (3:1 v/v). Carrier terpenoids (25µg β-carotene; 50µg phytoene; 25µg lanosterol; 75µg ergosterol; 100µg squalene) were added, and the lipids were extracted as described below.

Preparation of Carrier Carotenes: β-Carotene and phytoene were extracted from mycelia of P.blakesleeanus carS42 and carB10, respectively. After saponification the lipids were separated by chromatography on columns of neutral alumina (Brockmann activity grade III). β-Carotene was eluted with 0.25% (v/v) diethyl ether in light petroleum (b.p. 40-60°C), and was not purified further. Phytoene, eluted with light petroleum (b.p. 40-60°C), was rechromatographed on a column containing 10g neutral alumina (activity grade II). Most of the squalene was eluted from this column by 20ml of light petroleum, while phytoene was eluted by a further 40ml of this solvent. The spectra of the carotenes were the same as those reported by Davies (1963), while the low persistence of the peaks at 275 and 296nm indicated that the phytoene was predominantly the 15-cis isomer.

Lipid Extraction Procedures: Throughout these extractions the exposure of materials to heat and light was minimised. Lipids were extracted from whole mycelia by homogenisation in acetone, using an Ultra-Turrax homogeniser (Chemlab, London). The homogenate was filtered by suction through a sintered glass filter, rehomogenised in acetone, and then homogenised in diethyl ether until no more pigment was extracted. This method provides almost quantitative extraction of both carotenes and sterols (K.E. Davies et al., 1964). The lipids were partitioned into diethyl ether by addition of water, and then dried over anhydrous Na_2SO_4 in the dark for 1 to 2h.

Total lipids were extracted from cell fractions by the method of Bligh and Dyer (1959). Methanol/$CHCl_3$ (2:1 v/v; 3.75 vol.) was added to aqueous suspensions of cell fractions in 50ml polypropylene centrifuge tubes. These mixtures were left for 2h at room temperature and shaken occasionally. The samples were then centrifuged (2 000g; 10 min), and the pellets were re-extracted with MeOH/$CHCl_3$/H_2O (2:1:0.8 by vol.). After centrifugation the supernatants were combined,

and $CHCl_3/H_2O$ (1:1 v/v; 0.45 vol.) was added to separate the phases. The extracts were centrifuged to complete the partitioning, and the upper phase was removed. The lower, chloroform phase was washed with an equal volume of aq. NaCl (7.5% w/v), and then evaporated under a constant stream of N_2 at 70-80°C.

The extraction of carotenes and sterols from cell fractions and incubation mixtures was accomplished by sonication (MSE 150W sonicator; max. amplitude; 1 min) with ethanol/diethyl ether (3:1 v/v; 3 vol.). The extract was then partitioned with light petroleum (b.p. 40-60°C) until no more pigment was extracted.

Estimation of Lipids: Dry weights were determined after desiccation overnight in vacuo. β-Carotene and ergosterol were estimated spectrophotometrically in light petroleum (b.p. 40-60°C). An $A^{1\%}_{1cm}$ of 2500 at 451nm was used for assessing the concentration of β-carotene (Davies, 1965), while ergosterol was estimated at 281.5nm using an $A^{1\%}_{1cm}$ of 310. Correction was made for irrelevant u.v.-absorption using the formula $A_{281.5} = 0.654\ (5.A_{281.5} - 3.A_{276.5} - 2.A_{289.0})$ (Glover, 1964).

Phospholipids were determined by two methods. "Method 1" (Bartlett, 1959) involved digestion of the lipids for 3h at 150°C in 5M H_2SO_4 (0.5ml). Two drops of H_2O_2 were then added and the digestion continued for a further 30 min. If the digest was not clear a few more drops of H_2O_2 were added. The digestion was continued for a further 1½h after the addition of H_2O_2. The tubes were then cooled, water (4.4ml) was added, followed by 0.2ml aq. ammonium molybdate solution (5% w/v), and 0.2ml freshly-prepared 1-amino-2-naphthol-4-sulphonic acid reagent (Fiske and Subbarow, 1925). The tubes were thoroughly mixed between each addition, and they were then heated in a boiling water bath for 7 min. The absorbance at 830nm (730nm for more concentrated samples) was recorded and the weight of phospholipid determined from standard curves prepared using dipalmitoylphosphatidylcholine.

This method of phosphate determination, however, has the disadvantage of employing an unstable reagent, which must be prepared fresh each week. Consequently, variations from the standard curve occur, and these can only be corrected for by the inclusion of a number of standards with each sample.

The second method for phospholipid determination had the advantage of using a "universal, stable reagent" for colour development (Vaskovsky et al., 1975). However the published procedure had to be modified to accommodate samples which contained small amounts of phosphate in a large weight of lipid. Consequently a digestion procedure similar to that employed in Method 1 was employed, except that 1ml of 5M H_2SO_4 was used. After cooling, stock reagent (0.6ml), prepared as described by Vashovsky et al. (1975), and water (3.4ml) were added, and the colour was developed at 100°C for 15 min. The absorbance was read at 730 or 830nm as before. A linear relationship (correlation coefficient = 0.998) between absorbance and phospholipid concentration was obtained for lecithin using this procedure (Fig. 2.3).

Separation of Terpenoids: After removal of an aliquot for phospholipid determination lipids were saponified with methanolic KOH (6% w/v), either for 10 min in a boiling water bath, or overnight, under N_2, at room temperature. Unsaponifiable lipids were extracted by partition with diethyl ether, and the etheral extracts were washed with water to remove alkali and finally dried over anhydrous Na_2SO_4 for approximately 1h. This procedure resulted in the recovery of 90 to 95% of both β-carotene and terpenoids (as assessed by recovery of radioactivity when lipids, biosynthesized from [2-^{14}C] MVA, were resaponified).

The unsaponifiable lipids were dissolved in a minimum volume of light petroleum (b.p. 40-60°C) and chromatographed on columns of aluminium oxide (Woelm neutral alumina) deactivated by the addition of deionised water (6% w/v; Brockmann activity grade III). Unsaponifiable lipids obtained from up to 50mg of total lipids could be chromatographed on columns prepared from 10g of alumina, while larger amounts of material were chromatographed on 20g of alumina. Normally three fractions were collected: 30ml of light petroleum (b.p. 40-60°C) eluted squalene and phytoene; 30ml of diethyl ether in light petroleum (0.25% v/v) eluted β-carotene; and sterols were eluted with 30ml of diethyl ether. Using this procedure recoveries of 90 to 97% of all terpenoids were obtained (as assessed by recovery of radioactivity upon rechromatography of lipids biosynthesized from [2-^{14}C]MVA)

After column chromatography, radioactive lipids were further

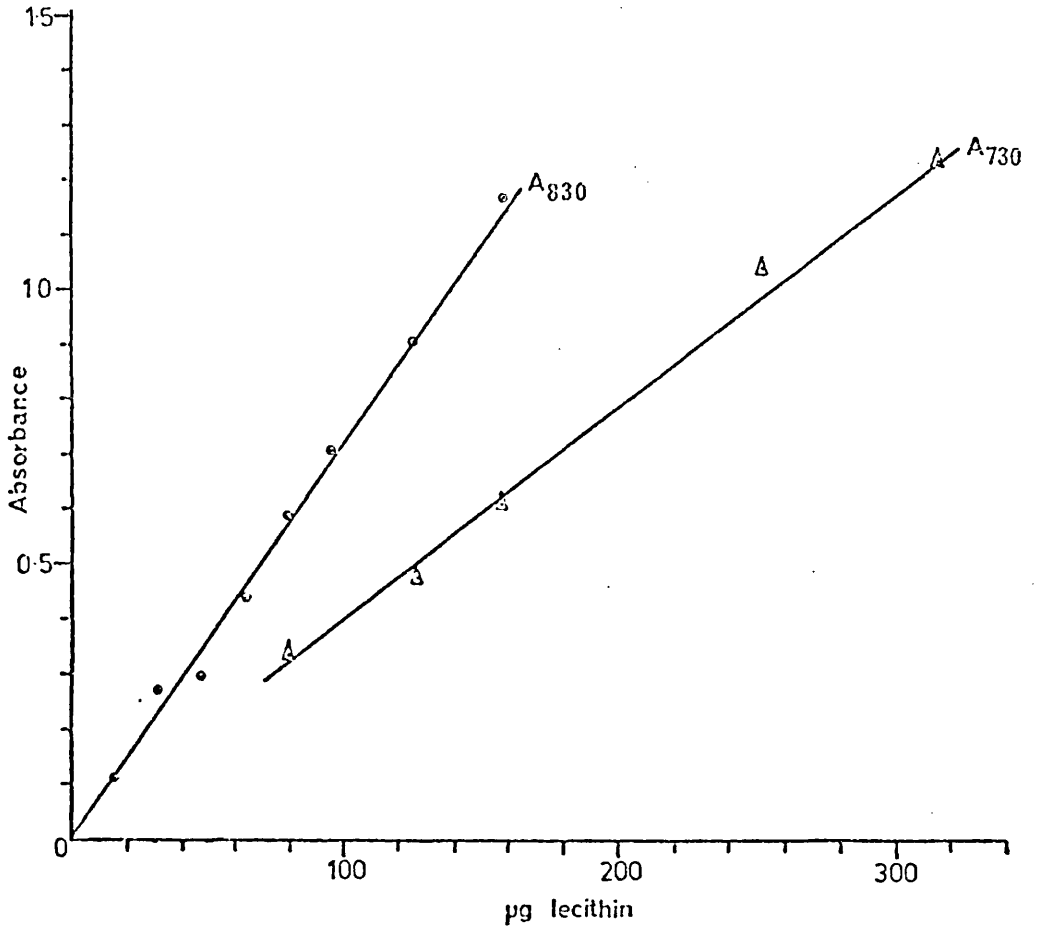


Fig. 2.3 Standard Curve for Determination of Phospholipids by a Revised Procedure

purified by t.l.c. (see below). Non-radioactive lipids were estimated as described previously, except that the spectrophotometric determination of ergosterol was no longer possible because of u.v.-absorbing compounds eluted from the alumina. Hence sterols were estimated by the method of Moore and Baumann (1952).

The sterols were dissolved in glacial acetic acid (1ml) and colour reagent (acetic anhydride; conc. H_2SO_4; 19:1 v/v; 2ml) were added. The colour was developed for 90 seconds, and the concentration of Δ^7 sterols determined from the absorbance at 620nm, using ergosterol as a standard (Glover, 1964).

Thin-layer Chromatography: All thin-layers were prepared with a gel thickness of 0.25mm. They were activated at 110°C for 2h, and stored in a desiccator. For argentation t.l.c. the gel contained the requisite percentage of $AgNO_3$, and the activated plates were stored in the dark. Some Kieselgel G plates were impregnated with paraffin by dipping them in liquid paraffin (5% v/v in light petroleum, b.p. 40-60°C) immediately before use.

All thin-layer chromatograms were developed in the dark in glass tanks lined with chromatography paper and saturated with solvent. The major solvent systems employed for the separation of lipids are shown in Table 2.2. Details of other systems are included in the relevant results sections.

Colourless compounds were visualized by spraying the plate with a solution of Rhodamine 6G in acetone (0.01% w/v), after which they appeared as brown or yellow areas on a green background when viewed under u.v. light. When greater sensitivity ($\geq$ 0.05µg) was required, and recovery of the lipids was not important, the plates were exposed to I_2 vapour (Davies et al., 1961). Sterols could be detected with great sensitivity ($\geq$ 0.5µg) by spraying with a solution of $SbCl_3$ in $CHCl_3$ (20% w/v), and heating the plate at 80°C for 5 min (Dawson et al., 1959). With this procedure lanosterol stained yellow, while ergosterol produced a blue-grey spot. Phospholipids were stained blue by spraying with the reagent of Vaskovsky and Kostetsky (1968).

Autoradiography: This technique was used to detect ^{14}C-labelled compounds on thin-layer chromatograms. A piece of X-ray film was cut to size, and placed on the top of the gel. A clean glass plate was then placed on top of the film, and the whole was wrapped in

TABLE 2.2 SOLVENT SYSTEMS EMPLOYED FOR THE SEPARATION OF LIPIDS

R_F Values

Solvent system / Lipid	1	2	3	4	5	6
β-Carotene	0.85	0.80	0.75	0.35		0.15
Phytoene				0.65	0.10	0.15
Squalene	0.85	0.80		0.80	0.55	0.25
Ergosterol	0.15	0.25	0.05	0.01		0.0
Lanosterol	0.20	0.45	0.20	0.05		0.0
Steryl esters	0.80	0.80				
Monoglycerides	0.0	0.05				
Diglycerides	0.40					
Triglycerides	0.45-0.70					
Phospholipids	0.0	0.0				
Fatty Acids	0.30		0.02			

Solvent Systems

1[a] 82:18:1 (by vol.) petrol*/diethyl ether/acetic acid

2[a] $CHCl_3$

3[b] 2:1 (v/v) petrol*/toluene

4[b] 4:1 (v/v) petrol*/toluene

5[c] 7:3 (v/v) petrol*/diethyl ether

6[a] petrol*

* light petroleum (b.p. 60-80°C)

a Adsorbent : Kieselgel G

b Adsorbent : 1:1 (w/w) Kieselgel G/MgO

c Adsorbent : Kieselgel G + 3% $AgNo_3$

aluminium foil and left in the dark for several months, after which the film was developed.

Elution of Bands from Chromatograms: Bands were scraped off thin-layer chromatograms with a spatula and collected on a sintered glass funnel. The lipids were eluted by washing the gel with diethyl ether (20-30ml). The elution of lipids from adsorbents containing MgO required the addition of a few drops of ethanol to the solvent. When samples of radioactive lipids (biosynthesized from [2-^{14}C]MVA) were subjected to this procedure quantitative recoveries were obtained.

Paper Chromatography: Compounds were applied to Whatman No. 1 chromatography paper using a micro-syringe, and the solvent was evaporated in a stream of warm air. Chromatograms were developed in a descending manner in a glass tank which had been pre-equilibrated with solvent. Radioactive bands were located using a 4π scanner (Nuclear Instrumentation Inc.), and the appropriate strips were assayed by scintillation counting.

Liquid Scintillation Counting: Radioactive lipids were counted in toluene containing PPO (5g/litre). Samples were pipetted into glass vials, the solvent was evaporated on a hot plate under a stream of N_2, and scintillant (10ml) was added. Aqueous samples were assayed in a scintillant consisting of Triton X-100:toluene/PPO (1:2 v/v). Up to 1.2ml of aqueous sample could be accommodated in 10ml of this scintillant.

Samples were counted in a Packard Tri-Carb liquid scintillation spectrometer for 10 min, or until at least 100 000 gross counts had been recorded. They were generally stored in the instrument overnight prior to counting in order to allow any luminescence to decay.

The efficiency of counting was determined from the external standard channels ratio, using quench correction curves. These curves were prepared by adding either up to 300µg of β-carotene (colour quenching) or up to 2.0ml of chloroform (chemical quenching) to 10ml of scintillant which contained a known amount of [^{14}C]hexadecane. In practice the colour and chemical quench curves were found to be identical.

Amino Acid Analysis: Analyses were conducted as described by Moore and Stein (1963). Washed fractions (containing approx. 0.5mg of protein) were suspended in deionised water and transferred to a thick-walled,

glass hydrolysis tube, to which an equal vol of conc. HCl was added. The contents of the tube were frozen in liquid N_2, and the tube was evacuated using an oil pump. Dissolved air was removed by thawing the sample and tapping the tube to release gas bubbles, then re-freezing the solution and re-evacuating the tube. This procedure was repeated once, and the tube was sealed and annealed over a Bunsen burner.

Identical samples were hydrolysed for 14 or 72 h at 110 ± 0.5°C. After hydrolysis the tubes were opened and their contents washed into a round-bottomed flask which was dried at 45°C on a horizontal rotary evaporator connected to an oil pump. The dry residue was dissolved in 0.01M HCl (5ml), clarified by filtration through a Millipore membrane, and analysed on a Jeol model JAC-6AH amino acid analyser.

The analyser was calibrated using a mixture of amino acids (0.1 μmole/ml of each), and the area of each peak on the chart recorder was calculated. The concentration of each amino acid at zero time was determined by extrapolation of the results obtained after 14 and 72 hours, using the formula:

$$\log A_o = \frac{t_2}{t_2-t_1} \log A_1 - \frac{t_1}{t_2-t_1} \log A_2$$

where A_1, A_2 and A_o are the quantities of amino acids present after t_1, t_2, and zero hours of hydrolysis, respectively.

Electron Microscopy: Samples were prepared for electron microscopy by methods described by Nunn (1970). Mycelia were cut into small blocks approximately 3mm long. Organelles were resuspended in warm agar (2% w/v), and small blocks were cut after the agar had set. They were then fixed overnight at 6°C in glutaraldehyde (3% w/v in 0.1M Na phosphate, pH 7.4). For the fixation of organelles 0.4M mannitol was added to this solution.

After glutaraldehyde fixation the samples were washed with buffer (3 x 15 min at 6°C), and then fixed with OsO_4 (2% w/v in 0.12M Na phosphate, pH 7.3, containing 0.54% w/v glucose; Millonig, 1961).

The samples were again washed with phosphate buffer (3 x 15 min at 6°C), and then dehydrated through a graded ethanol series (30%, 50%, 70%, 80%, 90%, 95% v/v ; 15 min each at 6°C) and finally washed in absolute ethanol (30 min at 6°C). They were stained overnight with uranyl acetate (2% w/v in ethanol), and washed with ethanol

(2 x 15 min at 6°C). In one experiment the samples were washed with buffer (3 x 15 min at 6°C) and then with deionised water (2 x 15 min at room temperature) after OsO_4 fixation. They were then dehydrated in 5 min by the addition of acidified dimethoxypropane (Muller and Jacks, 1975). This method is designed to reduce extraction of lipids by prolonged contact with organic solvents.

After dehydration samples were transferred to ethanol/propylene oxide (1:1 v/v) for 30 min, and then washed in propylene oxide (30 min). Samples dehydrated with dimethoxypropane were transferred directly to propylene oxide.

The blocks were then placed in a mixture of propylene oxide and resin (1:1 v/v), and left overnight at 6°C until all the propylene oxide was evaporated, after which they were transferred to fresh resin, and left for a further 24 h. To ensure adequate penetration of mycelia, samples had to be placed in several changes of fresh resin for one day each.

Finally the samples were placed at the bottom of plastic capsules, which were filled with fresh resin, and polymerised at 60°C for 48h.

In all these experiments the resin used was the Epon mixture of Luft (1961), as described by Nunn (1970). Solution A contained Epikote 812 (62ml) and dodecylsuccinic anhydride (100ml), while solution B consisted of methyl nadic anhydride (89ml) mixed with Epikote 812 (100ml). These solutions were stored dry at 6°C for up to six weeks. The final resin mixture consisted of solution A (6ml) and solution B (4ml) with N-benzyl-dimethylamine (0.15ml) as accelerator.

After polymerisation the blocks were removed from the capsules, and trimmed on an LKB Ultrotome to produce a truncated pyramidal face about 0.2mm square. Sections 90 to 150nm thick (interference colour gold) were cut using a glass knife, and floated onto sterile water. They were then swollen by exposure to trichloroethylene vapour, and picked up on Formvar-coated copper grids. Sections were viewed under a Jelco JEL T6S single condenser electron microscope operating at 60 kV.

ANALYSIS OF LIPIDS OF CELL ORGANELLES-PRELIMINARY EXPERIMENTS

Fungal carotenoids have been variously located in lipid droplets, in sedimentable cell organelles, and in hyphal walls (see pp. 77-78). In order to determine their subcellular distribution in P. blakesleeanus cell walls were prepared and analysed, while the protoplasm was fractionated by differential centrifugation.

Before these investigations could take place it was necessary to study the kinetics of growth and pigment production by the fungus, in order to ensure that subsequent experiments were performed on mycelia which were actively carotenogenic. The sizes, types, and distributions of organelles within the hyphae were also examined by electron microscopy, so that the results of cell fractionation experiments could be related to the morphology of the fungus.

Growth of Phycomyces blakesleeanus C115 carS42 mad-107(-)

The kinetics of growth and pigment production by the C115 mutant were examined in order to determine the optimum time for harvesting young, growing, carotenogenic mycelia.

A series of 250ml conical flasks, each containing 100ml of sterile growth medium, were inoculated and incubated as described in the Methods section. Two flasks were removed at random every three hours between 12 and 60 hours after inoculation, and thereafter at twelve-hour intervals up to 96 hours after inoculation. The mycelia were collected by filtration on a Buchner funnel, a portion was freeze-dried, and the remainder was extracted with acetone and ether as described previously. The pH of the filtrate was also measured.

After an initial lag phase the fungus continued to increase in dry weight for at least 96h (Fig. 3.1) although by the end of this period lysis of the cells appeared to have commenced since large amounts of lipid were visible floating on top of the medium. The decline in growth rate after 33h is probably due to the restriction on space discussed in the Introduction (p.58). The fall in pH during growth is probably due to the excretion of organic acids into the medium, a process which has been observed in a number of fungi (Cochrane, 1958). It is apparent that both β-carotene and ergosterol are produced throughout growth in shake-cultures (Fig. 3.2). This has previously been observed in wild-type mycelia grown in shake-culture (Olson and Knizley, 1962; Bartlett and Mercer, 1974).

As a result of this investigation mycelia grown for 40h were normally used in subsequent experiments.

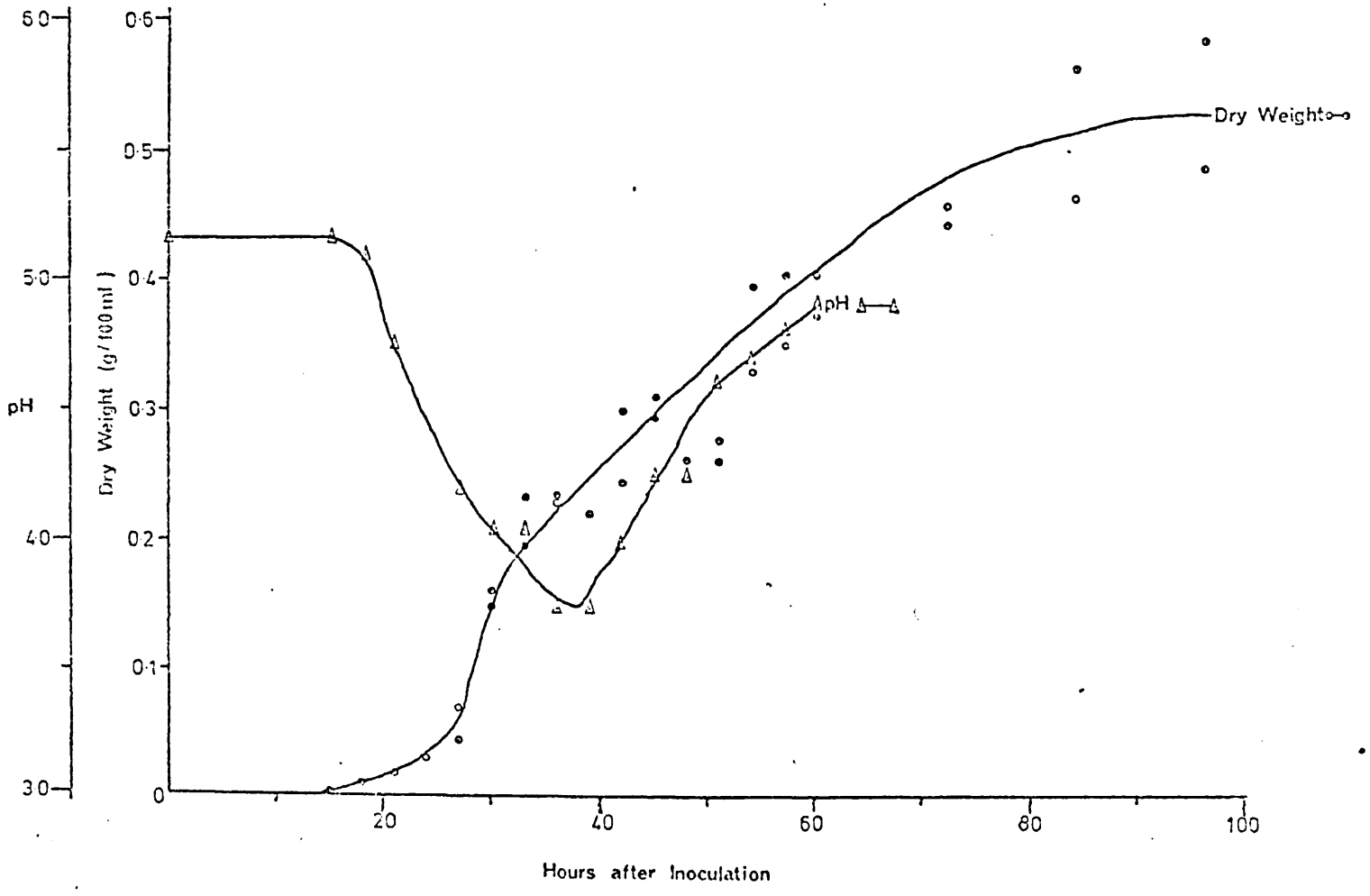


Fig. 3.1 Growth Curve for C115 Mutant in Shake Culture

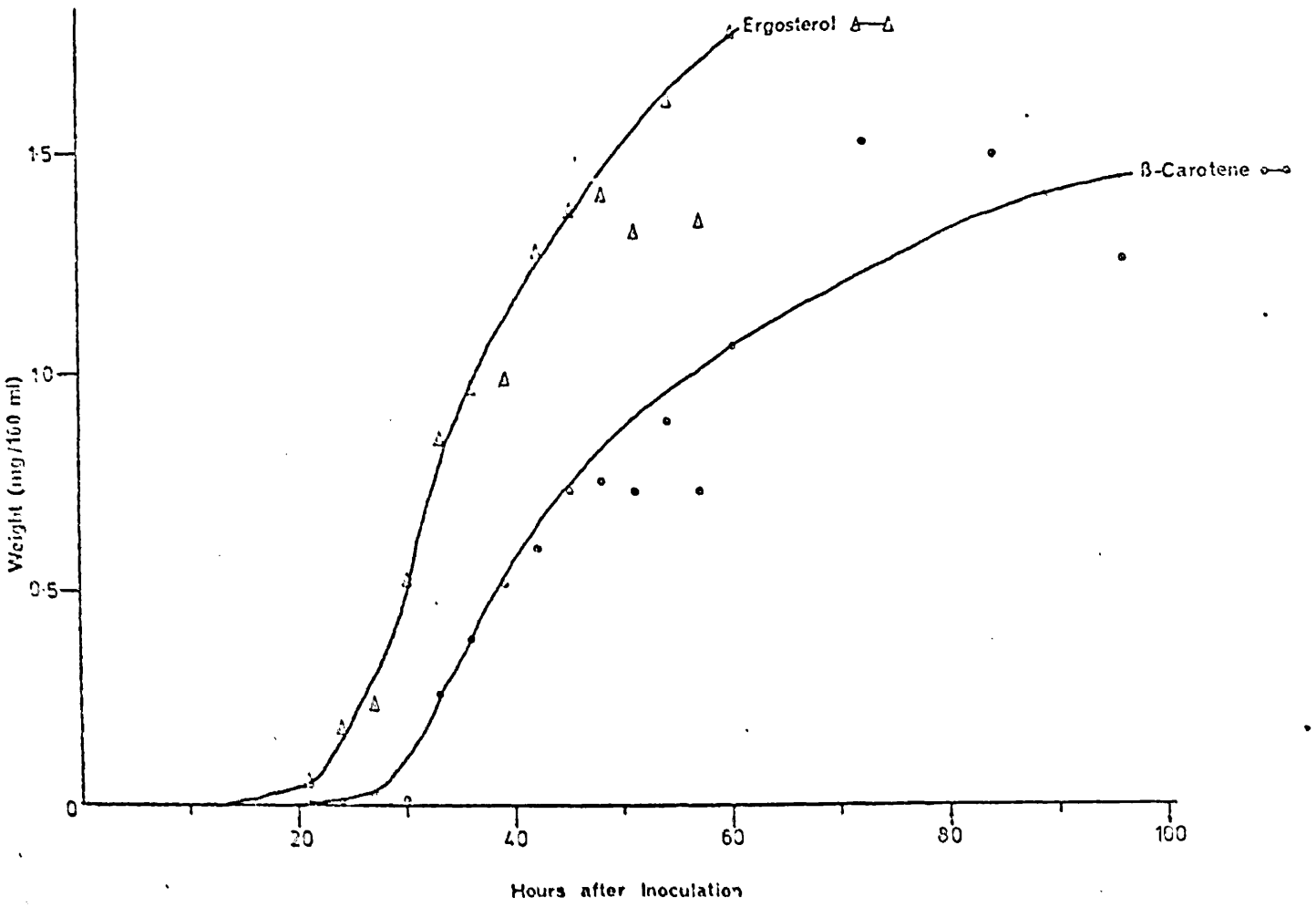


Fig. 3.2 Production of β-Carotene and Ergosterol by C115 Mutant in Shake Culture

Electron Microscopy

The morphology of the hyphae was examined by electron microscopy of mycelia from cultures grown for 40h.

In most of the sections examined the cell wall was 0.3 to 0.4μm thick, indicating that the samples came from areas behind the growing tip of the hypha (Figs. 3.5, 3.6, 3.7). Mitochondria were plentiful, and resembled those from plant and animal cells. They were about 0.6 x 0.4μm in size, and occupied 25 to 35% of the total cytoplasmic area (Figs. 3.3, 3.4, 3.5, 3.6). Glycogen was also abundant and was mainly in the form of α-rosettes, about 150nm in diameter (Drochmans, 1962; Figs. 3.3, 3.4, 3.6). Nuclei were about 2.8 x 1.8μm in size (Fig. 3.7). Osmiophilic globules, approximately 0.3 to 0.8μm in diameter, were also visible in some sections, especially close to, or within, vacuoles (Figs. 3.5, 3.7). No membrane profiles due to E.R. or Golgi bodies could be discerned in these sections, probably because they were too thick.

Transverse sectioning showed that about 70% of the hypha was filled with a large vacuole, the cytoplasm being appressed against the cell wall (Fig. 3.7). Numerous smaller vacuoles and vesicles were present within the cytoplasm (Figs. 3.4 and 3.5). In very old zones (cell wall 0.75μm thick) the hyphae were completely filled with a disorganised mass of vacuoles, osmiophilic globules and senescing mitochondria (Fig. 3.8).

These results are very similar to those obtained by Hawker and Abbott (1963), who studied the fine structure of vegetative hyphae of Rhizopus sexualis and R. homothallicus, which are of the same order as Phycomyces. They were also able to show the presence of presumed Golgi cisternae and of a membrane separating the growing region into two zones, but their observations on the distribution of organelles in older zones agree closely with the results described above.

Preparation of Cell Walls

Most procedures for cell disruption do not completely break all the hyphae, and hence debris must be removed from the homogenate before cell fractionation. Since this debris includes hyphal walls, and since the presence of carotenoids in the cell walls of Dacryopinax spathularia has been reported (Vail and Lilly, 1968), cell walls were isolated from Phycomyces and analysed for the presence of carotenes. In this experiment the wild-type strain was used, since it contains a smaller amount

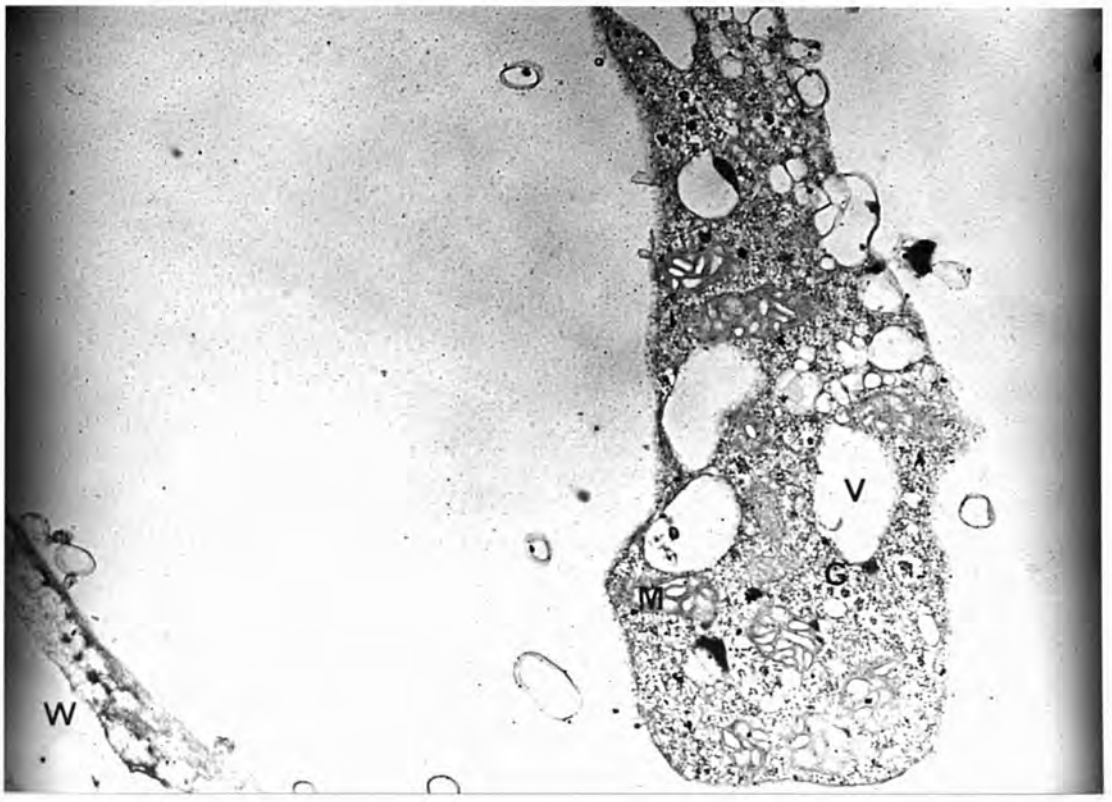


Fig. 3.3 Mycelium : transverse section through growing zone (x 17 000). Fixed in glutaraldehyde and osmium tetroxide. Stained with uranyl acetate

Abbreviations: G, glycogen; M, mitochondrion; V, vacuole; W, cell wall

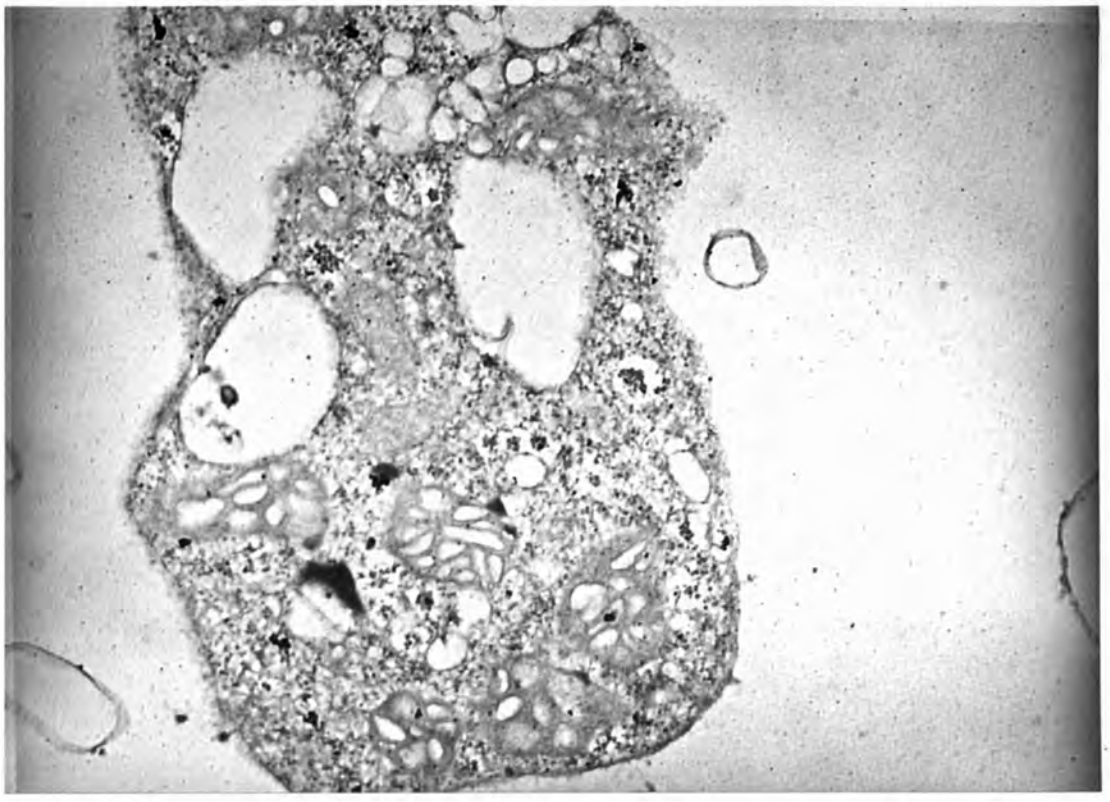


Fig. 3.4 Mycelium : transverse section through growing zone (x 27 500). Fixation and staining as for Fig. 3.3

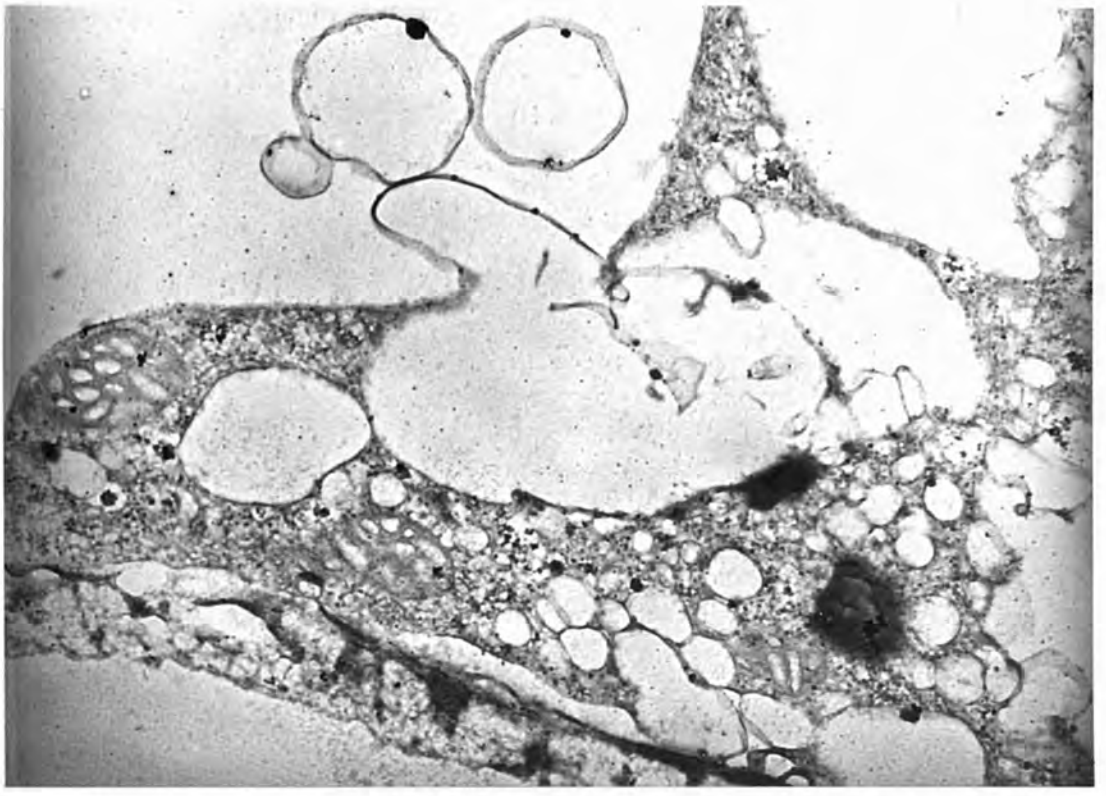


Fig. 3.5 Mycelium : glancing section (x 32 000). Fixation and staining as for Fig. 3.3

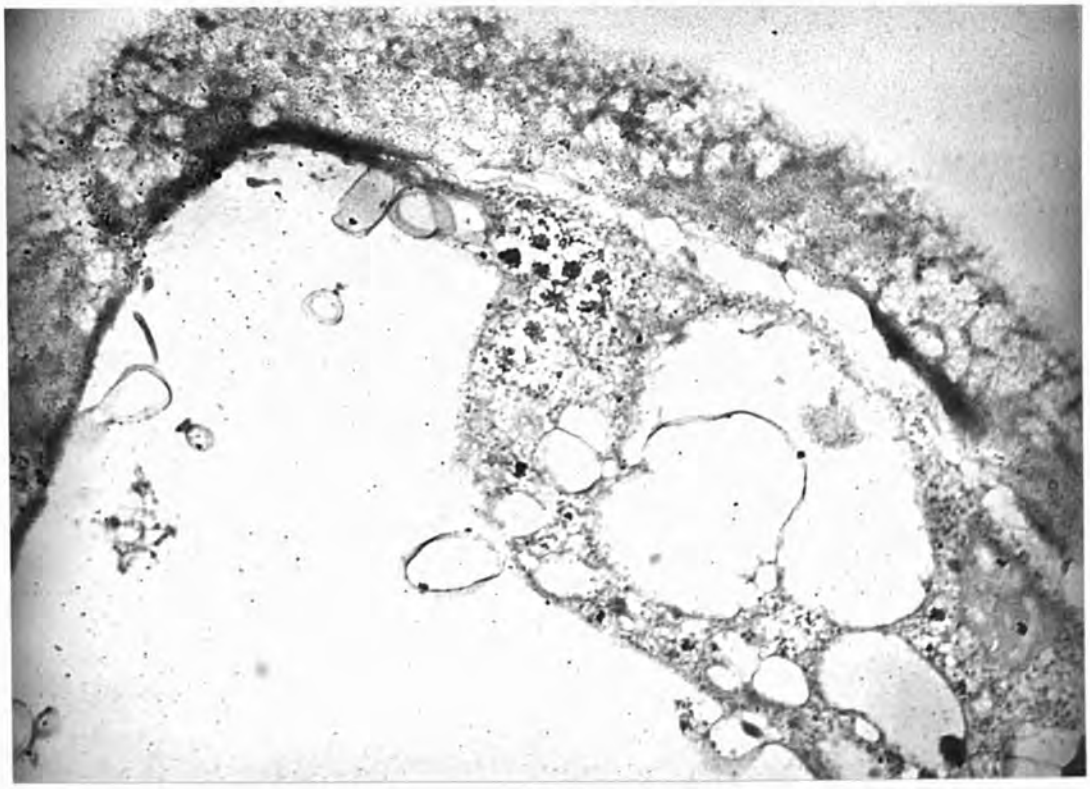


Fig. 3.6 Mycelium : glancing section (x 40 000). Fixation and staining as for Fig. 3.3

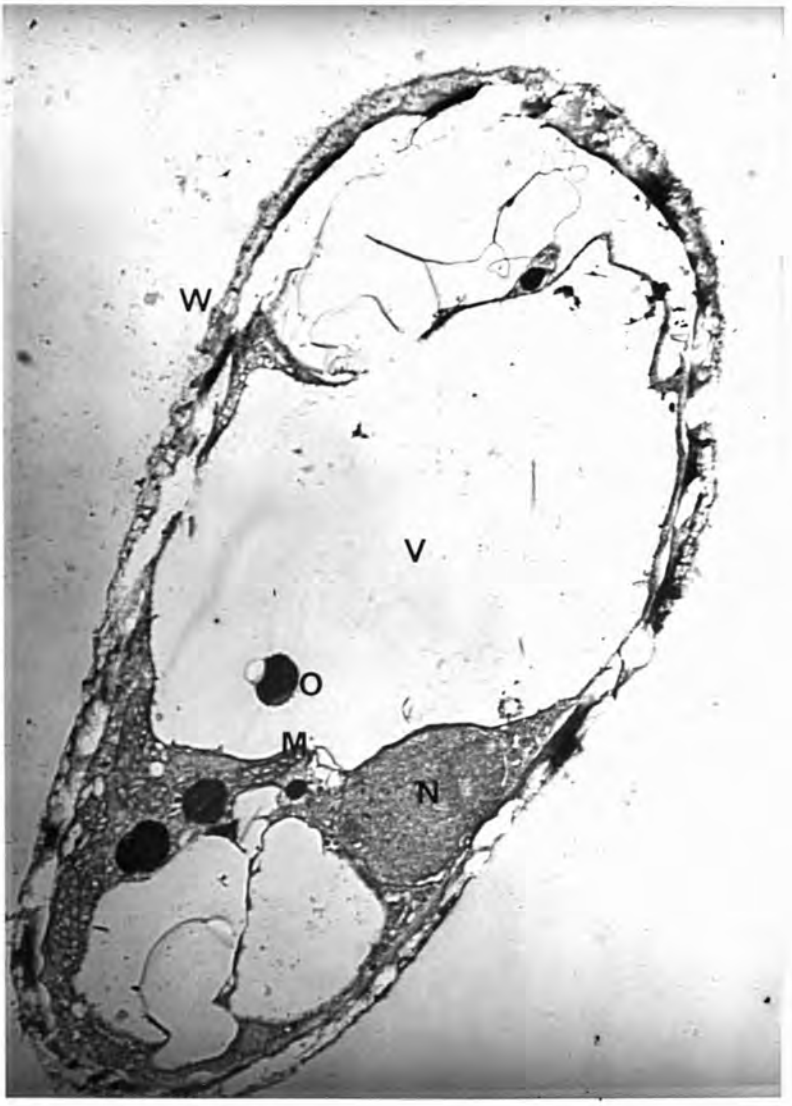


Fig. 3.7 Mycelium : transverse section through growing zone (x 10 000). Fixation and staining as for Fig. 3.3

Abbreviations: M, mitochondrion; N, nucleus; O, osmiophilic globule; V, vacuole; W, cell wall

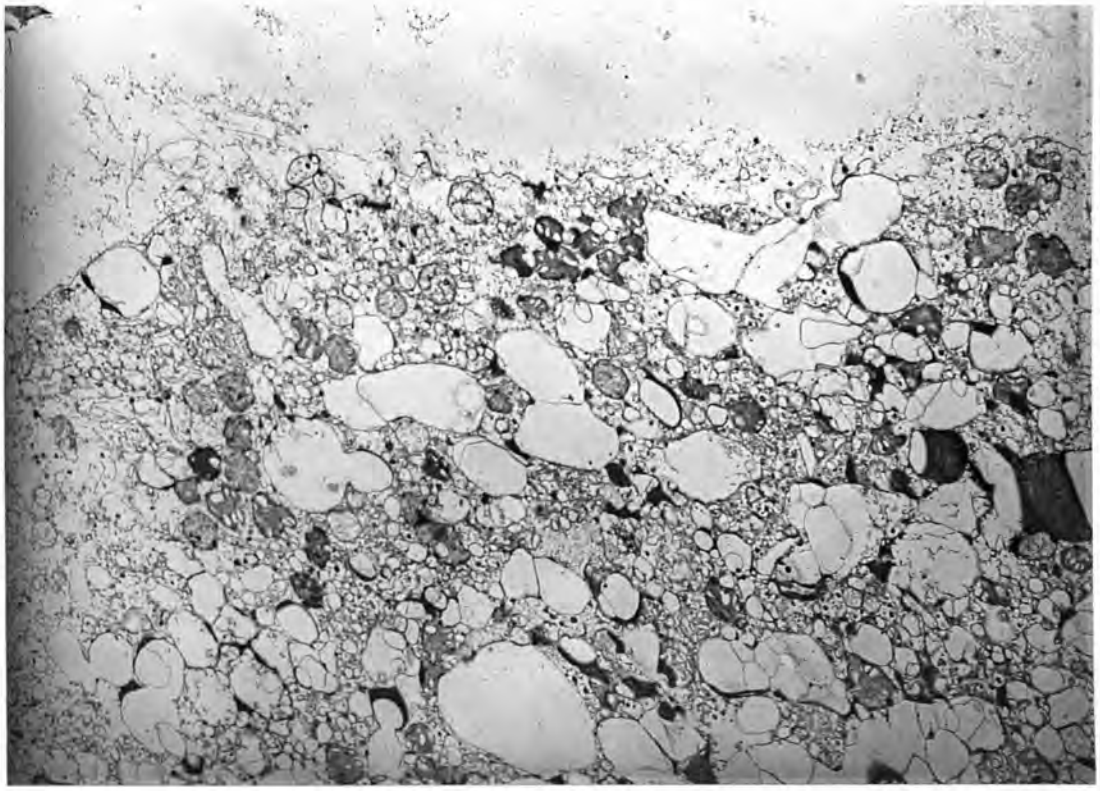


Fig. 3.8 Mycelium : section through senescent tissue (x 10 000). Fixation and staining as for Fig. 3.3

of carotenoids than the C115 mutant, and hence any contamination of the cell walls by protoplast carotenoids should be reduced.

Mahadevan and Tatum (1965) published a method for the preparation of cell walls from N.crassa by stirring lyophilized mycelia in an aqueous solution of SDS (sodium dodecyl sulphate). When this method was applied to lyophilized mycelia of P.blakesleeanus, however, the hyphae were not lysed, and microscopic examination showed that most of them retained their cytoplasm. Consequently lyophilized mycelia were first disrupted by passing them through a 40-mesh sieve, and then washed with aqueous SDS (1.5% w/v) on a sintered glass funnel until no more colour was released into the filtrate. The washed mycelia were stirred with SDS solution for 6h at 8°C, and then washed with water. Microscopic examination showed that this procedure yielded almost entirely small fragments of clean cell walls which were essentially free from cytoplasm (Fig. 3.9).

β-Carotene was extracted from the SDS solution with light petroleum (b.p. 40-60°C), and from the isolated cell walls using acetone and diethyl ether. A recovery of 98% of β-carotene was obtained, and of this only 1.1% was found in the washed cell walls. Thin-layer chromatography of the cell wall extract (System 1; Table 2.2) showed that free fatty acids, acylglycerols, and sterols were present.

Cell Fractionation by Differential Centrifugation

Since β-carotene is not located in the cell walls, experiments were designed to discover its location within the protoplasm. This was first attempted by differential centrifugation of cell homogenates.

Previous results have shown that cell disruption techniques involving lyophilisation and sieving, or the use of an X-press (L.K.B., Ltd.), are too severe for the isolation of intact cell organelles (Bramley, P.M., unpublished). Consequently an alternative technique was employed. This involved homogenisation in a glass Potter-Elvehjem homogenizer with a teflon pestle (total clearance 0.4mm).

In a preliminary experiment the liberation of protein and β-carotene during homogenisation was investigated: they were released from mycelia at the same rate, with maximal yields after 40 passes of the teflon pestle (Fig. 3.10). In most experiments, however, only 15 passes were employed in order to reduce damage to cell organelles.

Having established a viable method for cell disruption, attempts were made to isolate pigmented organelles by differential centrifugation.

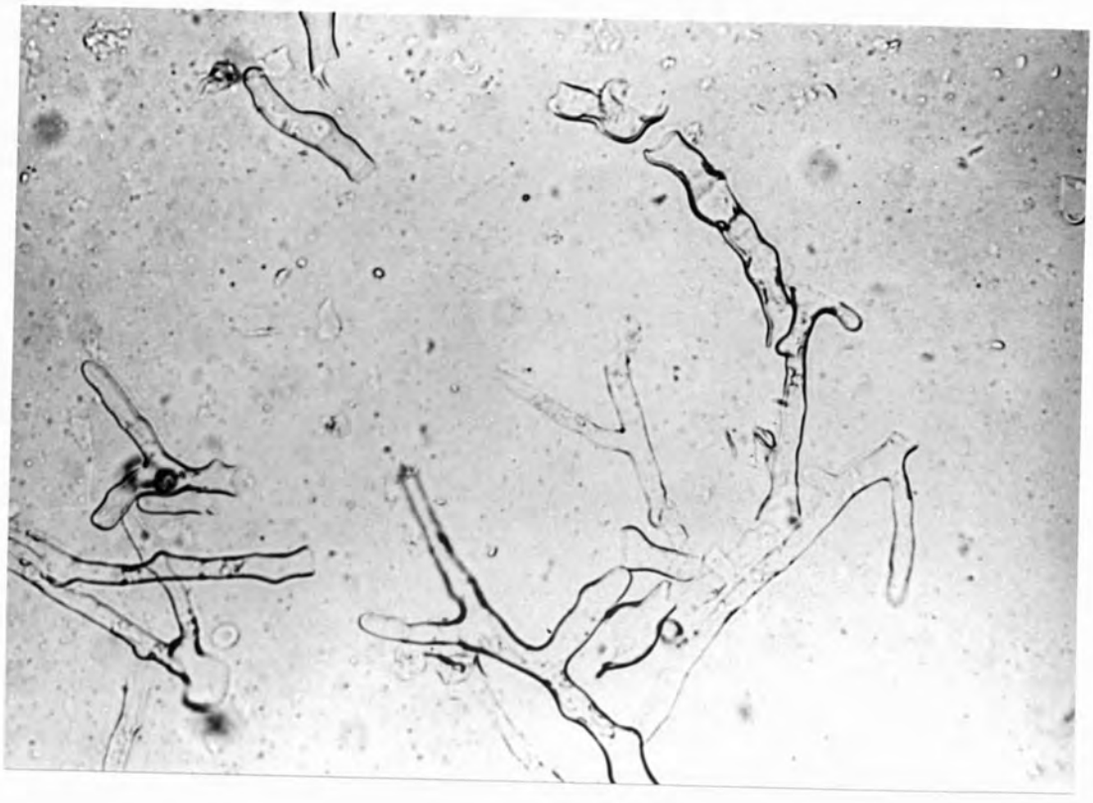


Fig. 3.9 Hyphal Walls after Extraction with SDS
(approx. magnification, x 800)

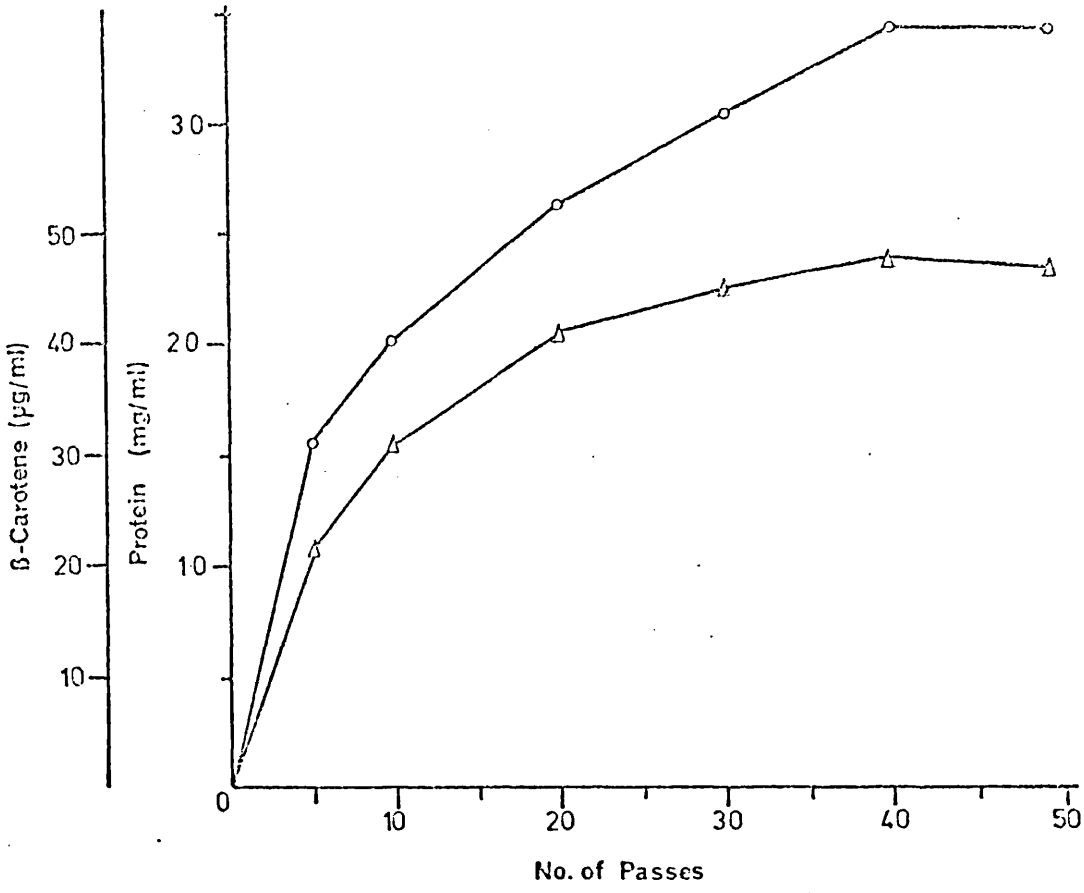


Fig. 3.10 Release of Protein and β-Carotene During Homogenisation

Mycelia suspended in homogenisation buffer (8:1 v/w) were subjected to varying numbers of passes of the teflon pestle, and the quantities of protein (o) and β-carotene (Δ) estimated after filtration through muslin

In an experiment similar to that of Cederberg and Neujahr (1970) a mycelial homogenate was filtered through two layers of muslin, and then centrifuged successively at 800g (5 min), 2 100g (10 min), 5 000g (10 min), 10 000g (10 min), 25 000g (15 min) and 100 000g (1h). Each pellet was assayed for β-carotene and protein, while the lipid globules, which floated to the top of the tubes during centrifugation, were collected on pieces of muslin from which the pigment was extracted with ether.

The results (Fig. 3.11) are comparable to those of Cederberg and Neujahr (1970) in that most of the recovered β-carotene (88%) was present in the floating fat fraction, while the sedimentable β-carotene was found predominantly in the fractions sedimenting at 800g (cell debris) and 5 000g. However, no conclusion can be reached as regards the identity of the latter fraction unless the presence of known marker enzymes can be demonstrated.

Consequently another experiment was designed in which "nuclear", "mitochondrial", "microsomal" and "supernatant" fractions were prepared by the conventional differential centrifugation procedure (Claude, 1946), and the distribution of enzymes characteristic of the mitochondria and cytosol respectively were assayed.

Mycelia (10g wet wt.) were homogenised in 50mM tris-HCl, pH 7.6, (90ml), containing 5mM EDTA, 0.6M mannitol, and 0.2% (w/v) BSA. After filtration through 2 layers of muslin the homogenate was centrifuged at 800g (10 min). The pellet was resuspended in buffer (10ml) and recentrifuged at 800g. The washed sediment was resuspended in buffer (5ml) and designated the nuclear fraction, while the supernatants were combined and a 12 000g (15 min) pellet was prepared. This was washed and designated the mitochondrial fraction, and the combined supernatants were centrifuged at 100 000g_{av} (1h) to produce microsomal and 100 000g supernatant fractions.

The distributions of succinate:DCPIP reductase and glucose 6-phosphate dehydrogenase, enzymes which are respectively characteristic of the mitochondria and cytosol of animal cells (de Duve et al., 1962), was determined in these fractions (Table 3.1). The highest specific activities of these enzymes were found in the mitochondrial and supernatant fractions, but significant amounts of each enzyme were also detected in the other fractions. The large amount of succinate:DCPIP reductase in the 100 000g supernatant indicates that a number of mitochondria were damaged during homogenisation.

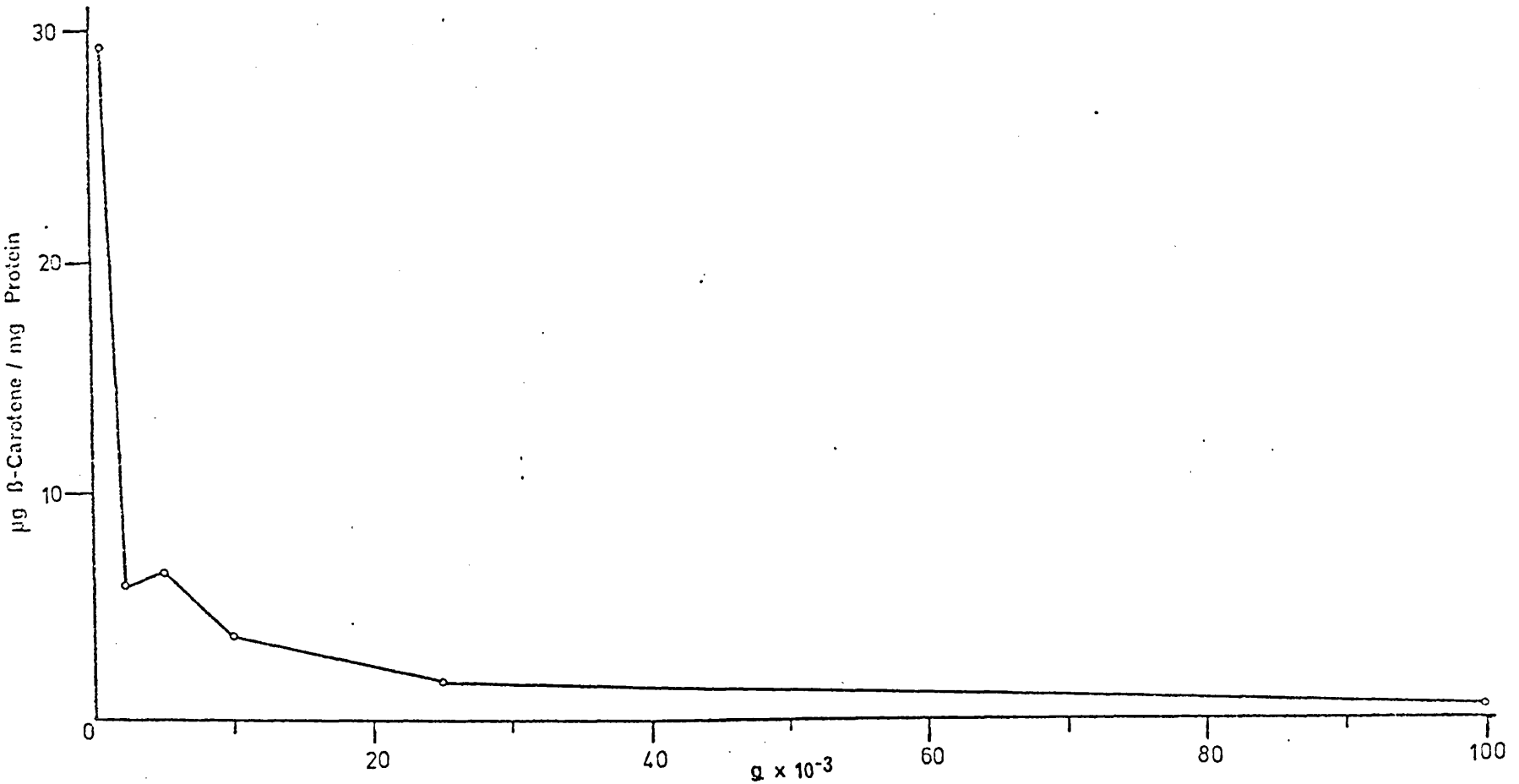


Fig. 3.11 Distribution of β-Carotene in Fractions Prepared by Differential Centrifugation

Recoveries: β-Carotene 74% *

Protein 92%

* includes lipid globule fraction

TABLE 3.1 DISTRIBUTION OF MARKER ENZYMES IN FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION

Fractions isolated by the methods described in the text. All figures are from duplicates of four separate experiments and are quoted ± S.E.M. Units of enzyme activities are nmoles of substrate converted per minute and specific activities are units per mg protein

Fraction	Protein		Succinate : DCPIP reductase			Glucose 6-phosphate dehydrogenase		
	Total (mg)	Total (%)	Units	Sedimentable activity (%)	Specific activity	Units	Units (% of total)	Specific activity
Nuclear	1.21 ± 0.18	1.4	25.0 ± 0.6	4.3	0.83	19.0 ± 0.6	0.06	15.7
Mitochondrial	7.51 ± 0.19	8.6	404.0 ± 13.8	69.2	53.9	200.0 ± 7.1	0.61	26.6
Microsomal	8.70 ± 1.04	9.9	155.0 ± 3.9	26.5	17.8	261.0 ± 6.4	0.80	30.0
Cytosol (100 000g supernatant)	70.20 ± 5.72	80.1	458.0 ± 10.7		6.5	3 229.0 ± 90.4	98.5	460.0

No glucose 6-phosphatase (E.C.3.1.3.9) activity could be detected in any fraction using the methods of either Swanson (1955) or Morré (1971). Neither could 5'-nucleotidase (E.C.3.1.3.5; assayed as described by Morré, 1971) or nucleoside diphosphatase (E.C.3.6.1.6; assayed by the method of Plaut, 1963) be detected. Consequently no microsomal marker was assayed in these experiments.

Examination of the mitochondrial pellet by electron microscopy (Figs. 3.12 and 3.13) indicated that the organelles were spherical, but were otherwise morphologically similar to the mitochondria present in mycelia (Figs 3.3 to 3.6). They were considerably contaminated, however, by glycogen and by unidentified vesicles. Similar heterogeneity of fungal "mitochondrial" pellets has been described by Viglietti et al. (1973).

The distribution of β-carotene within these cell fractions was essentially the same as that observed in the previous experiment, with the majority (> 80%) being contained in the lipid droplet fraction. Of the sedimentable β-carotene, slightly more than 50% was in the mitochondrial pellet, but significant amounts were also present in the microsomal fraction. However, this distribution did not mirror that of the mitochondrial marker enzyme (Fig. 3.14), and it was concluded that the sedimentable β-carotene was not located in the mitochondria, but more probably among the contaminating vesicles visible under the electron microscope.

The differential centrifugation procedure was modified, so that two mitochondrial fractions were sedimented at 8 000g (15 min) and 18 000g (15 min) respectively. The heavier fraction contained 75% of the sedimentable succinate:DCPIP reductase activity, but both fractions were still heavily pigmented, and it was concluded that differential centrifugation alone was not able to resolve mitochondria from pigmented organelles.

Density Gradient Centrifugation of Mitochondria

Since separation by sedimentation rates alone was insufficient for the separation of mitochondria and pigmented organelles, an attempt was made to resolve these organelles on the basis of differences in their densities. A mitochondrial fraction, sedimented at 12 000g, was resuspended in sucrose solution (17% w/v, 1ml) containing 1mM EDTA, and layered over a linear sucrose gradient (30% to 70% w/v, 12ml) containing 1mM EDTA. This was centrifuged for 1½h at 110 000g_{max}, and

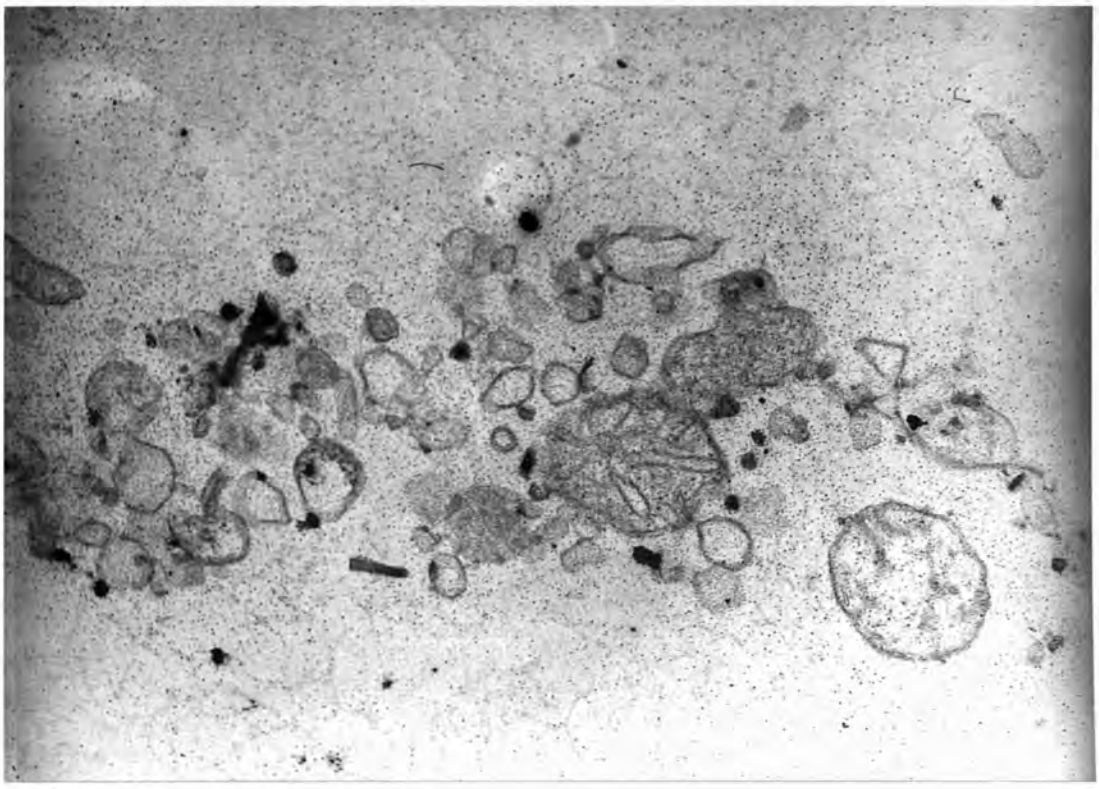


Fig. 3.12 Mitochondria (x 25 000). A 15 000g (10 min) pellet was dispersed in molten agar, and fixed in glutaraldehyde followed by osmium tetroxide. The samples were stained with uranyl acetate

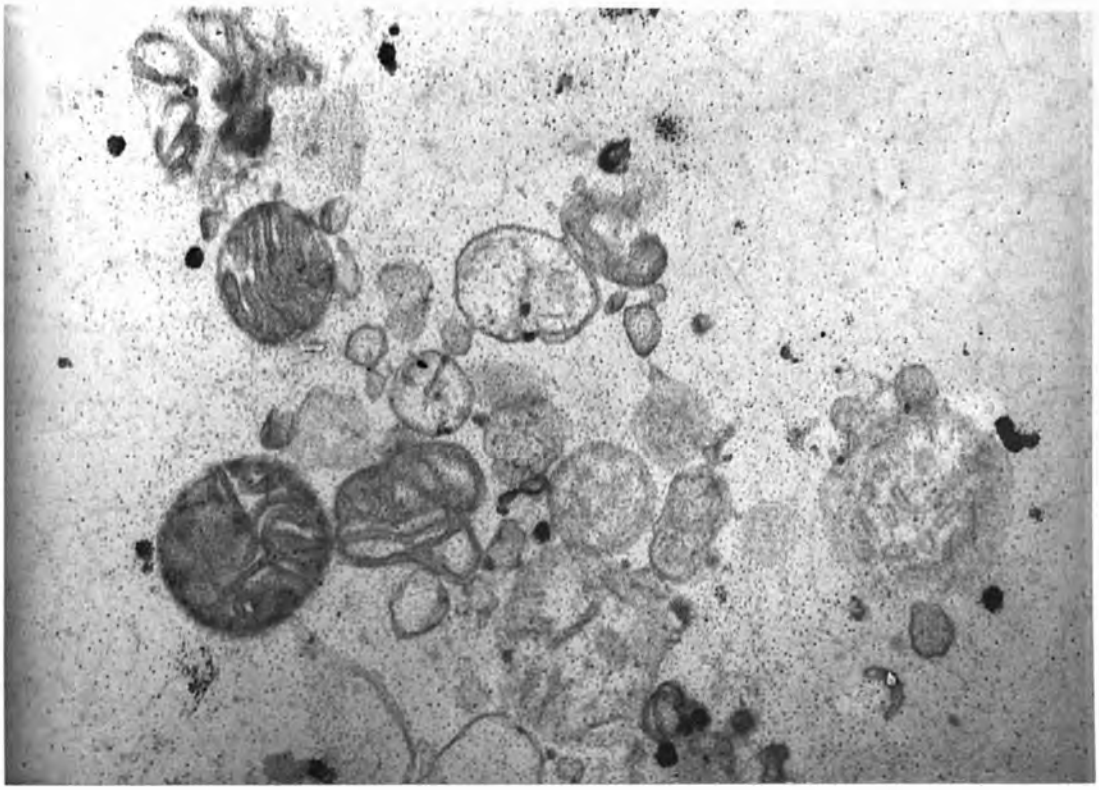


Fig. 3.13 Mitochondria (x 25 500). For details of preparation see Fig. 3.12

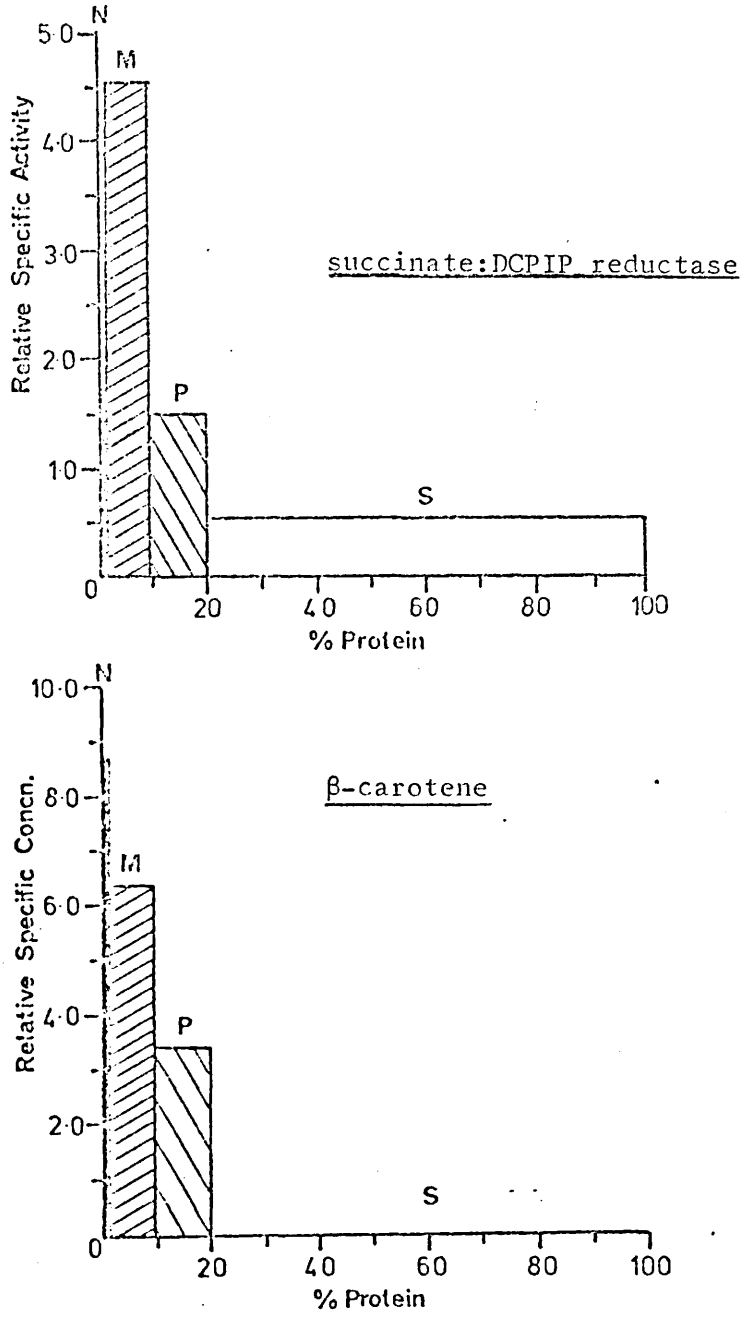


Fig. 3.14 Distributions of Succinate:DCPIP Reductase and β-Carotene in Fractions Prepared by Differential Centrifugation

N = nuclear fraction P = microsomal fraction

M = mitochondrial fraction S = 100 000g supernatant

$$\text{Relative Specific Activity} = \frac{\%\text{ total activity}}{\%\text{ total protein}}$$

$$\text{Relative Specific Concn.} = \frac{\%\text{ total sedimentable β-carotene}}{\%\text{ total protein}}$$

then analysed as described in the Methods section. Fractions (0.4ml) were assayed for succinate:DCPIP reductase activity and for the presence of β-carotene (absorbance at 462nm).

The major peaks of protein (absorbance at 258nm) and enzyme activity coincided at a density (ρ) of 1.185g/ml (Fig. 3.15), which is the density observed for mitochondria isolated from both N.crassa (Luck, 1963) and P.blakesleeanus (Zalokar, 1969). Another peak of protein coincided with a pigmented fraction at ρ=1.130. The sample zone (fractions 32-35) contained some material which had not entered the gradient. When a 10 000g pellet was resuspended in sucrose (80% w/v, containing 1mM EDTA) and floated upwards through the same gradient, the less dense pigmented fraction was again observed at ρ=1.130, indicating that this is its equilibrium density.

The spectrum of this pigmented organelle was obscured by light-scattering, but it showed three distinct peaks at 435, 462 and 492nm. These maxima are the same as those determined for the absorption spectrum of sporangiophores of P.blakesleeanus (Bergman et al., 1969). Upon storage they showed a bathochromic shift of approximately 20 to 30nm. The large degree of light scattering at wavelengths below 330nm indicates that this fraction contained large, membrane-bounded vesicles.

Unfortunately, although this experiment was repeated successfully, further attempts were less successful, since the pigmented material appeared to aggregate with the mitochondria so that it could not be removed by subsequent density gradient centrifugation. This aggregation was apparent in the visual appearance of the mitochondrial band, which appeared "granular", and also in the lower densities (1.150 to 1.165g/ml) at which this fraction banded. Furthermore the aggregation phenomenon was not reproducible, since on some occasions almost all the pigment was associated with the mitochondria, whereas in other cases most of it was at ρ=1.13. A similar irreversible contamination of fungal mitochondria by carotene-containing organelles was observed by Herber (1974), who attributed it to aggregation with lipid droplets. However, the previous observation that sedimentable, pigmented organelles (ρ=1.130g/ml) are present in the C115 mutant of P.blakesleeanus suggests that these are the contaminants in this case.

Since the pigmented mitochondrial aggregate appeared to be granular it was suspected that the agglutination was due to mitochondrial deterioration during centrifugation. In an endeavour to

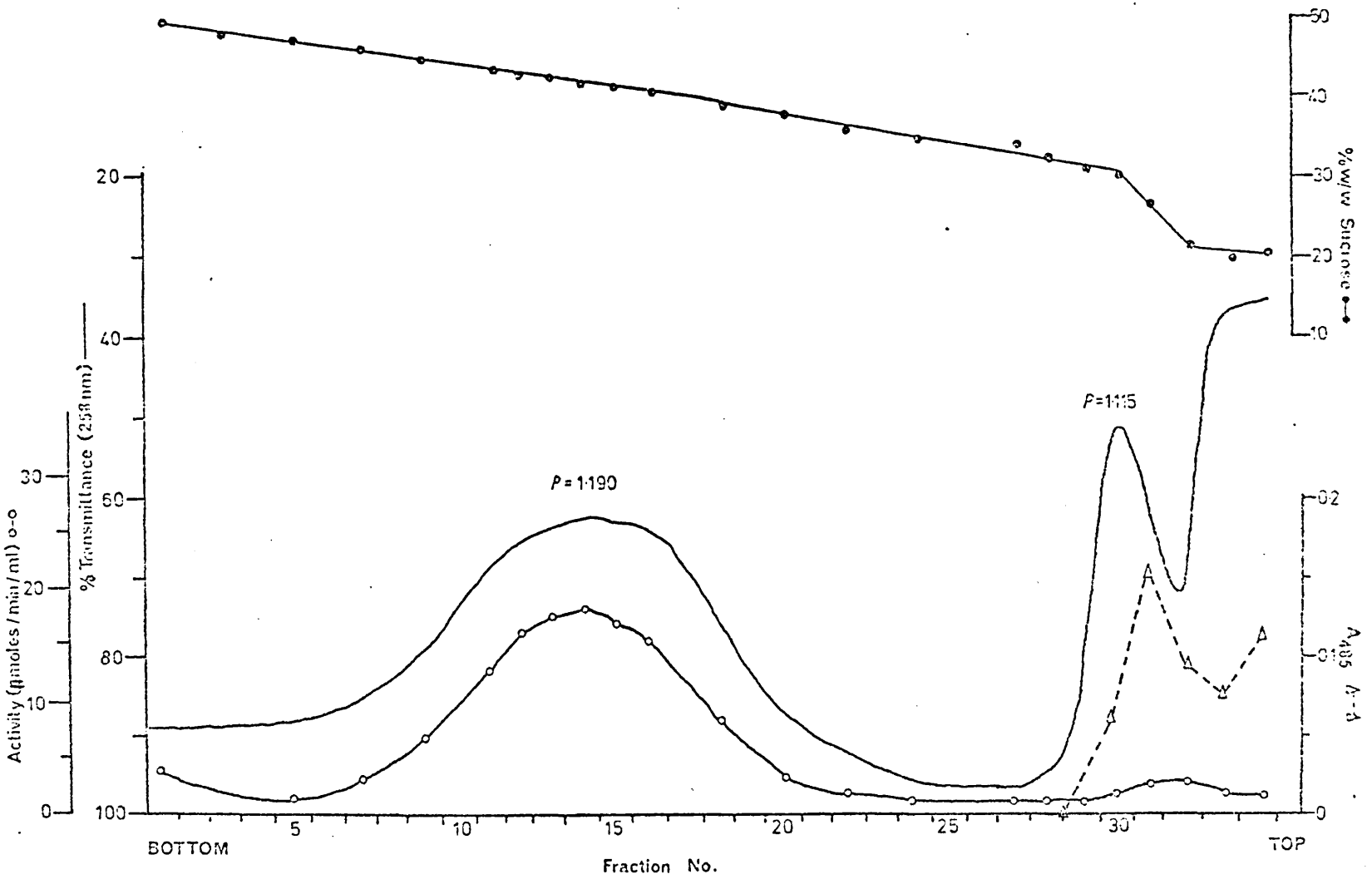


Fig. 3.15 Density Gradient Centrifugation of Mitochondria

A 12 000g pellet was resuspended in sucrose solution and layered over a linear sucrose gradient (30-70% w/v in 1mM EDTA; 12ml). Centrifugation was at 110 000g_{max} for 1½h. Fractions (0.4ml) were assayed for succinate:DCPIP reductase activity and carotenoid (A_{485})

overcome this problem several experimental alterations were made. Gradients were buffered to pH 7.6; sorbitol was used as the gradient solute in place of sucrose, since it has been observed to preserve ultrastructure better than sucrose (Price, 1974); and the tris-HCl buffer was replaced by 50mM Hepes-KOH, pH 7.4. The latter buffer has negligible binding constants for cations (Jan, 1974), and divalent cations are known to be important in stabilising mitochondrial membranes (Carafoli, 1975). However none of these approaches enabled the separation of the mitochondria and the β-carotene-containing fraction; and furthermore aggregation of the two types of particle still occurred even when the resuspended pellet was dialysed overnight, or when incompletely resuspended material was removed by a preliminary centrifugation at 1000g (3 min).

When the ionic strength of the buffer was increased by the addition of either EDTA (disodium salt) or NaCl, this aggregation increased. Similar problems have been reported in the preparation of mitochondria from animal cells, since microsomes tend to adsorb onto them during homogenisation and fractionation of the cells (Allfrey, 1959; Dallner and Ernster, 1968). These problems are due to the high net negative surface charge of microsomal vesicles, and are hence aggravated by the presence of cations in the buffer (Dallner and Nilsson, 1966; Dallner, 1974). The agglutination of microsomes isolated from animal cells has been reduced by the addition of lithium bromide (0.5M) or heparin (50 i.u./ml) to the homogenisation buffer (Katz et al., 1970; Headon and Duggan, 1971), but both these additives have deleterious effects on enzyme activities, and consequently they were not used in the present investigations.

Because of the difficulties caused by the aggregation of lipid-containing particles with other organelles, it seems that density gradient centrifugation of particles previously isolated by sedimentation is inadequate for the preparation of uncontaminated organelles from mycelia of the C115 mutant of P.blakesleeanus.

ANALYSIS OF LIPIDS OF PURIFIED CELL ORGANELLES

A. DEVELOPMENT OF METHODS FOR THE PREPARATION OF PURIFIED CELL ORGANELLES

Since differential centrifugation followed by density gradient sedimentation was not adequate for the preparation of purified organelles an alternative approach was sought. Dallner (1974) suggested that although aggregation of organelles can occur during homogenisation it was most noticeable when cell fractions were pelleted. Consequently, methods were designed for the separation of organelles by using density gradient centrifugation alone.

Preparation of Mitochondria

Mycelia were homogenized in 50mM Hepes-KOH buffer, pH 7.4, containing 5mM EDTA, 0.4M mannitol and 0.2% (w/v) BSA, as described previously. After centrifugation at 1000g (5 min) crude homogenate (10ml) was layered over a gradient prepared from 20% and 30% (w/v) buffered sorbitol solutions (10ml of each), above a cushion of 70% (w/v) sorbitol (2ml). After sedimentation (12 500g $_{av}$, 50 min; 3 x 40ml swing-out rotor) a band of organelles had collected at the interface between the gradient and the 70% sorbitol cushion. This band was only slightly pigmented and contained succinate:DCPIP reductase activity, suggesting that this method was potentially suitable for the preparation of mitochondria which were not contaminated with pigmented organelles.

After several further experiments a gradient composed of sample (10-12ml) and 50%, 57.5% and 70% (w/v) sorbitol solutions (5ml of each) was found to be the best method for isolating mitochondria from the homogenate. Most of the sedimentable mitochondrial activity was found at the interface between the 57.5% and 70% sorbitol solutions (Fig. 4.1), while coloured material collected at the sample/gradient interface and in the floating lipid fraction. In some experiments mitochondrial activity was also found at the interface between the 50% and 57·5% sorbitol solutions, but since this band was most prominent in preparations of damaged mitochondria it is probably due to ruptured organelles.

Properties of Mitochondria

Mitochondria prepared by this method had a density of 1.175g/ml when subsequently sedimented in a linear sorbitol gradient. They were not coupled (respiratory activity measured using an oxygen electrode,

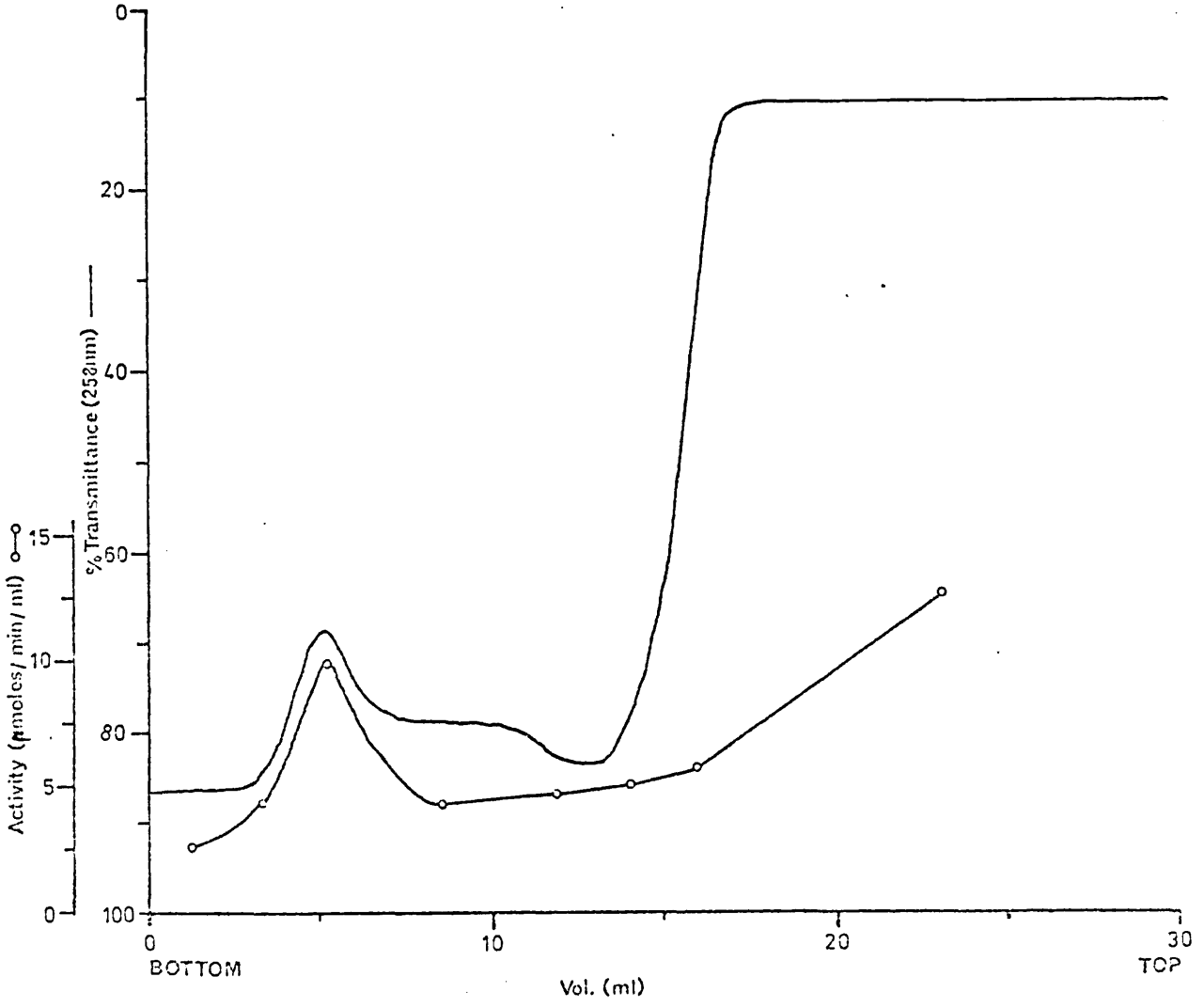


Fig. 4.1 Preparation of Mitochondria by Isopycnic Centrifugation

A 1 000g (5 min) supernatant (15ml) was loaded onto a gradient consisting of 70%, 57.5%, and 50% (w/v) buffered sorbitol (5ml of each). Sedimentation was at 12 500g_{av} for 50 min. Fractions were assayed for succinate:DCPIP reductase activity (o).

as described by Douce et al., 1972), but did possess activity of all the mitochondrial enzymes assayed (Table 2.1), as well as catalase, an enzyme characteristic of microbodies (de Duve, 1969). The presence of catalase was to be expected, since the density of fungal microbodies is very similar to that of mitochondria (Perlman and Mahler, 1970; Kobr and Vanderhaeghe, 1973).

Since microbodies are involved in lipid metabolism (p.63), it was possible that the absence of pigment from mitochondria prepared by isopycnic centrifugation was caused by their association with microbodies which could oxidize the carotenoids. In order to assess whether degradation of β-carotene could occur during centrifugation, the mitochondrial band was mixed with the pigmented band visible at the sample/gradient interface, and incubated at $0^{o}C$ in the dark for two hours. No detectable loss ($<0.5\mu g$) of β-carotene occurred during this period.

Since the outer membranes of mitochondria isolated from N.crassa apparently contain carotenoids (Neupert and Ludwig, 1971), it was important to determine whether the unpigmented mitochondria isolated in these experiments retained their outer membranes. Douce et al. (1972) suggested that the degree of intactness of mitochondrial outer membranes could be estimated by assaying the enzymes succinate: cytochrome c oxidoreductase and NADH:cytochrome c oxidoreductase (both inner membrane enzymes), using exogenous cytochrome c, which is not able to pass across intact outer membranes. By comparing the activities of these enzymes when they were assayed in the presence and absence of 0.3M sorbitol it is possible to estimate the proportion of mitochondria in the preparation possessing an intact outer membrane.

A homogenate was fractionated by discontinuous gradient centrifugation as described above, and the mitochondrial zone was diluted slowly (25-30 min) with buffer until a concentration of approximately 0.4M sorbitol was obtained. Mitochondria were then sedimented (10 000g, 15 min), and resuspended in homogenisation buffer. According to Douce et al. (1972) intact plant mitochondria are able to withstand such a dilution, although mitochondria isolated from rat liver do not survive this procedure.

A comparison of the activities of the enzymes succinate:cytochrome c oxidoreductase and NADH:cytochrome c reductase in the presence and absence of 0.3M sorbitol indicated that only 30 to 40% of the mitochondria

retained an intact outer membrane, although 50 to 60% of the activity of these enzymes was latent in mitochondria prepared by differential centrifugation. This could indicate that some mitochondria lose their outer membranes during isopycnic centrifugation, but the reduction in enzyme latency could also be due to damage during dilution, or by the high hydrostatic pressures involved in centrifugation through density gradients (Wattiaux, 1974).

Separation of Mitochondrial Membranes

In order to demonstrate conclusively that mitochondria prepared by density gradient centrifugation possess both inner and outer membranes enzymes characteristic of these membranes were assayed.

The inner membranes of mammalian mitochondria contain the enzymes of the respiratory chain, such as succinate dehydrogenase, while malate dehydrogenase is found in the matrix. The outer membranes possess rotenone-insensitive NADH:cytochrome c reductase, monoamine oxidase (E.C.1.4.3.4) and kynurenine 3-hydroxylase activities (Ashwell and Work, 1970). These locations have been confirmed for mitochondria isolated from N.crassa (Cassady and Wagner, 1971; Neupert and Ludwig, 1971) and S.cerevisiae (Bandlow, 1972; Bednarz-Prashad and Mize, 1974), although monoamine oxidase has only been detected by the latter workers.

Sedimentation on a linear sorbitol gradient (30-75% w/v), of mitochondria prepared by discontinuous gradient centrifugation (p. 125) showed that the activities of succinate:DCPIP reductase, malate dehydrogenase and antimycin A-insensitive NADH:cytochrome c reductase all coincided with a peak of protein at ρ=1.175 (Fig. 4.2). Fractions 1 to 3 represent the original sample zone, and contain material which did not enter the gradient. This probably includes mitochondria ruptured during centrifugation and dilution.

This experiment demonstrated that mitochondria prepared from Phycomyces by density gradient centrifugation possess enzymes characteristic of both inner and outer membranes of mitochondria of other organisms. In an attempt to show that they possess the same intramitochondrial location in Phycomyces experiments were conducted in which the membranes were separated by physical methods, and then assayed for the presence of these enzymes.

Preliminary experiments showed that neither the "sonication with stirring" nor the "osmotic shock and homogenisation" procedure of

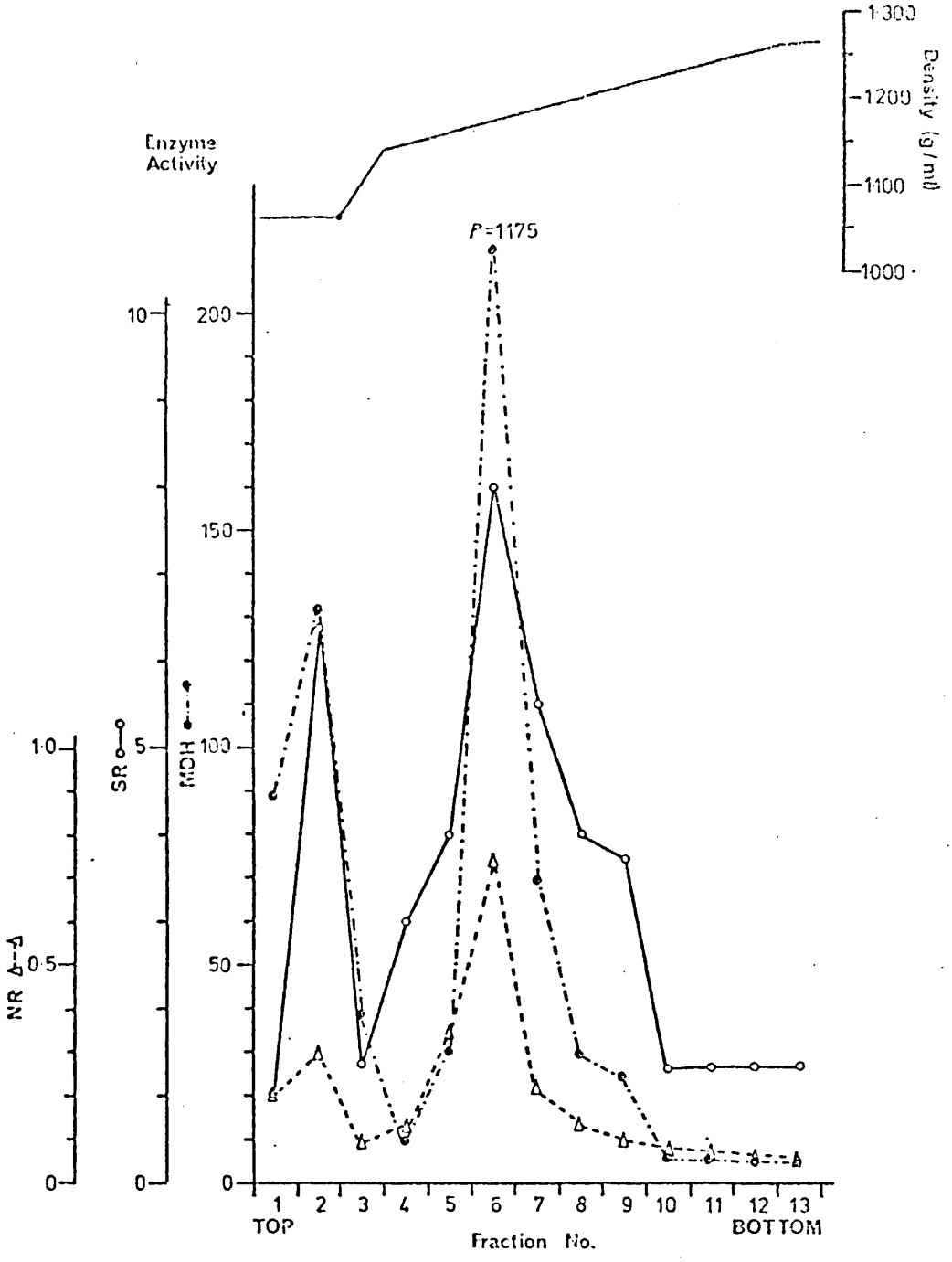


Fig. 4.2 Purification of Mitochondria by Density Gradient Centrifugation

800g (3 min) supernatant (15ml) was layered over a discontinuous sorbitol gradient (18ml) and centrifuged as described in Fig. 4.1. The 70-57.5% sorbitol interface was collected, diluted slowly with buffer to 15% (w/v) with respect to sorbitol, and sedimented at 35 000g (20 min). The pellet was resuspended in homogenisation buffer (1ml), and centrifuged through a linear gradient of 75-30% (w/v) buffered sorbitol (12ml) at 110 000g_{max} for 1.5h. Fractions (1ml) were assayed for succinate:DCPIP reductase (o;SR) malate dehydrogenase (•;MDH) and antimycin A-insensitive NADH:cytochrome c reductase (Δ ;NR). Enzyme activities are quoted as nmoles substrate converted per min per ml.

Bednarz-Prashad and Mize (1974) was sufficiently vigorous to completely separate the membranes of mitochondria prepared from Phycomyces, so the procedure devised by Cassady and Wagner (1971) for the separation of mitochondrial membranes of N.crassa was applied.

Mitochondria were prepared by density gradient centrifugation, and collected by slow dilution, followed by sedimentation at 10 000g (15 min). They were resuspended in homogenisation buffer which did not contain mannitol, and homogenised (10 strokes). The mitochondrial suspension was left at 0°C for 30 min to permit the organelles to swell. They were then rehomogenised for 2 min, and shrunk by the addition of 1.8M sucrose solution ($\frac{1}{3}$vol.; adjusted to pH 7.6 with KOH). This contained 8mM ATP and 8mM $MgCl_2$, since contraction requires an active cation/proton exchanger (Brierley and Jurkowitz, 1976). After the mitochondria had been allowed to shrink for 5 min at 0°C they were sonicated (3 x 5 sec, MSE 150W sonicator, max. amplitude), and the membranes were collected by centrifugation at 100 000g $_{av}$ (45 min). The pellet was resuspended in homogenisation buffer, layered over a linear sorbitol gradient (30-75% w/v, 13ml), and centrifuged at 110 000g $_{max}$ (90 min). Fractions were assayed for malate dehydrogenase, succinate:DCPIP reductase, antimycin A-insensitive NADH:cytochrome c reductase and kymurenine hydroxylase. A clear separation between free outer membranes (ρ=1.02 to 1.06) and inner membranes (ρ= 1.24) was obtained (Fig. 4.3). Intact mitochondria were also found at ρ=1.19. Malate dehydrogenase showed a complex distribution, most of the activity being at the bottom of the gradient, suggesting that it had been retained within the isolated inner membranes.

As a result of these experiments it was possible to conclude that the carotenoids of the C115 mutant of P.blakesleeanus are not located in the mitochondria.

Constitution of Buffer for the Preparation of Mitochondria

At the commencement of the experiments described in this chapter mycelia were homogenised in a buffer which contained 50mM Hepes-KOH, 5mM EDTA, 0.4M mannitol and 0.2% (w/v) BSA. If a lower concentration of BSA was used the activity of succinate:DCPIP reductase declined quickly upon storage of the mitochondria, while flotation of a 24h-old "mitochondrial" pellet through a linear sucrose gradient (65-30% w/v, 12ml) showed no peak of light absorption in the expected area if the mitochondria had been prepared and stored in the absence of BSA (Fig. 4.4).

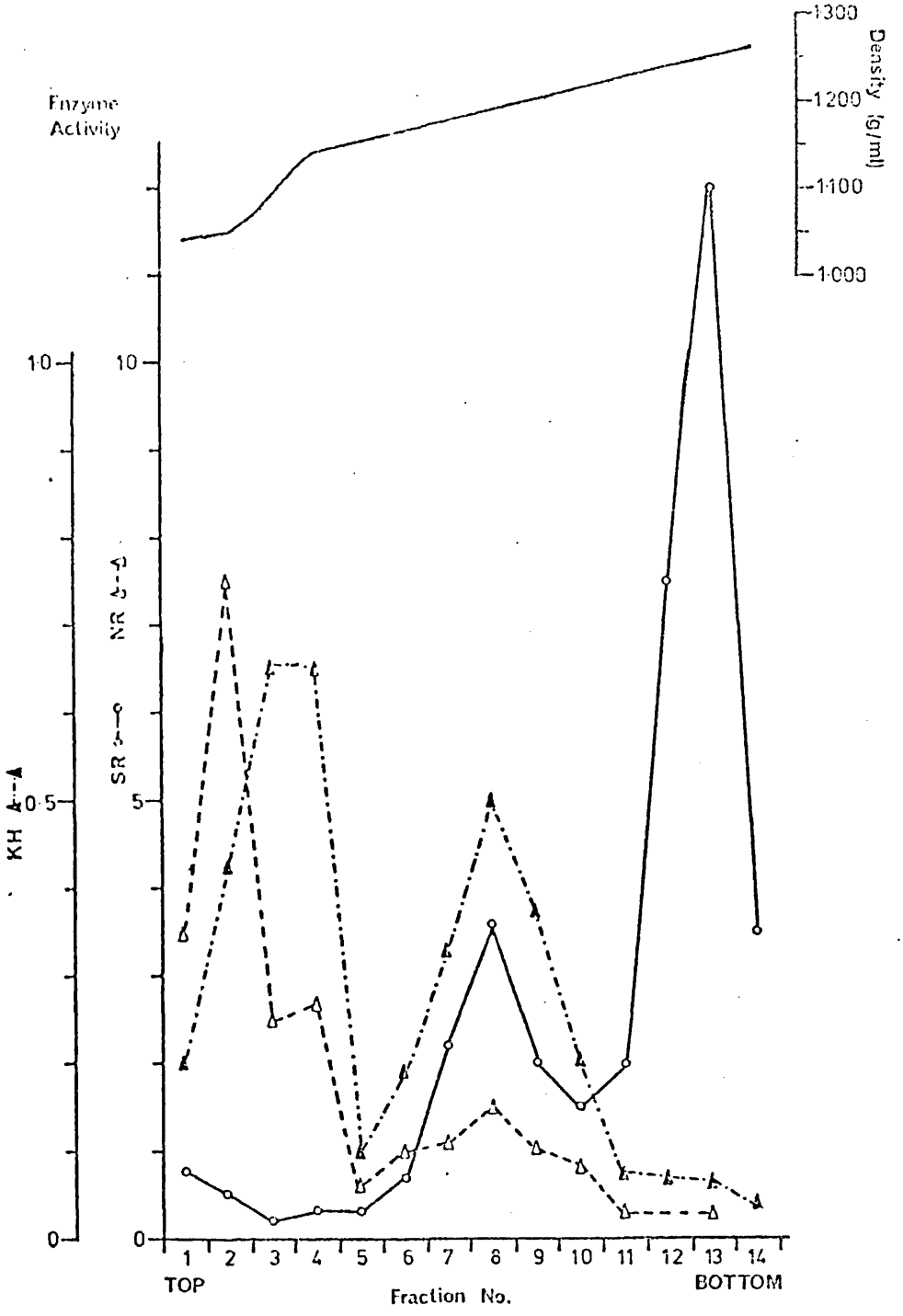


Fig. 4.3 Separation of Outer and Inner Mitochondrial Membranes

Mitochondria were broken by methods described in the text, and the membranes were collected by centrifugation at 100 000g (45 min). The pellet was resuspended in homogenisation buffer (1ml), and centrifuged through a linear gradient of 25-30% (w/v) buffered sorbitol (13ml) at 110 000g_{max} for 1.5h. Fractions (1ml) were assayed for succinate:DCPIP reductase (o;SR), antimycin A-insensitive NADH:cytochrome c reductase (Δ;NR) and kynurenine 3-hydroxylase (▲;KH). Enzyme activities are quoted as nmoles substrate converted per min per ml.

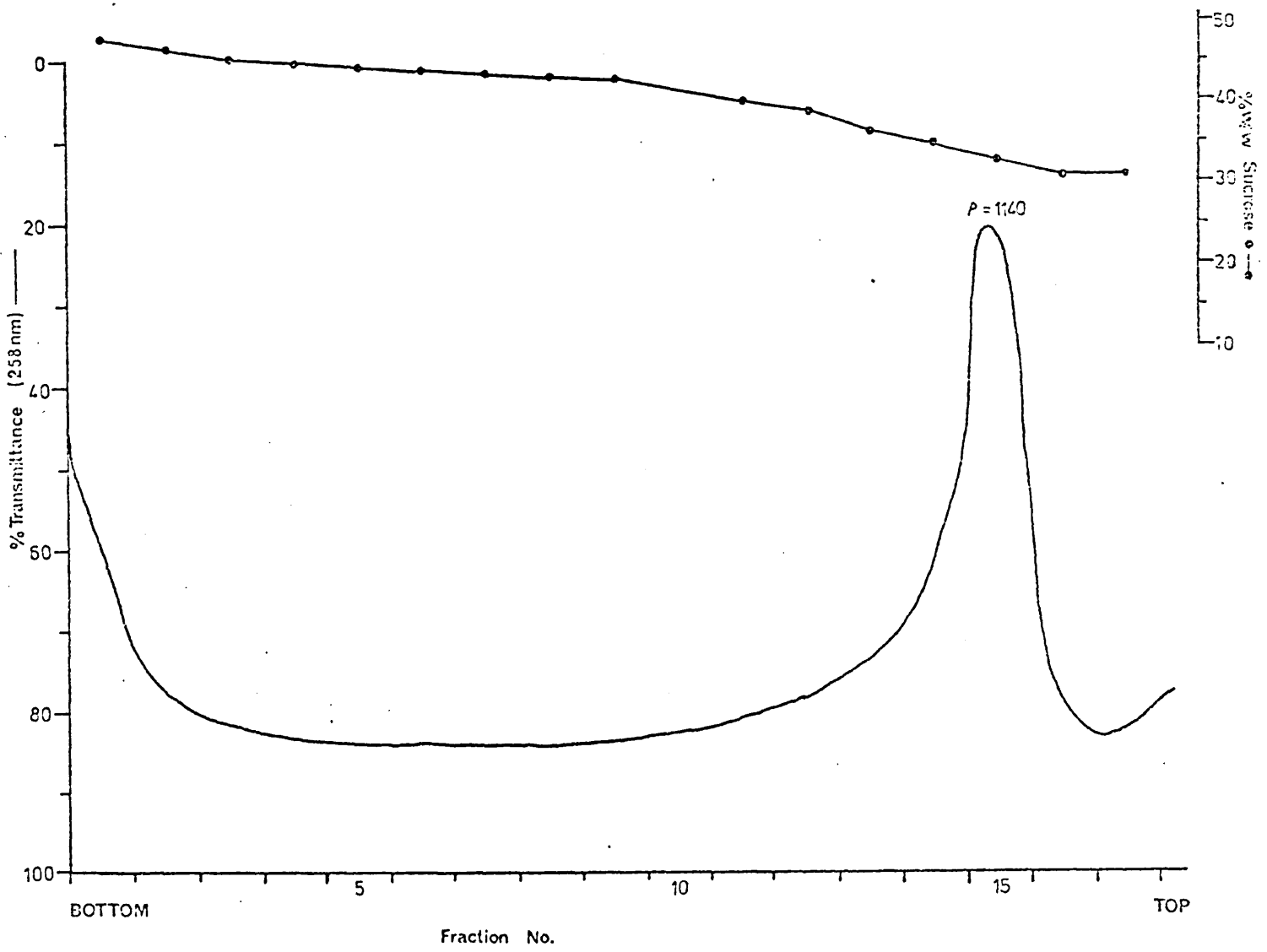


Fig. 4.4 Density Gradient Centrifugation of Mitochondria Prepared in Buffer Without Bovine Serum Albumin

Mycelia were homogenised in 50mM tris-HCl, pH 7.6, containing 5mM EDTA and 0.6M mannitol. Mitochondria were sedimented at 10 000g (15 min) and stored for 24h in homogenisation buffer. They were collected by centrifugation and resuspended in 70% (w/v) sucrose containing 1mM EDTA (1ml). This was floated through a linear sucrose gradient (65-30% w/v, 12ml) at 110 000g_{max} for 3h.

However, during the course of this investigation the buffer was modified, (see pp. 134 & 140) so that it also contained 1mM $MgCl_2$ and 10mM KCl. Under these conditions BSA was no longer required to ensure mitochondrial stability.

In one experiment mycelia were homogenised in Hepes/mannitol buffer alone and also in Hepes/mannitol containing either 5mM EDTA, 5mM EDTA plus BSA (0.2% w/v), or 10mM KCl plus 2mM $MgCl_2$. Mitochondrial fractions were prepared by sedimentation, resuspended and stored for 24h in the appropriate buffer. They were then analysed by density gradient centrifugation. Very little material (A_{258}) was found at ρ=1.17-1.19 in the case of the pellet suspended in buffer plus 5mM EDTA, while considerably larger amounts were present in each of the other gradients. This suggests that BSA is able to maintain the structure of mitochondria which have been destabilized by the effects of EDTA. This destabilizing effect can also be counteracted by the presence of Mg^{2+} and K^+ ions.

Carafoli (1975) has discussed the importance of Ca^{2+} ions in stabilizing mitochondrial membranes, and it is also known that Ca^{2+} may be replaced by Mg^{2+}, or by monovalent cations such as Rb^+ and K^+ (Scarpa and Azzi, 1968; Earnshaw, 1975). Hence EDTA probably exerts its destabilizing effect by removing Ca^{2+} from the membranes.

The mode of action by which BSA overcomes this effect of EDTA is unclear, but its action on another membrane, the plasmalemma of yeast spheroplasts, has been extensively studied.

Indge (1968) noted that yeast spheroplasts could be lysed by chelating agents such as EDTA and citrate, but that K^+ and Na^+ opposed the destabilizing effect of these anions, indicating the presence of cation binding sites which were essential for membrane stability. Schlenk and Dainko (1965) and Ottolenghi (1967) have both observed that basic proteins (e.g. ribonuclease, BSA, lysozyme, and cytochrome c) will bind to yeast plasma membranes under conditions of low ionic strength, and that this binding is inhibited by the presence of cations. This indicates that basic proteins, such as BSA, are able to bind to anionic sites on the membrane if these sites are depleted of their normal cations. In yeast cells this binding causes lysis of the cell (Schlenk and Dainko, 1965; Ottolenghi, 1967), but the results in this thesis indicate that an analogous binding of BSA to anionic sites in mitochondrial membranes may have a stabilizing effect.

Since BSA has been observed to affect the incorporation of precursors into unsaponifiable lipids in a cell-free carotenogenic system (Bramley, 1973) later experiments were conducted in buffers which did not contain either EDTA or BSA.

Preparation of Nuclei

Nuclear membranes are particularly sensitive to destabilization by cation removal (Allfrey, 1959), and differential centrifugation of mycelia, homogenised in a buffer containing 5mM EDTA, showed that under these conditions very little DNA was in a 1 500g (10 min) pellet, while 60% of it was sedimented at 100 000g (60 min; Bramley, P.M. and Waters,S. unpublished). Dusenbery (1975) prepared nuclei from sporangiophores of P.blakesleeanus homogenized in 20mM Hepes, pH 7.5, containing 0.5M sucrose, 25mM ascorbic acid, 0.5mM EDTA, 15mM $MgCl_2$, 40mM KCl, and 1% (w/v) BSA. In these experiments, however, the presence of 5mM $MgCl_2$ in 50mM Hepes caused flocculation of lipid globules, so a buffer containing 50mM Hepes, pH 7.4, 2mM $MgCl_2$, 10mM KCl, and 0.4M mannitol was employed in these studies.

Differential centrifugation of mycelia homogenized in this buffer showed that a 1 500g (10 min) pellet was enriched with DNA (Fig. 4.5), indicating that this buffer had maintained the integrity of some nuclei. The large amount of DNA sedimented at 25 000g (20 min) may represent molecules released from damaged cells which have aggregated due to the high ionic strength of the buffer (A. Mackenzie, personal communication).

The large amount of both DNA and protein in the 800g pellet was due to the presence of cell debris. Several methods are available for the separation of cell debris from homogenates, but these also remove a large number of nuclei. A commonly employed technique is the filtration of a homogenate through "Miracloth" (Chicopee Mills Inc., New York), but when this was tried very low yields of DNA were found in the low-speed pellets. An alternative method is to pass the homogenate through "Nybolt Normal 6" nylon mesh (Swiss Silk Bolting Cloth, Zurich). This has a mesh size of 212μm, and it is believed to retain some nuclei from higher plant tissue but to permit the majority to pass through (W.J. Owen, personal communication). However, nearly twice as much sedimentable DNA passed through two layers of muslin as through the nylon mesh, so in the experiments described below the homogenate was subjected to filtration through two layers of muslin

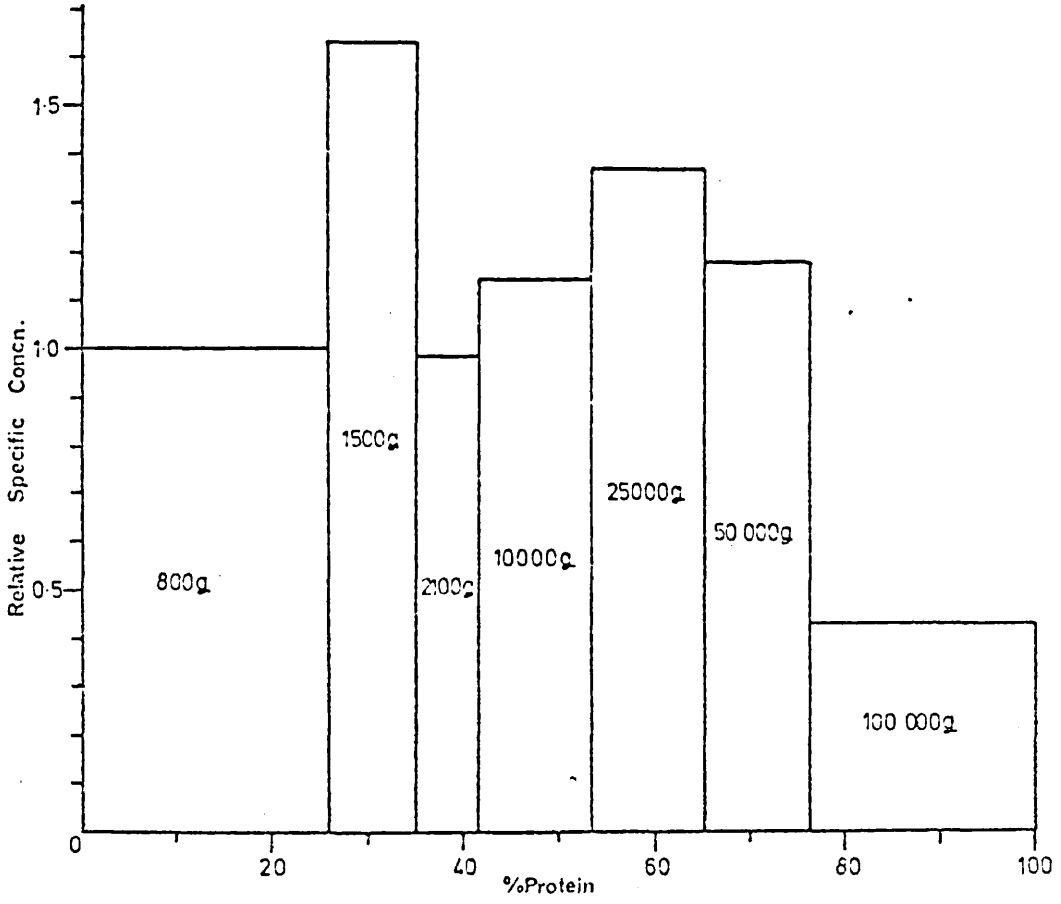


Fig. 4.5 Sedimentation of DNA-Containing Structures

Mycelia were homogenised in 50mM Hepes, pH 7.4, containing 2mM $MgCl_2$, 10mM KCl and 0.4M mannitol. The homogenate was centrifuged successively at 800g (5 min), 1 000g (10 min), 2 100g (10 min), 10 000g (15 min), 25 000g (20 min), 50 $000g_{av}$ (30 min) and 100 $000g_{av}$ (60 min). Pellets were assayed for DNA by the method of Ogur and Rosen (1950). The 100 000g supernatant contained 38% of the recovered DNA. The relative specific concn. of DNA is the percentage of the total DNA found in one fraction divided by the percentage of the total protein present in that fraction

to remove whole cells and cell walls.

When a 1 500$\underline{g}$ (10 min) pellet was resuspended and sedimented on a linear sucrose gradient (35 to 75% w/v; 100 000$\underline{g}_{max}$, 3h) a DNA-containing peak was present at ρ = 1.20-1.23. This peak was coloured, but the presence of pigment was attributed to the agglutination problems discussed above.

A discontinuous gradient, used for the purification of a nuclear pellet prepared from M.rouxii (Young and Whiteley, 1975), was modified slightly so that it consisted of a cushion of sucrose (75% w/v) with 75% and 68% (w/v) sorbitol steps above it. In preliminary experiments 5ml of each of these solutions were overlayed with homogenate (10-15ml) and centrifuged at 27 000$\underline{g}_{max}$ (2h; 3 x 40ml swing-out rotor). Under these conditions nuclei (assayed by u.v.-absorption and DNA) were present at the sucrose/sorbitol interface, while mitochondria (assayed for succinate:DCPIP reductase activity) had only just entered the 68% sorbitol step. No colour was associated with the nuclear band.

Unfortunately a large amount of DNA remained in the sample zone, and much of this could be collected by sedimentation at 2 000$\underline{g}$, indicating that the centrifugal force acting on the sample was too low to move many of the nuclei through the viscous sorbitol solutions.

In order to overcome this problem the gradients were prepared in larger tubes (70ml) and overlayed with sorbitol (5% w/v, 30ml). Nuclei were sedimented at 100 000$\underline{g}_{max}$ (equivalent to 85 000$\underline{g}$ at the sample zone) for 2h (3 x 70ml rotor). Under these conditions 16% of the DNA of the homogenate was found at the sucrose/sorbitol interface, while most (85%) of the remaining DNA was in the sample zone and the 5% sorbitol overlay, indicating that this was material solubilized by homogenisation (Fig. 4.6). The DNA:RNA ratio of the purified nuclei was 7.6, indicating that it was relatively free from contamination by E.R. fragments (Reid, 1969), although a low activity of NADPH:cytochrome c reductase was detected in this zone. Succinate:DCPIP reductase was confined mainly to the sample zone and the upper sorbitol layer. A band of coloured material was visible at the 75%/68% sorbitol interface. This had a low DNA content, and examination under a phase-contrast microscope showed that it contained mainly cell wall fragments, many of which were derived from the growing tips of the hyphae.

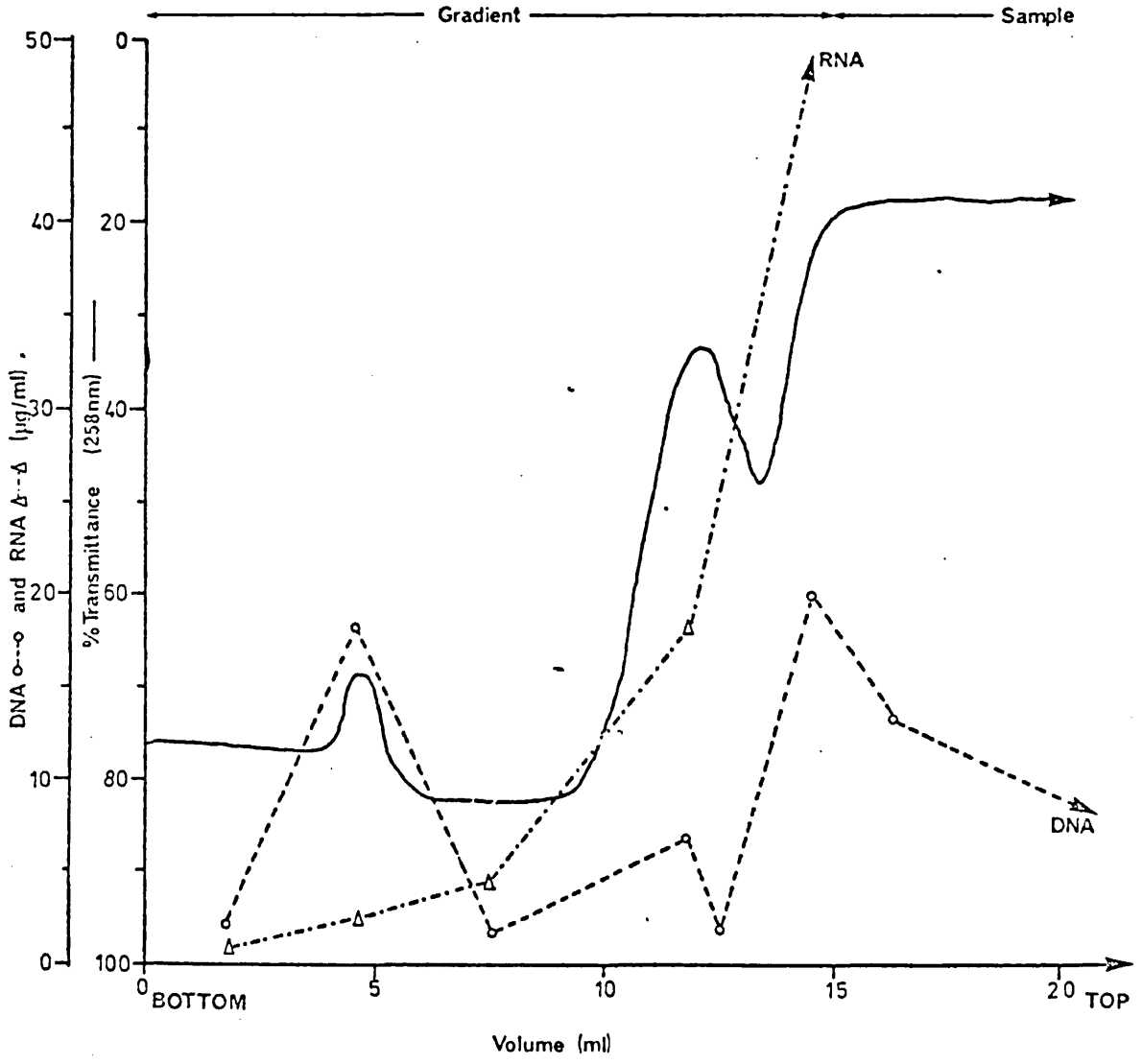


Fig. 4.6 Preparation of Nuclei by Isopycnic Centrifugation

Mycelia were homogenised in 50mM Hepes, pH 7.4, containing 2mM $MgCl_2$, 10mM KCl, and 0.4M mannitol. 800g (3 min) supernatant (15ml) was layered over 5ml each of 68% and 75% (w/v) sorbitol and 75% (w/v) sucrose, and centrifuged at 100 000g_{max} (2h). DNA (o) and RNA (Δ) were estimated by the method of Ogur and Rosen (1950)

Properties of Isolated Nuclei

Nuclei prepared by the method described above were sedimented, and their microscopic appearance examined after staining as described by Duffus (1969). The pellet was resuspended in aq. acetic acid (1% v/v) and fixed onto a slide. This was washed with water and stained overnight in Giemsa R66 (4% w/v in 50mM tris-HCl, pH 7.6). Excess stain was washed off, and the slide was mounted in dilute Giemsa and viewed under phase contrast. Each nucleus appeared as a pale purple matrix, containing an intensely stained nucleolus. Membranous contamination was not observed.

In order to determine the density of the nuclei they were sedimented on a linear sucrose gradient (30-70% w/v; 100 000g_{max}, 3h). Nuclei, with a DNA:RNA ratio of 15:1, were found at a density of 1.22g/ml (Fig. 4.7). A similar density (1.23g/ml) was found when the nuclear band from a discontinuous gradient was mixed with Metrizamide (to increase the density of the nuclear fraction) and floated through a sucrose gradient.

These experiments have shown that nuclei may be prepared from P.blakesleeanus in low yield, but apparently free of membrane contaminants. No pigment is associated with these organelles except when they are prepared by differential centrifugation.

Separation of other Organelles

As discussed earlier (p. 117) the marker enzymes usually considered to be characteristic of E.R. fragments were absent from mycelia of P.blakesleeanus grown under the conditions described in the Methods section. Similar problems have been reported with yeast cells (Colson et al., 1974; Nurminen et al., 1976), where NADPH:cytochrome c reductase has been employed as a microsomal marker (Schatz and Tzagoloff, 1964). However, when this enzyme was assayed in fractions from homogenates of P.blakesleeanus there was interference oxidation of cytochrome c by an unknown process. Fortunately also possible to assay the enzyme using DCPIP as an electron (Williams and Kamin, 1962), and under these conditions were experienced, provided that freshly-prepared buffer and solutions were used.

For the isolation of ribosomes and polysomes high concentrations of both Mg^{2+} and K^{+} ions are required, together with some EDTA

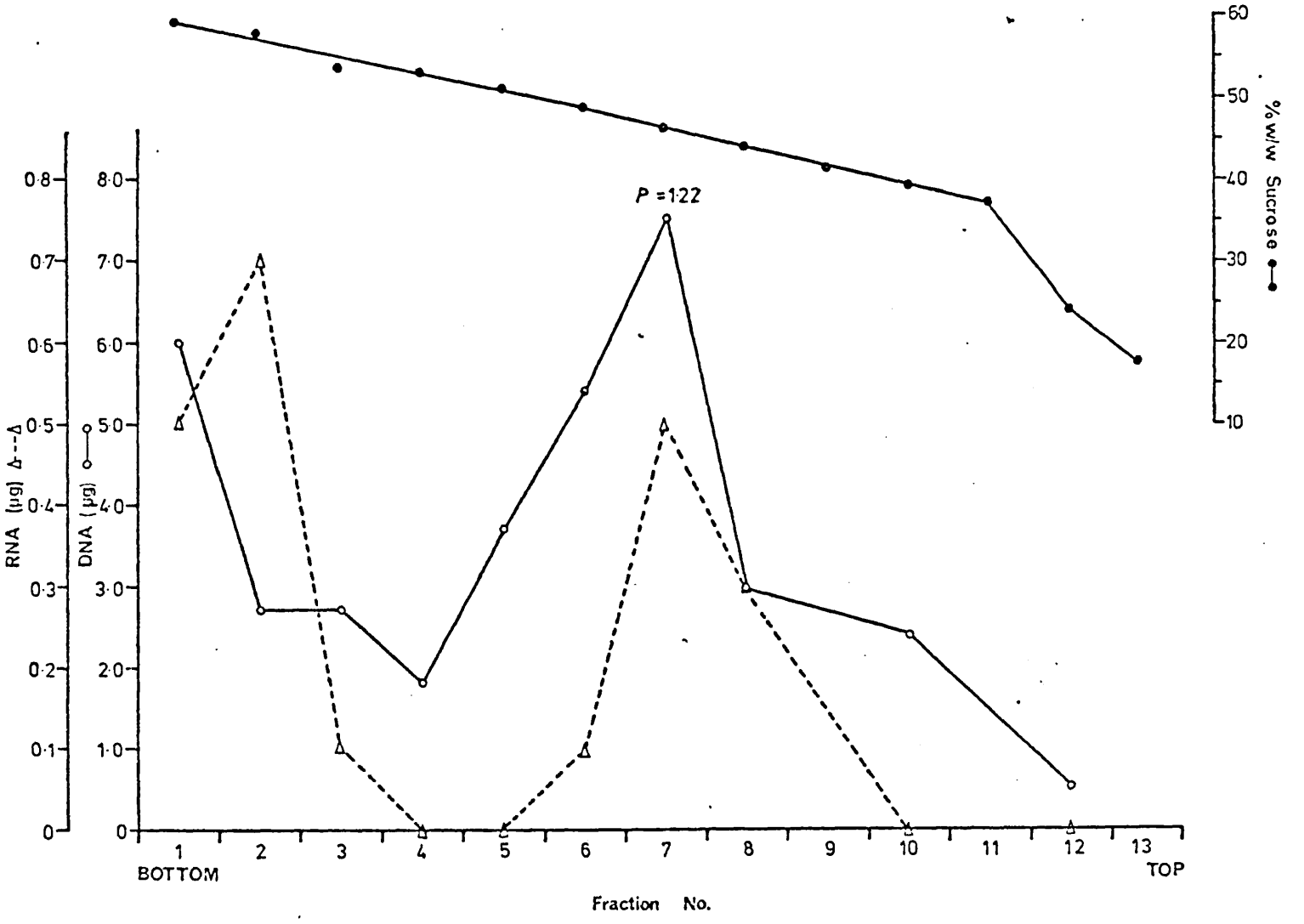


Fig. 4.7 Purification of Nuclei by Linear Gradient Centrifugation

Nuclei were prepared as described in Fig. 4.6. They were resuspended in homogenisation buffer (1ml) and sedimented through a linear sucrose gradient (30-70% w/v, 13ml; 110.000g_{max}, 3h). DNA and RNA were estimated by the method of Ogur and Rosen (1950)

(Arlinghaus and Ascione, 1972). In view of the flocculation problems experienced with 5mM $MgCl_2$ (p. 134) most of these experiments were carried out using a buffer containing 50mM Hepes, 0.4M mannitol, 10mM KCl, and 1 or 2mM $MgCl_2$. In some experiments 5mM EDTA was also included.

A microsomal fraction was prepared by centrifuging a 15 000$\underline{g}$ (15 min) supernatant at 40 000$\underline{g}$ for 30 min. The pellet was layered over a linear sorbitol gradient (25-65% w/v), and centrifuged at 100 000$\underline{g}_{max}$ for 3h (Fig. 4.8). The u.v.-absorbing material near the top of the gradient showed an absorption spectrum with a λ_{max} of 262nm, and hence corresponds to either polysomes or ribosomal aggregates. Since this pattern was not obtained in the absence of EDTA, which inhibits non-enzymic RNA degradation (Arlinghaus and Ascione, 1972), these peaks are probably genuine polysomes. However, the material at ρ=1.185 was pigmented, and possessed succinate:DCPIP reductase activity, suggesting that the microsomal pellet was contaminated with mitochondria, which were in turn associated with pigmented organelles.

In an attempt to overcome this contamination problem 6ml of a mitochondria-free supernatant (the sample zone remaining after separation of mitochondria by isopycnic centrifugation, p. 125) was layered over a discontinuous gradient comprising 70% and 45% (w/v) sorbitol (4ml of each). This corresponds to the gradient employed by Brooker and Russell (1975) for the separation of "light" and "heavy" microsomes from higher plants. The gradient was centrifuged at 110 000$\underline{g}_{max}$ for 5 to 21h (6 x 16.5ml swing-out rotor). A slight separation was obtained between ribosomes (estimated spectrophotometrically from the ratio of A_{260} to A_{250}; median density 1.245g/ml) and microsomal membranes (NADPH:cytochrome c reductase activity; median density 1.200g/ml), but the fractions overlapped considerably. The pigmented zone was contaminated by succinate:DCPIP reductase, which had presumably been solubilized from mitochondria during homogenization.

Attempts to improve the separation by modifying the discontinuous gradient were unsuccessful due to diffusion of the gradient during the long periods of centrifugation necessitated by the relatively lower maximum speed of the rotor compared to that used by Brooker and Russell (1975; $\underline{g}_{max}$ = 284,000).

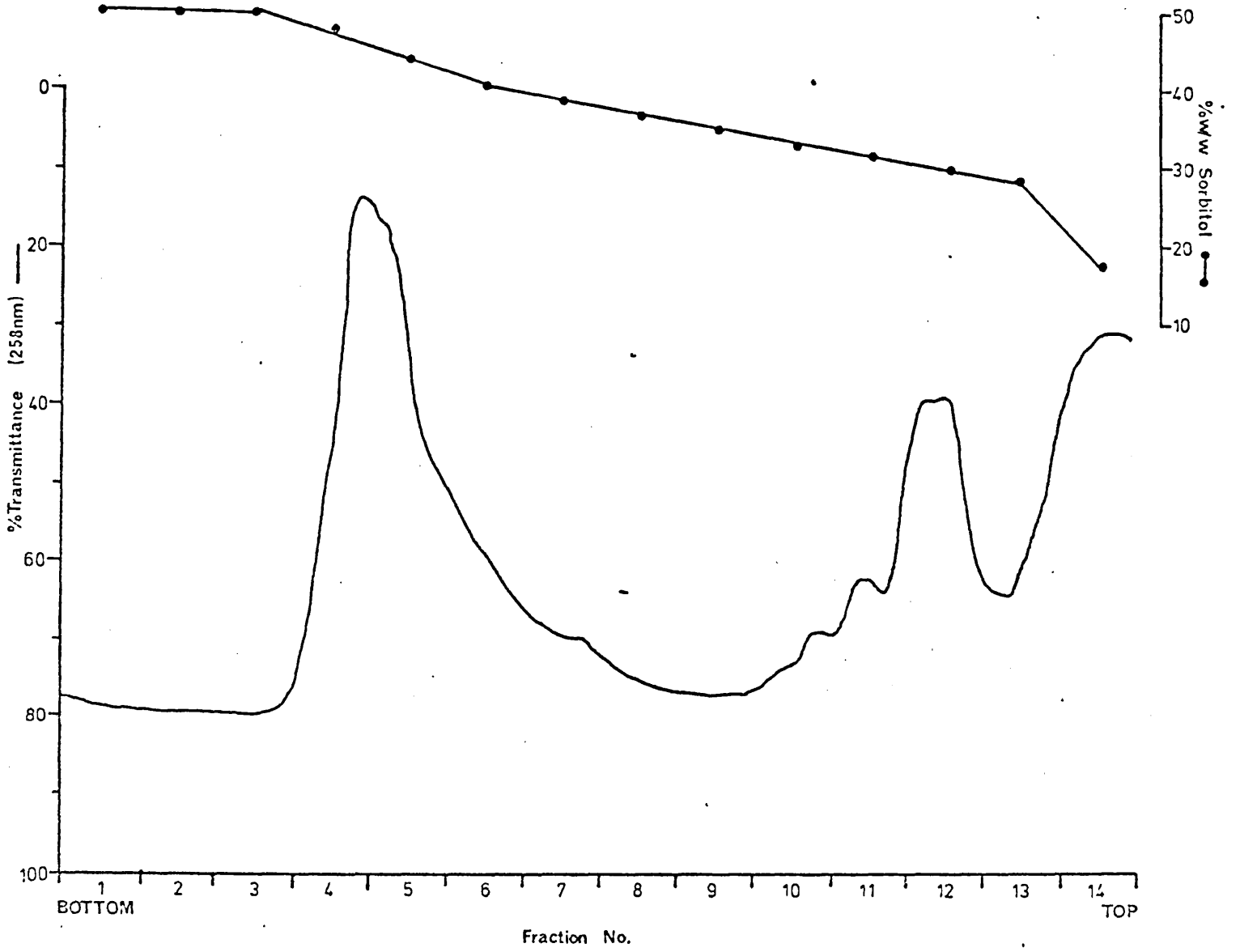


Fig. 4.8 Separation of Organelles from a Microsomal Pellet

Mycelia were homogenised in 50mM Hepes, pH 7.4, containing 5mM EDTA, 10mM KCl, 1mM $MgCl_2$, and 0.4M mannitol. A 15 000g (15 min) supernatant was centrifuged at 40 000g (30 min), and the pellet was resuspended in homogenisation buffer (1ml) and sedimented on a linear sorbitol gradient (25-65% w/v, 13ml; 110 000g_{max}, 3h)

Because isopycnic separation of the various components of the post-mitochondrial supernatant was not possible, a separation based on sedimentation rates was devised. The rate of sedimentation of a particle is proportional to the product of its size and the difference between the density of the particle and that of the medium. Although most organelles have densities between 1.10 and 1.20g/ml their sizes may vary by a factor of 100 or more, so that rate zonal techniques permit greater differentiation between particles.

Early experiments (e.g. Fig. 4.4) had shown that the coloured organelles floated to their isopycnic point very quickly. The post-mitochondrial supernatant (from a discontinuous gradient such as that described on p. 125) was mixed with a concentrated solution of sorbitol to produce a final concentration of 70% (w/v). This sample (5ml) was layered below a linear sorbitol gradient (25-60% w/v, 2ml), overlayed with 5% (w/v)sorbitol solution (0.5ml), and centrifuged at 110 000$\underline{g}_{max}$ (1.5h; 6 x 16.5ml swing-out rotor). A complete separation between pigmented material and other microsomal and soluble constituents was effected (Fig. 4.9).

Later experiments showed that this separation could also be performed in a larger rotor (3 x 70ml swing-out), in which up to 15ml of sample was layered below 16ml of gradient (20-60% w/v) and 5 to 10ml of overlay. Microsomal and 100 000$\underline{g}$ supernatant fractions could then be prepared by diluting the original sample zone with buffer (6 vol.) and centrifuging at 100 000$\underline{g}_{av}$ for 1h. It was also possible to prepare the pigmented fraction directly by homogenising mycelia in sorbitol (70% w/v) and layering this homogenate below the same gradient.

Sedimentation of these pigmented organelles on a linear sorbitol gradient (15-35% w/v; 110 000$\underline{g}_{max}$, 1.5h) indicated that they possess a density of 1.105-1.115g/ml.

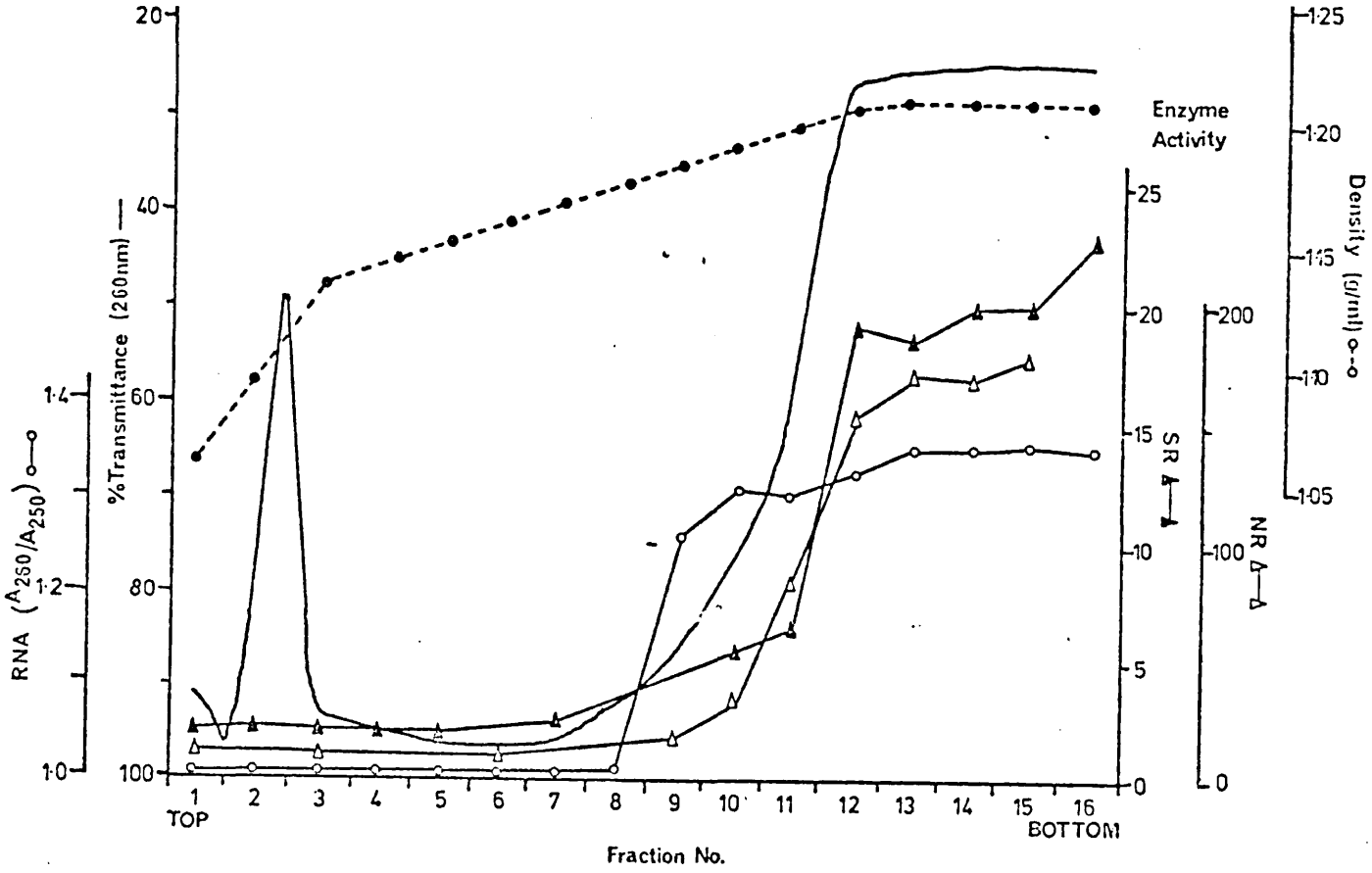


Fig. 4.9 Isolation of a Pigmented Particulate Fraction

Post-mitochondrial supernatant (5ml) was floated through a linear sorbitol gradient (25-60% w/v, 12ml) overlayed with 5% (w/v) sorbitol (1ml). Centrifugation was for 1.5h at 110 000g_{max}. Fractions were assayed for RNA (o, A_{260}/A_{250}), succinate:DCPIP reductase (▲, SR) and NADPH:cytochrome c reductase (△, NR). Enzyme activities are nmoles substrate hydrolysed/min/ml. β-Carotene was located in fractions 1 and 2 only.

B. LIPID DISTRIBUTION IN PURIFIED SUBCELLULAR FRACTIONS

Methods

The techniques described in section A permit the preparation of subcellular fractions which are not contaminated by pigmented organelles, and which were judged to be suitable for lipid analysis. Since only very small amounts of nuclear material could be prepared, and since the nuclei are not pigmented they were not studied further, and cell fractionation was carried out on an 800g (3 min) supernatant.

Mycelia (5.5g) were homogenised in 50mM Hepes, pH 7.4, containing 2mM $MgCl_2$, 10mM KCl, and 0.5M mannitol. Mitochondrial, microsomal, pigmented, and 100 000g supernatant fractions were prepared by the density-gradient centrifugation procedures described in Table 4.1. Fractions were assayed for marker enzymes, protein, and RNA. Lipids were extracted with $CHCl_3$/MeOH as described in the Methods section. Phospholipids were assayed by "Method 1" (p. 92) while unsaponifiable lipids were chromatographed on columns of grade III alumina. β-Carotene was estimated spectrophotometrically, and sterols were determined using the Moore and Baumann procedure (p. 95).

Results and Conclusions

The significant amounts of all the marker enzymes in the 100 000g supernatant (Table 4.2) indicate that organelles were broken during homogenization. The amount of soluble succinate:DCPIP reductase activity found after these procedures (78%) is greater than that present in the supernatant fraction prepared by differential centrifugation (44%), indicating that the high hydrostatic pressures caused by the sedimentation of organelles through density gradients have disrupted many mitochondria (Wattiaux, 1974). However, the distribution of sedimentable enzyme activities indicates that there are sufficient intact organelles for the determination of their lipid contents. The lipid droplet fraction was contaminated by small quantities (0 to 2%) of marker enzymes, but these were due to incomplete separation of the globules from the supernatant (see p. 154). The sedimentable, pigmented fraction represents only 13% of the total homogenate protein, and contains only approximately 1% of each marker enzyme assayed. The high specific activity of glucose 6-phosphate dehydrogenase in the microsomal fraction is surprising, since differential

TABLE 4.1 SUMMARY OF METHODS USED FOR THE PREPARATION OF SUBCELLULAR FRACTIONS

Centrifugation Procedure			Zone	Processing	Fraction
(1)			Floating Fraction	Collected on a circular metal spatula. Resuspended in buffer.	LIPID DROPLETS
	15ml	Homogenate	Sample Zone	Stock sorbitol soln. added to concn. of approx. 70% (w/v).	Sample for gradient (2)
	5ml	50%(w/v) sorbitol			
	5ml	57.5% " "	57.5/70% Interface	Diluted slowly to approx. 8%(w/w). Pelleted at 10 000g (15 min).	MITOCHONDRIA
	5ml	70% " "			
(2)	5ml	5% (w/v) sorbitol	5/20% Interface	Pelleted at 40 000g (20 min).	PIGMENTED FRACTION
	16ml	20% " "			
		↓			
		60% " "			
	15ml	Sample	Sample Zone	Diluted to approx. 10%(w/v).	MICROSOMES
				Centrifuged at 100 000g_{av} (1h).	SUPERNATANT

12 500g_{max}	1h	
60 000g_{max}	1h	

TABLE 4.2. DISTRIBUTION OF MARKER ENZYMES IN FRACTIONS OBTAINED BY DENSITY GRADIENT CENTRIFUGATION

Fractions were isolated as described in Table 4.1. All figures are from 3(*), 4(**) or 5(+) separate experiments, and are quoted ± S.E.M. Units of enzyme activity are nmoles substrate converted per min, and specific activities (S.A.) are units/mg protein. RNA is quoted as μg/mg protein. S.U.(%) is the percentage of sedimentable enzyme activity, S.Q.(%) the percentage of sedimentable RNA. H is the homogenate (800g supernatant), M the mitochondrial fraction, P the microsomal fraction, V the pigmented fraction, S the 100 000g supernatant, and G the lipid droplets. N.A. means not assayed.

Fraction	Protein (*)		RNA(+)			Succinate dehydrogenase (**)			Catalase (+)			NADPH:cytochrome c reductase (*)			Glucose 6-phosphate dehydrogenase (+)		
	Total (μg)	Total (%)	Total (μg)	Quantity	S.Q. (%)	Units	S.A.	S.U. (%)	Units	S.A.	S.U. (%)	Units	S.A.	S.U. (%)	Units	S.A.	Total (%)
H	83.7 ± 8.2		10675 ± 870	127.8		1040 ± 75	12.4		130 ± 25	1.5		9310 ± 600	110.3		2350 ± 245	28.2	
M	4.05 ± 0.23	4.9	190 ± 20	46.9	8.9	110 ± 15	29.6	60.0	18 ± 2	4.9	62.5	120 ± 35	29.3	12.5	80 ± 30	19.5	3.4
P	8.57 ± 1.37	10.3	1890 ± 100	220.5	88.5	70 ± 5	8.2	35.0	8 ± 4	1.2	31.3	780 ± 75	90.7	81.3	590 ± 15	68.8	25.1
V	1.05 ± 0.21	1.3	55 ± 10	52.4	2.6	10 ± 5	9.5	5.0	2 ± 1	1.9	6.8	60 ± 45	54.5	6.3	0	0	0
S	63.6 ± 0.5	76.3	4640 ±1180	73.0		760 ±300	11.9		105 ± 80	1.7		3930 ± 50	61.8		1900 ± 335	29.9	80.9
G	0.47 ± 0.10	0.6	N.A.			20 ± 10	21.3		0	0	0	70 ± 0	149.0		20 ± 10	1.8	0.9
Recovery (%)	93		63			93			102			53			110		

centrifugation has shown that it is a supernatant enzyme (Table 3.1). Since the recovery of this enzyme was between 97 and 119% in each of the four experiments averaged in Table 4.2 this anomalous result is unlikely to be due to the presence of an activator or inhibitor in either the microsomal or supernatant fractions. However, Dallner (1974) has observed that during cell fractionation significant amounts of certain cytoplasmic proteins become attached to microsomal membranes, and this may explain the presence of glucose 6-phosphate dehydrogenase in the particulate fraction.

β-Carotene was found only in the light particulate fraction and in the lipid globules (Table 4.3). The low recovery of β-carotene is due mainly to incomplete recovery of lipid droplets, which adhere to the walls of plastic centrifuge tubes, and also to glassware. The loss does not appear to be due to oxidation, since when a homogenate was stored on ice in the dark, no detectable loss ($<5\%$) of β-carotene occurred in 12h.

In addition to β-carotene, the lipid droplet fraction also contained large amounts of sterols and a small percentage of phospholipids (Table 4.3). Sterols were distributed throughout the membranous structures of the mycelia with a constant sterol:phospholipid molar ratio of 0.10. This value compares with cholesterol:phospholipid molar ratios of 0.11 to 0.33 for mitochondrial and microsomal membranes of rat liver (Demel and de Kruyff, 1976), and ergosterol:phospholipid ratios of 0.05 to 0.35 for mitochondrial membranes isolated from Ophiobolus graminis, Aspergillus niger and N.crassa (Olsen, 1973; Hallermayer and Neupert, 1974).

Previous microscopic studies have indicated that, in the Mucorales, mycelial carotenes are located in lipid globules (Schopfer, 1928; Prevost, 1968), but cell fractionation experiments have suggested a partially particulate location (Cederberg and Neujahr, 1970; Keyhani et al., 1972; Herber, 1974). The preparation of a β-carotene-containing particulate fraction from P.blakesleeanus in a relatively pure form (as judged by the absence of enzymes specific for other organelles) confirms the dual location of carotene in Mucorales mycelia, and permits further experiments designed to characterise these pigmented organelles.

TABLE 4.3 β-CAROTENE, PHOSPHOLIPID AND STEROL COMPOSITION OF FRACTIONS OBTAINED BY DENSITY GRADIENT CENTRIFUGATION

Subcellular fractions were isolated and their lipid content estimated as described in the text. Lipid weights are μg ± S.E.M. from 5 experiments. Abbreviations are the same as those for Table 4.2

Fraction	Lipid Content (μg)					
	β-Carotene		Phospholipids		Sterols	
	Total	Total (%)	Total	Total (%)	Total	Total (%)
H	280 ± 45		3765 ± 560		1725 ± 305	
M	0.1	0	345 ± 45	9.2	60 ± 15	3.5
P	0.1	0	630 ± 85	16.8	110 ± 25	6.4
V	4.0 ± 0.6	1.4	165 ± 30	4.4	30 ± 5	1.7
S	0.1	0	3665 ±1330	97.6	1100 ± 220	63.8
G	142 ± 16	50.7	220 ± 45	5.9	425 ± 100	24.6
Recovery (%)	52		133		100	

CHARACTERIZATION OF β-CAROTENE-CONTAINING ORGANELLES

A. ENZYMES OF PIGMENTED FRACTIONS

Particulate Fraction

The density of the pigmented particulate fraction (1.105 to 1.115g/ml; p. 142) does not correspond to that observed for any organelles present in mammalian cells (Anderson et al., 1966), but it is similar to the density of vacuoles of S.cerevisiae (Matile and Wiemken, 1974) and the lighter "spherosomes" present in maize seedlings and wheat seeds (Semadeni, 1967; Jelsema et al., 1975).

Vacuoles isolated from fungi have been found to contain hydrolytic enzymes (see p.66). The vacuoles of both Coprinus lagopus and N.crassa contain proteinases, glycosidases, phosphatases and ribonuclease (Iten and Matile, 1970; Matile, 1971). Consequently p-nitrophenylphosphatase was assayed, at pH 4.7 and 8.5, in the pigmented fraction. A very small amount of acid phosphatase was detected (10nmoles substrate hydrolysed/min/mg protein at pH 4.7), but the specific activity of alkaline phosphatase was much higher (400nmoles/min/mg).

In order to confirm the location of alkaline phosphatase in this fraction mycelia were homogenised in buffered sorbitol (70% w/v) and floated through a linear gradient as described in Table 4.1. Glucose 6-phosphate dehydrogenase and NADPH:cytochrome c reductase remained predominantly in the original sample zone, but a peak of alkaline phosphatase activity coincided with the band of pigmented material (Fig. 5.1).

The distribution of a number of hydrolytic enzymes in subcellular fractions was also determined. Neutral proteinase is present in the vacuoles of Coprinus lagopus, N.crassa and S.cerevisiae (Iten and Matile, 1970; Matile, 1971; Wiemken, 1975), while arylsulphatase is contained in a sedimentable organelle in N.crassa (Scott et al., 1971). β-N-Acetylhexosaminidase has been found in "lysosomes" isolated from Dictyostelium discoideum myxamoebae (Wiener and Ashworth, 1970). Ribonuclease was not assayed, as it has been observed to bind non-specifically to membranes during cell fractionation (Parish, 1975c).

The distribution of these enzymes among the fractions (Table 5.1) confirmed the lysosomal nature of the pigmented fraction, suggesting that it contains vacuoles, but it also shows that this fraction is not the only repository of lysosomal enzymes. Both arylsulphatase and

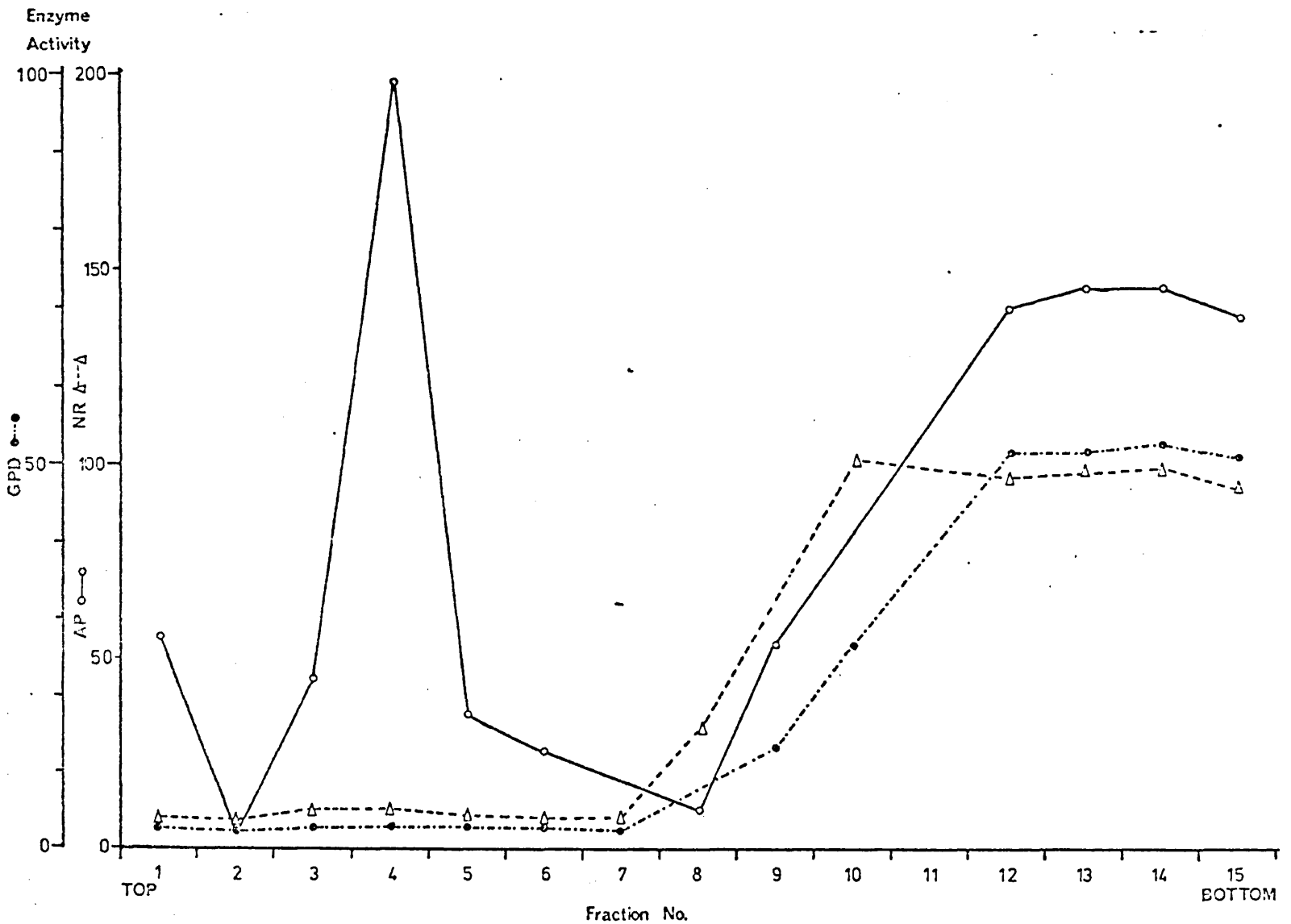


Fig. 5.1 Association of Alkaline Phosphatase Activity with the Pigmented Particulate Fraction

Mycelia were homogenised in buffered sorbitol (70% w/v). Homogenate (15ml) was layered below a linear sorbitol gradient (20-60% w/v, 16ml) and a 5% (w/v) sorbitol overlay (5ml). Centrifugation was at 100 000g_{max} for 1h. Fractions were assayed for alkaline p-nitrophenylphosphatase (o,AP), NADPH:cytochrome c reductase (Δ,NR) and glucose 6-phosphate dehydrogenase (●,GPD). Activities are expressed in nmoles substrate converted/min/ml. Fractions 1 and 2 represent the overlay, 3-9 the gradient, and 10-15 the sample zone. Only fractions 1 and 3-5 contained β-carotene

TABLE 5.1 DISTRIBUTION OF HYDROLYTIC ENZYMES IN FRACTIONS OBTAINED BY DENSITY GRADIENT CENTRIFUGATION

Fractions were isolated as described in Chapter 4. Enzyme units are nmoles substrate hydrolysed per min, except for proteinase, which is quoted as ng azocasein hydrolysed per min. Specific activities (S.A.) are units per mg protein. Figures for alkaline phosphatase and proteinase are quoted ± S.E.M. of 3 experiments, while those for arylsulphatase and hexosaminidase are the means of duplicates from a single experiment. H is the homogenate, M the mitochondrial fraction, P the microsomal fraction, V the pigmented particulate fraction, S the 100 000g supernatant, and G the lipid droplets. S.U. (%) is the percentage of sedimentable enzyme activity.

Fraction	Enzyme in Fraction											
	Alkaline p-nitrophenyl-phosphatase			Proteinase			Arylsulphatase			N-Acetyl-β-D-hexosaminidase		
	Units	S.A.	S.U. (%)	Units	S.A.	S.U. (%)	Units	S.A.	S.U. (%)	Units	S.A.	S.U. (%)
H	11000 ± 110	130		3320 ± 65	39.8		84.4	0.75		150.0	1.33	
M	1900 ± 60	470	28.6	260 ± 40	64.2	29.9	7.3	1.22	47.0	2.3	0.38	14.3
P	4100 ± 75	480	64.0	450 ± 10	52.3	51.7	4.6	0.33	29.0	13.3	0.96	83.3
V	410 ± 20	390	6.4	160 ± 15	145.5	18.4	3.7	6.92	24.0	0.4	0.75	2.5
S	5500 ± 430	90		1230 ±195	19.3		69.7	1.21		70.0	1.22	
G	440 ± 15	940		260 ± 20	553.2		5.0	7.9		0		
Recovery (%)		112			71			107			59	

proteinase have high specific activities in the presumed vacuole fraction, but considerable amounts were also present in the other particulate fractions; while hexosaminidase had the same distribution as the endoplasmic reticulum marker, NADPH:cytochrome c reductase (Table 4.2). Alkaline p-nitrophenylphosphatase activity was present in all the particulate fractions.

The multiple locations of alkaline phosphatase, however, are not unexpected since the assay is not necessarily specific for only one enzyme (HÜbscher and West, 1968; Gerlach and Hilby, 1974; Lee et al., 1975), and cytochemical studies have shown that most membranes and vesicles of Candida albicans and Phytophthora palmivora possess alkaline phosphatase activity (de Nollin et al., 1975; Meyer et al., 1976). In an attempt to discover whether the phosphatases in each fraction had similar properties, the enzyme activity of each fraction was investigated between pH 4.0 and pH 9.0. In each case no activity was detected below pH 6.5, and maximum activity was obtained with 0.2M tris-HCl buffer, pH 8.1.

Matile and Wiemken (1967) and Hasilik et al. (1974) have concluded that, in S.cerevisiae, hydrolytic enzymes are found almost exclusively within the vacuoles. Other workers, however, have obtained multiple distributions of hydrolytic enzymes similar to those found in the C115 mutant. Cartledge and Lloyd (1972) fractionated homogenates of S.carlsbergensis by zonal centrifugation, and found a complex distribution of acid hydrolases, while sedimentable alkaline phosphatase was found at ρ =1.18 and 1.22. Scott et al (1971) found arylsulphatase and acid phosphatase in particles of density 1.15g/ml isolated from N.crassa, while Meyer et al. (1976) found that hydrolytic enzymes in Phytophthora palmivora were associated with organelles which banded at densities of 1.18 and 1.09g/ml. Similar results were obtained for the location of α-L-arabinofuranosidase (E.C.3.2.1.55) and acid phosphatase in mycelia of Sclerotinia fructigena (Hislop et al., 1974).

Part of the explanation for the diverse locations reported by these authors is probably the aggregation of mitochondria and "vacuoles" observed in the experiments described in Chapter 3. Similar associations of mitochondria and vacuoles have been reported by Parish (1975c) and Susani et al. (1976). Furthermore, the density of vacuoles can vary with the isolation conditions employed;

vacuoles from S.cerevisiae swell during isolation to assume a low density, while vacuoles from S.carlsbergensis do not swell, and consequently have a greater density. Vacuoles also shrink irreversibly at high osmolarities (Cabib et al., 1973).

Consequently, the heterogenous distribution of hydrolytic enzymes observed in the present experiments may reflect an in vivo heterogeneity of the lysosomal system, or the incomplete separation of vacuoles from other sedimentable organelles. In an attempt to answer this question the distribution of α-mannosidase was investigated. This enzyme is characteristic of the tonoplast itself in S.cerevisiae (van der Wilden et al., 1973), unlike most of the other hydrolytic enzymes which are soluble. The distribution of this enzyme and another α-glycosidase amongst organelles separated on a density gradient (Fig. 5.2) suggests that many vacuole membranes remained in the sample zone, either because they had been shrunk by the high sorbitol concentration at the sample zone (3.9M), or because they had lost their soluble contents during homogenisation. However, both the absence of β-carotene from the sample zone, and the variations in distribution between different hydrolases (Table 5.1) indicate that there is an in vivo heterogeneity of hydrolytic enzyme distribution, and also that the α-mannosidase present in the sample zone is probably due to tonoplast fragments rather than intact vacuoles.

Lipid Globules

Unpurified lipid droplets contained several hydrolytic enzymes (Table 5.1). In order to determine if these enzymes were genuinely located in the globules, this fraction was resuspended in sorbitol (10% w/v in 50mM phosphate buffer, pH 7.4), and purified by flotation through buffered sorbitol (5% w/v; 140 000g_{av}, 45 min). The lipid droplets were collected from the surface of the solution and resuspended in buffered sorbitol (10% w/v).

This washing procedure was repeated several times, and the specific activities of various enzymes and the β-carotene content were determined after each wash. This procedure removed all contamination due to mitochondrial, microsomal and cytosolic enzymes, but both the specific activity of alkaline phosphatase, and the β-carotene: protein ratio, reached constant values (Table 5.2). Arylsulphatase

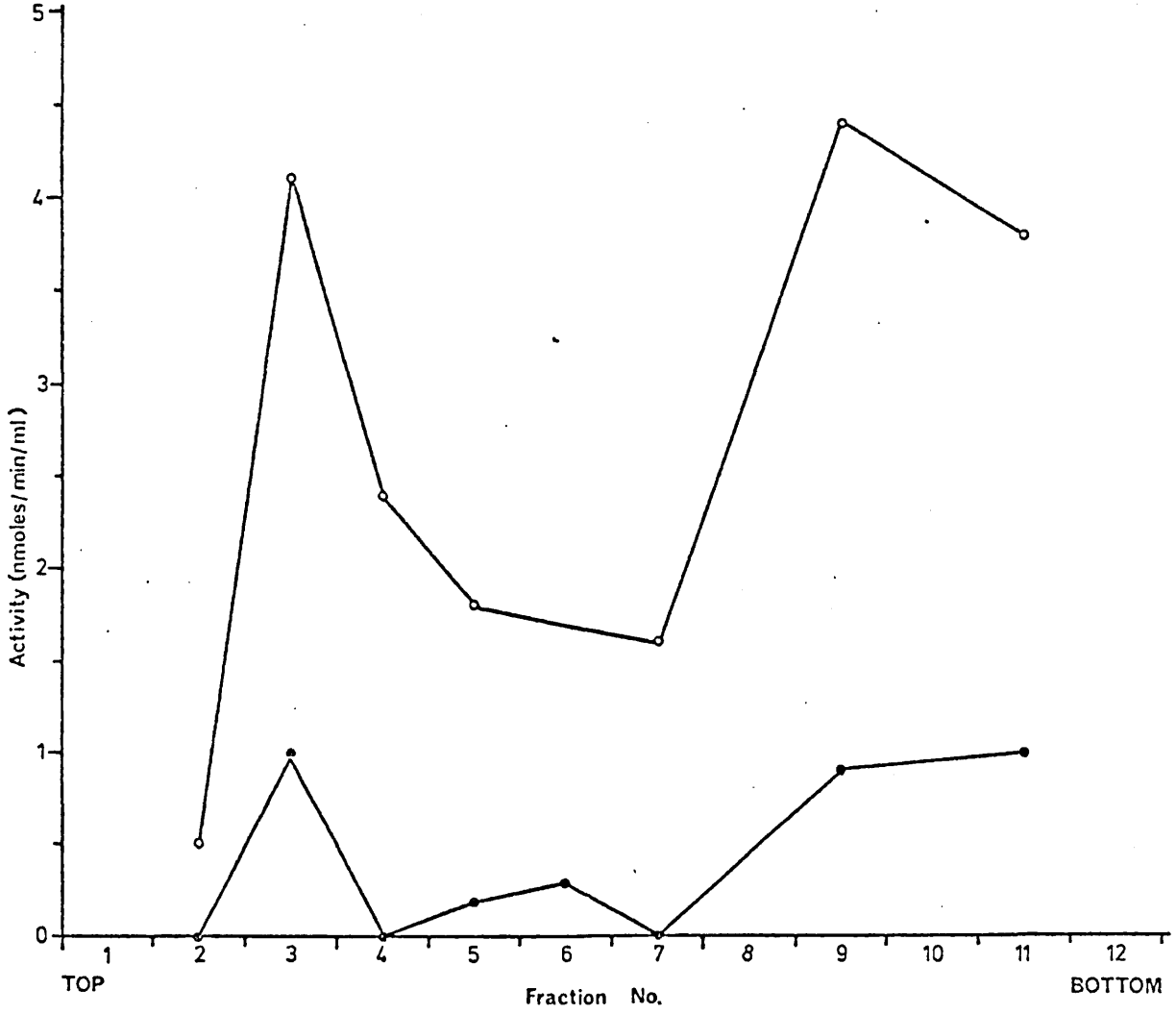


Fig. 5.2 Distribution of α-Glycosidases in Cell Homogenates

Mycelia were homogenised and fractionated as described in Fig. 5.1 Fractions were then assayed for α-mannosidase (o) and α-galactosidase (•). Fraction 1 represents the overlay, fractions 2-7 the gradient and 8-12 the sample zone. Only fractions 1-4 contained β-carotene

TABLE 5.2 PURIFICATION OF LIPID PARTICLES BY REPEATED FLOTATION

Lipid particles were washed as described in the text. Enzyme activities are nmoles substrate converted per min per mg protein. N.A. means not assayed.

	Number of Flotations				
	1	2	3	4	5
β-Carotene:protein ratio (w/w)	0.049	0.066	0.132	0.133	0.130
Alkaline p-nitrophenylphosphatase	136	96	171	179	179
Arylsulphatase	N.A.	2.25	3.92	0	0
Succinate:cytochrome c reductase	21.3	0	0	N.A.	N.A.
NADPH:cytochrome c reductase	149	0	0	N.A.	N.A.
Glucose 6-phosphate dehydrogenase	1.8	0	0	N.A.	N.A.

and proteinase activities were removed by this procedure, indicating that these enzymes are not located on the lipid globules.

The presence of alkaline phosphatase in lipid droplets is difficult to explain, since the function of this enzyme is not known (Fernley, 1971), but it has previously been demonstrated histochemically in the lipid globules of both Achyla flagellata and S.cerevisiae (Palczewska and Jagodzka, 1972; Bauer and Sigarlakie, 1975).

B. CHEMICAL COMPOSITION OF PIGMENTED FRACTIONS

Amino Acid Analyses

In one of the first studies of the membranes of lipid droplets Yatsu and Jacks (1972) published an analysis of the amino acid composition of fat globules isolated from peanut cotyledons. Since that time the amino acid contents of lipid droplets isolated from M.hiemalis and Lipomyces starkeyi have been reported (Herber, 1974; Uzaka et al., 1975). Consequently the amino acid compositions of proteins of lipid droplets and vacuoles isolated from the C115 mutant were analysed and compared with these results.

Lipid droplets and vacuoles were prepared as described earlier, and the droplets were washed by flotation five times (see Table 5.2). An attempt was made to extract structural protein from these organelles using dilute acetic acid (1.4% v/v) as described by Zahler et al. (1968), but the yields were too low for analyses to be conducted, so that total proteins were used instead. Hydrolyses were carried out as described in the Methods section; duplicate samples were hydrolysed for 14 and 72 hours and the results extrapolated to zero-time. The analysis of lipid droplets was repeated on four different samples, all with essentially the same results. The reproducibility with which the major constituent amino acids were measured is approximately ± 5%.

The amino acid analyses of lipid droplets and vacuoles isolated from P.blakesleeanus were similar (Table 5.3), the major difference being the greater serine content of the lipid droplets. In addition, approximately 4 moles of hydroxyproline were present per 100 moles of lipid globule amino acids, while no hydroxyproline was detected

TABLE 5.3 A COMPARISON OF THE AMINO ACID COMPOSITIONS OF VARIOUS FUNGAL ORGANELLES

AMINO ACID	MOLES PER 100 MOLES OF AMINO ACIDS				
	Lipid Globules	Vacuoles	Lipid Globules from M.hiemalis(1)	L.starkeyi(2)	Mitochondria from N.crassa(3)
Lys	6.8	7.8	8.6	7.8	6.3
His	2.5	2.5	3.5	2.5	2.3
Arg	3.3	4.0	4.6	4.3	5.4
Asx	8.5	8.8	12.3	10.1	8.9
Thr	5.5	5.9	4.8	5.5	5.4
Ser	11.2	5.3	5.7	6.7	5.8
Glx	9.0	9.4	13.3	12.6	10.0
Pro	4.0	4.4	0	trace	4.1
Gly	7.0	8.6	11.1	11.2	8.6
Ala	8.3	8.8	10.2	9.5	10.2
½ Cys	2.3	2.4	3.2	trace	1.8
Val	7.6	8.4	2.2	7.7	7.1
Met	2.0	1.6	1.7	3.7	2.1
Ile	6.7	6.7	2.3	5.6	5.4
Leu	9.1	9.1	8.3	7.0	8.6
Tyr	1.8	1.6	3.6	2.4	4.0
Phe	4.3	4.7	4.6	3.4	3.9

References:

1 - Herber (1974)

2 - Uzaka et al. (1975)

3 - Woodward and Munkres (1966)

TABLE 5.4 A COMPARISON OF THE AMINO ACID COMPOSITIONS OF VARIOUS ORGANELLES USING KOSHLAND'S HOMOLOGY SET A (Haber and Koshland, 1970)

AMINO ACIDS	MOLES PER 100 MOLES OF AMINO ACIDS					
	Lipid Globules from:				Vacuoles from:	Mitochondria from:
	P. blakesleeanus	*M. hiemalis*[1]	*L. starkeyi*[2]	Peanuts[3]	*P. blakesleeanus*	*N. crassa*[4]
Gly, Ala	15.4	21.3	20.7	25.1	17.4	18.8
Ser, Thr	16.7	10.5	12.2	17.2	11.2	11.2
Val, Ile, Leu, Met	25.4	14.5	24.0	19.5	25.8	23.2
Phe, Tyr	6.1	8.2	5.8	8.6	6.3	7.9
His, Arg, Lys	12.6	16.7	14.6	9.7	14.3	14.0
Asx, Glx	17.5	25.6	22.7	15.8	18.2	18.9
½ Cys	2.3	3.2	trace	-	2.4	1.8
Pro	4.0	-	trace	4.1	4.4	4.1

References:

1 - Herber (1974)

2 - Uzaka *et al.* (1975)

3 - Yatsu and Jacks (1972)

4 - Woodward and Munkres (1966)

in the vacuoles. The "difference index of compositional relatedness" (D.I.) between these proteins is approximately 7.5, suggesting a high degree of sequence homology (Metzger et al., 1968), but the difference indices for the comparison of proteins of Phycomyces lipid droplets with those from other sources are between 11 and 17, suggesting that they are not so closely related. However, if these results are compared using Koshland's Homology Set A (Haber and Koshland, 1970; Table 5.4), then a similarity between the lipid droplets of P.blakesleeanus and those of L.starkeyi is apparent. There is also a very close relationship (D.I. = 6.5) between the proteins of vacuoles from P.blakesleeanus and mitochondria from N.crassa (Woodward and Munkres, 1966).

Scanning electron microscopy has shown that the lipid globules of L.starkeyi are surrounded by a membrane (Uzaka et al., 1975). The similarity between the amino acid compositions of these globules and those isolated from P.blakesleeanus suggests that a membrane is also present around the lipid droplets of the latter organism, while the correspondence between the structural protein of mitochondria from N.crassa and the proteins of vacuoles of Phycomyces indicates that this fraction may possess a normal tripartite membrane.

Distribution of β-Carotene between Globules and Vacuoles

In an attempt to discover the site(s) of carotenoid biosynthesis the distribution of carotene in hyphae of different ages was investigated.

When mycelia, harvested 24h after inoculation, were examined by light microscopy large numbers of refractive oil globules were visible in the hyphae, but insufficient pigment was present for its distribution to be assessed. Mycelia grown for 48 to 72h contained large deposits of β-carotene, distributed throughout the hyphae (Figs. 5.3 and 5.4). These deposits were more highly coloured in older zones away from the growing tip. However in 96h-old mycelia the pigment was concentrated in the hyphal tips, and the older zones were practically colourless (Fig. 5.5). This agreed with the decline in the rate of carotenogenesis previously observed in older cultures (Fig. 3.2). Alternatively, it is possible that degradative enzymes are present in older zones of the hyphae.

These observations suggest that β-carotene accumulates in lipid droplets as they age, but no conclusion is possible about the site of

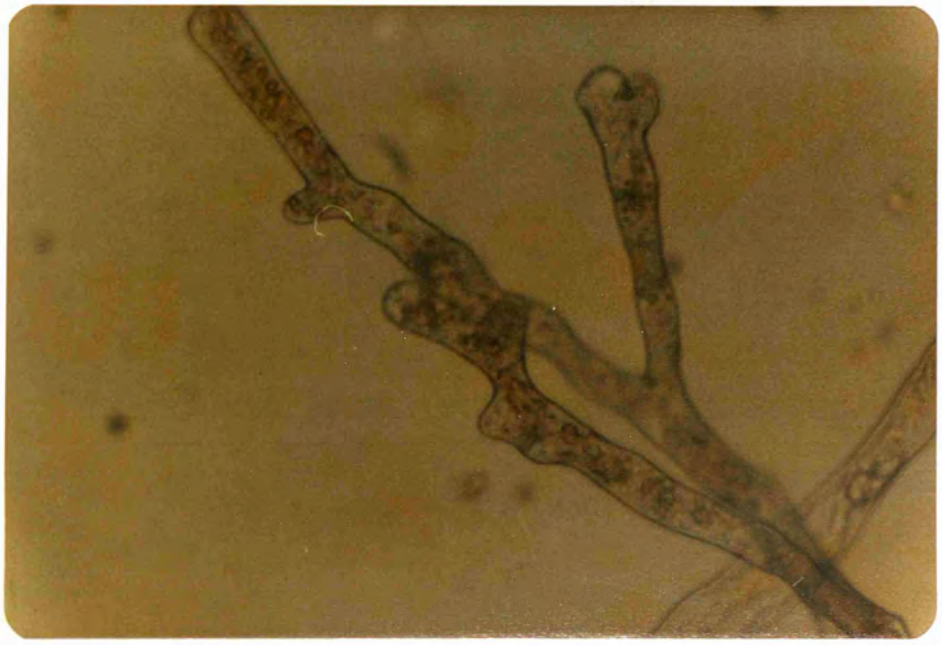


Fig. 5.3 Mycelia Grown for 48h after Inoculation
(approx. magnification, x 2000)

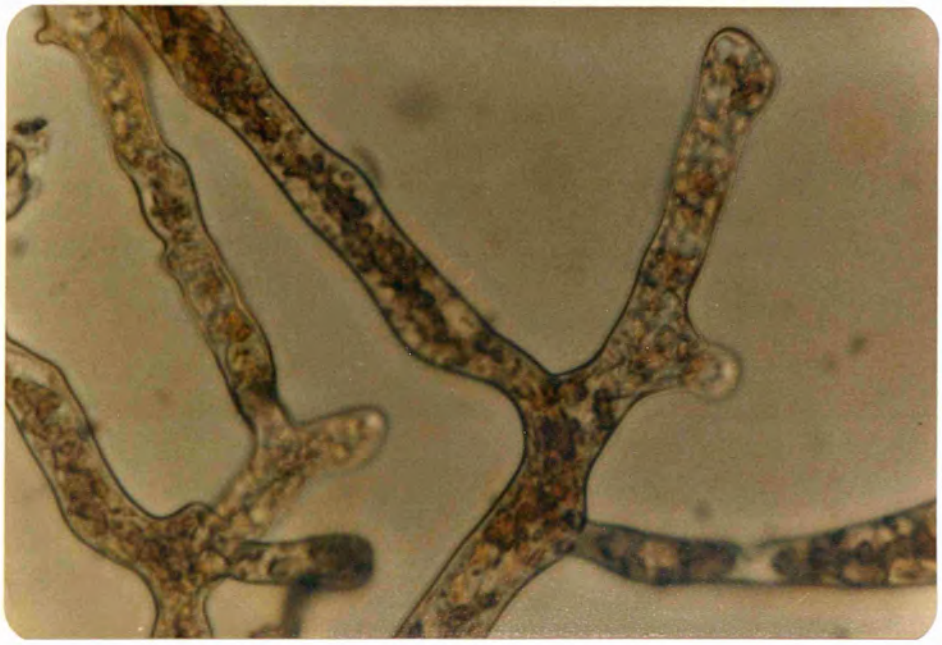


Fig. 5.4 Mycelia Grown for 72h after Inoculation
(approx. magnification, x 2000)

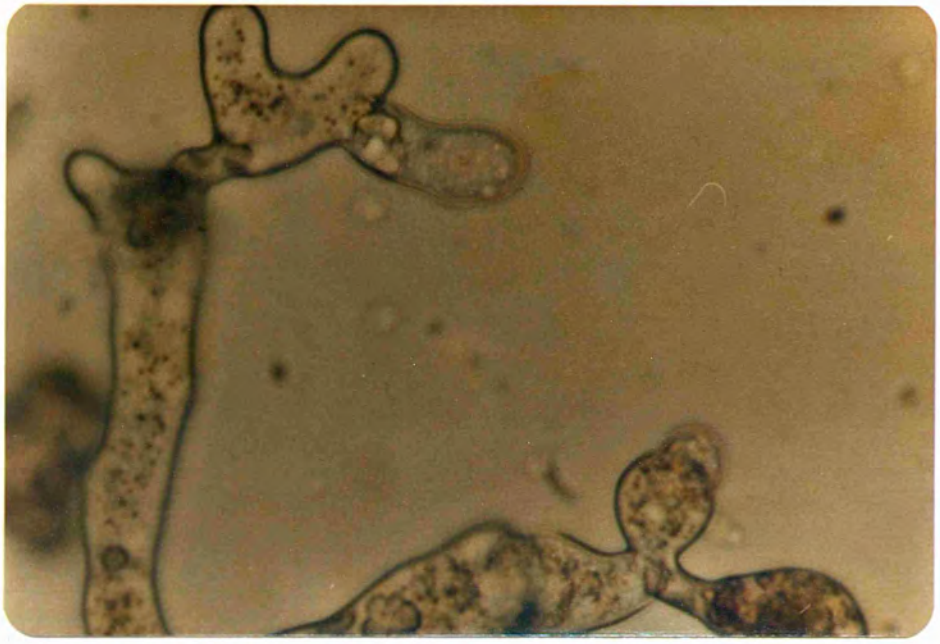


Fig. 5.5 Mycelia Grown for 96h after Inoculation
(approx. magnification, x 2000)

carotenoid biosynthesis, since organelles containing only small amounts of pigment would not be visible under the light microscope.

In order to overcome this difficulty in the detection of slightly-pigmented organelles cell fractionation techniques were employed. In a preliminary experiment mycelia were homogenised with 5, 15 and 30 passes of the teflon pestle. Aliquots were removed at each stage and lipid droplets and vacuoles separated by centrifugation (130 000g_{av}, 1h). The distribution of β-carotene between the pellet and the floating lipid pellicle was not changed by the degree of homogenisation, indicating that the observed distribution probably resembles that in the intact mycelia.

Mycelia, grown for between 24 and 72h after inoculation, were homogenised in sorbitol (70% w/v in 50mM Hepes., pH 7.4, containing 10mM KCl and 2mM $MgCl_2$). Vacuoles and lipid globules were separated by floating homogenate (20ml) through a linear sorbitol gradient, as described in Table 4.1. The vacuoles were collected by sedimentation (40 000g, 15 min), while the lipid droplets were collected on muslin pads, which were then extracted with diethyl ether. β-Carotene was extracted from the vacuoles and from the homogenate by sonication with ethanol:diethyl ether (3:1 v/v) as described in the Methods section.

As the mycelia developed the amount of β-carotene in the lipid droplets increased, but the amount in the vacuoles remained fairly constant, as did the β-carotene:protein ratio (Table 5.5).

Mycelia cultured for 24 hours showed the expected distribution of pigment between floating lipid droplets and vacuoles (collecting at the interface between the gradient and the overlay), but in addition a yellow band was visible at the lower interface, i.e. between the sample zone and the gradient. The identity of this band could not be established due to lack of material, but it is possible that the vacuoles of younger hyphae are less resistant to shrinking, and hence they assume the density of the medium in which they are suspended (see p. 154).

Since the amount of β-carotene in the globules increases as the mycelia age while that in the vacuoles remains static, carotenogenesis probably occurs in the former organelle, and the pigmentation of the vacuoles may be due to the transfer of material to them from the lipid globules.

TABLE 5.5 DISTRIBUTION OF β-CAROTENE BETWEEN GLOBULES AND VACUOLES AS A FUNCTION OF AGE OF MYCELIA

The figures quoted are the results of one experiment, in which recoveries of β-carotene were between 60 and 85%. Similar results were obtained in another experiment.

AGE (hours after inoculation)	Mycelia	Vacuoles			Globules
	β-Carotene (µg/g dry wt.)	Protein (µg/g dry wt.)	β-Carotene (µg/g dry wt.)	Carotene: protein ratio	Carotene (µg/g dry wt.)
24	25	205	1.2	.006	12
			(denser) 2.5		
40	480	290	13.5	.019	245
48	540	75	7.0	.025	355
63	1170	175	11.0	.021	750
72	930	95	9.0	.024	785

Lipid Content of Lipid Droplets

Thin-layer chromatography of extracts of the lipid globule fraction demonstrated that they contained β-carotene and ergosterol, together with large amounts of triglycerides and free fatty acids, and smaller quantities of diglycerides and more polar lipids.

Extracts were also chromatographed on grade III alumina columns. Compounds eluted with 6% (v/v) diethyl ether in light petroleum (b.p. 40-60°C) were separated by t.l.c. (system 2, Table 2.2). A spot (R_F 0.75) stained grey-blue with $SbCl_3$, showing the presence of steryl esters in the lipid droplets.

Phospholipids were still present in the lipid globule fraction after repeated washing of the droplets in an analogous manner to that described in Table 5.2. After three flotations a protein: phospholipid ratio (w/w) of 5.25 was obtained. This was not altered by further flotation.

The lipid contents of globules isolated from mycelia grown for 40, 48 and 72 hours were determined by the techniques described in the Methods section. The percentage (w/w) content of β-carotene and ergosterol in the lipid globules increased linearly with the age of the mycelia (Table 5.6), but the phospholipid content was more variable.

These results are comparable to those obtained for lipid droplets isolated from other plant sources (Table 1.2). The amount of β-carotene present in these globules, however, is much greater than that reported for droplets from zoospores of Blastocladiella emersonii (0.04% w/w; Mills and Cantino, 1977), but this probably reflects only the greater amount of β-carotene in mycelia of the C115 mutant of P.blakesleeanus compared to most other fungi.

Size of Lipid Droplets

Lipid globules were isolated from homogenates of mycelia grown for 40, 48 and 72 hours, and were then dispersed in molten agar (2% w/v) and fixed in glutaraldehyde followed by osmium tetroxide. Dehydration was accomplished rapidly using dimethoxypropane (see p. 99), and the samples were embedded and sectioned as described in the Methods section.

Staining (due to OsO_4) was confined to the centre of the globules (Fig. 5.6), indicating that some lipids had been extracted during preparation. However the agar in which the samples were embedded should

TABLE 5.6 LIPID COMPOSITION OF OIL DROPLETS ISOLATED FROM P.BLAKESLEEANUS

Results are the averages of two experiments. Fractions were extracted with $CHCl_3$/MeOH, followed by a final extraction with diethyl ether to remove the last traces of pigment. β-Carotene and ergosterol were estimated spectrophotometrically in the crude extract, while phospholipids were assayed using the revised procedure (p. 93). "Neutral lipids" are the difference between the total lipid dry wt. and the amounts of the other components estimated.

AGE OF MYCELIA (hours after incubation)	LIPID COMPOSITION (% w/w)			
	Neutral lipids	Sterols/steryl Esters	β-Carotene	Phospholipids
40	95.3	3.25	0.78	0.67
48	93.7	3.77	0.98	1.56
72	90.7	5.27	1.89	2.16

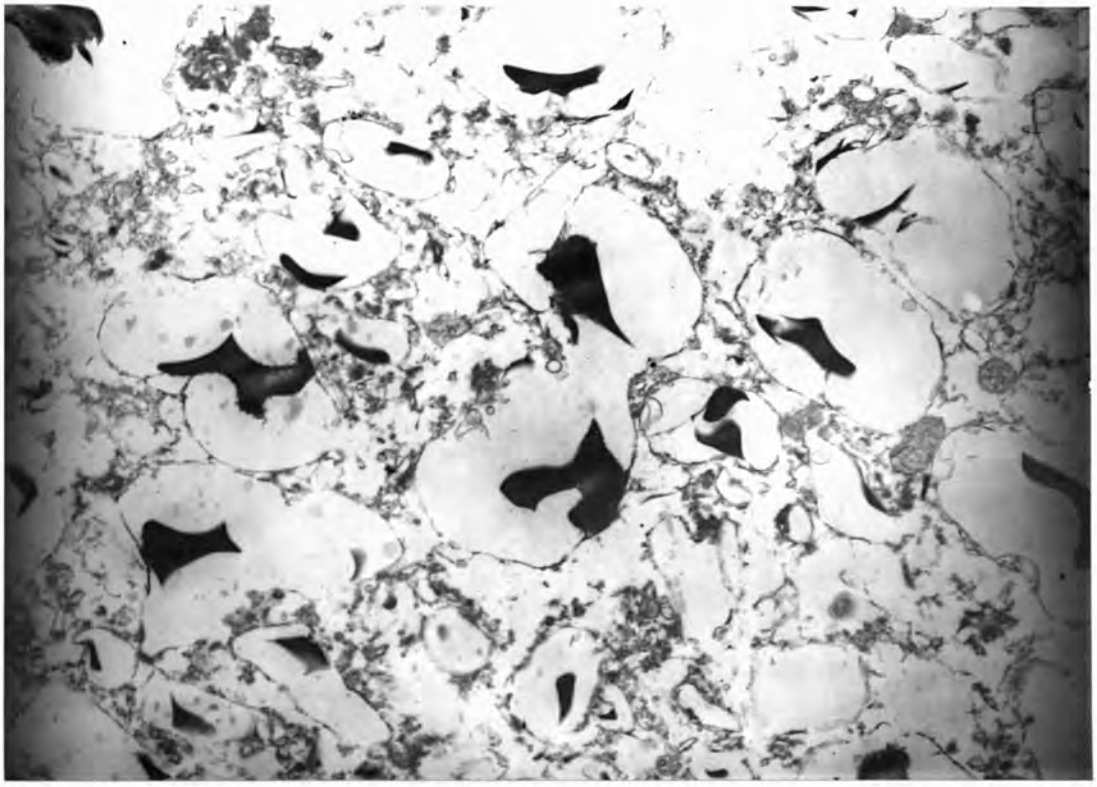


Fig. 5.6 Lipid droplets (x 8 300). Lipid droplets were dispersed in molten agar and fixed in glutaraldehyde followed by osmium tetroxide

have preserved their original size and shape. Under higher magnification (x 35 000) an osmiophilic boundary about 15nm thick was visible around the droplets. Some of the larger globules appear to be dividing, but they might alternatively have begun to coalesce during preparation.

In order to determine the average diameter of the droplets the geometric means of at least 40 globules of each sample were measured on prints enlarged to a total magnification of 11500 times. Measurements were accurate to 0.5mm (0.04μm).

The apparent diameters of the sectioned droplets are not their true diameters, since not all of them were sectioned through the centre. In order to overcome this problem the arithmetical procedure of Wicksell (1925) was used to convert a frequency distribution of apparent diameters into the frequency distribution of real diameters.

The mean diameters were determined from these distributions as 0.52μm, 0.51μm and 0.55μm for globules isolated from mycelia grown for 40, 48 and 72 hours, respectively. These dimensions are of the same order as those observed in whole mycelia (Fig. 3.7), and also those reported for droplets isolated from other organisms (Table 1.2). The insignificant increase in size between 40 and 72 hours agrees with observations on seeds of Crambe abyssinica, where the increase in triglyceride content of the seed is due to the formation of new oil bodies, rather than an increase in the volume of those already present (Smith, 1974).

Protein Content of Lipid Droplets

Mackenzie et al (1966) devised formulae for the calculation of the amount of protein and phospholipid necessary to surround corpuscles of various sizes.

The area occupied by a fully compressed monolayer of protein is aproximately 0.8 m^2/mg (Bull, 1947). Assuming a density of 1.4 g/ml for globular proteins, this corresponds to a thickness of approximately 8.9×10^{-4}μm. The percentage of the volume of a sphere occupied by a surface layer of protein 8.9×10^{-4}μm thick is

$$\frac{5.3 \times 10^{-3}}{\text{diameter of sphere in microns}} \times 100$$

(This approximation holds when the diameter is > 0.1μm).

In order to determine whether sufficient protein to form a membrane was present in lipid globules isolated from P.blakesleeanus the protein content of droplets was estimated, and lipids were extracted with $CHCl_3$/MeOH, dried in vacuo, and weighed. Assuming a density of 0.91 to 0.95g/ml for lipids the amount of protein present in the lipid droplets is just sufficient to cover them with a monolayer (Table 5.7)

TABLE 5.7 SIZE AND PROTEIN CONTENT OF OIL DROPLETS ISOLATED FROM PHYCOMYCES BLAKESLEEANUS C115 carS42 mad-107(-)

	Age of Mycelia (hours after inoculation)		
	40	48	72
Mean Diameter (μm)	0.52	0.51	0.55
Protein required for a Monolayer (%v/v)	1.02	1.04	0.95
Protein present (%v/v)	1.2	0.9	1.0

Lipid droplets 0.50 to 0.55μm in diameter should contain 2.50 to 2.25% (v/v) phospholipid if they are surrounded by a monolayer membrane (Mackenzie et al., 1966). Although this is greater than the amount found in lipid droplets isolated from P.blakesleeanus (Table 5.6), neutral lipids are also involved in membrane structure in fungi (Keith et al., 1973).

Because of the possible sources of error in these experiments (e.g. protein contents were determined assuming identical colour development for lipid droplet proteins and for BSA; the droplets may have shrunk during fixation; very small droplets may have been overlooked), and because of the approximations made in the calculations, it is impossible to conclude that lipid droplets are surrounded by only a half-unit membrane. However these results do provide some evidence for the existence of a membrane around the droplets, and they also show that fat globules do not contain large amounts of non-structural proteins and phospholipids.

INCORPORATION OF [2-^{14}C] MEVALONIC ACID INTO THE TERPENOIDS OF GROWING MYCELIA

In an attempt to determine the subcellular site of β-carotene biosynthesis the incorporation of [2-^{14}C]MVA into β-carotene and sterols of growing mycelia was investigated.

Preliminary Experiments

In order to study the effect of concentration on the incorporation of MVA by growing cultures of the C115 mutant DL-[2-^{14}C]MVA (0.5 to 2.0μCi, specific activity 13mCi/mmole) was added at inoculation to flasks containing 50ml of medium. After 40h the mycelia were harvested and extracted with acetone and ether as described previously. In each case 2.0 to 2.5% of the active isomer was incorporated into lipids, indicating that the incorporation of MVA was directly dependent upon the concentration in the medium. Some inhibition of growth (approx. 30%) was observed at the highest MVA concentration.

The types of lipids synthesized from MVA in this experiment were determined by saponification, followed by t.l.c. (System 1, Table 2.2) and autoradiography (p. 95). The gel contained about 165 d.p.s. in a band 15cm long, and the film was exposed for 3 months. Theoretically, under these conditions a band 0.2cm wide containing only 1 to 2% of the total radioactivity should be detectable (assuming an overall efficiency of 1.5 to 2%, and a limit of detection of 10^5 β-interactions per cm^2; Faires and Parks, 1973). Two major radioactive bands were observed (R_F values 0.15 and 0.8), and also a minor component (R_F 0.2). These correspond to ergosterol, β-carotene and lanosterol, respectively. Very faint bands were also visible (R_F 0.0, 0.02, 0.17 and 0.23), but these were all very close to the limit of detection, and are possibly due to incorporation of MVA into terpenoid quinones.

Because of these possible sources of contamination the sterols were purified by t.l.c. (System 2, Table 2.2) in most experiments. In order to assess the recovery of compounds after each t.l.c. separation the areas corresponding to β-carotene, sterols, and other radioactive materials were eluted and counted.

Two samples of unsaponifiable material were resaponified overnight, and the recoveries of β-carotene and radioactivity were compared. The apparent specific activity of the β-carotene was the same before and after saponification in each case (Table 6.1). Consequently, the recovery of material after saponification was assessed

from the recovery of β-carotene.

TABLE 6.1 RECOVERY OF MATERIAL AFTER SAPONIFICATION

Samples containing [^{14}C] unsaponifiable lipids were resaponified overnight at room temperature. The concentration of β-carotene and the content of radioactivity were assessed on aliquots removed before and after saponification. Sample 1 contained material synthesized from [2-^{14}C]MVA *in vivo*; Sample 2 material synthesized from MVA *in vitro* (see Chapter 7). Apparent specific activities (S.A.) are d.p.s./μg β-carotene.

SAMPLE	β-Carotene (μg)	Radioactivity (d.p.s.)	S.A.
1, Before saponification	74.8	127	1.7
After saponification	68.2	122	1.8
2, Before saponification	9.9	3 812	385
After saponification	8.8	3 510	400

β-Carotene was purified by the methods employed by Bramley (1973). Unsaponifiable lipids were separated by t.l.c. (System 1, Table 2.2), and the specific activity of the β-carotene was determined. The pigment was then chromatographed in System 3 (Table 2.2) and again radioassayed. In order to verify that these t.l.c. systems yielded radiochemically pure β-carotene, approximately 0.5mg of carrier β-carotene (purified by chromatography on grade III alumina) was added to two samples, and these were dissolved in the minimum volume of benzene, to which MeOH (3 vol.) was then added. The solutions were left in the dark at room temperature for 3h, followed by 2h at -20°C, during which time crystals formed. Both the mother liquor and the crystals were radioassayed, and found to be of the same specific activity (Table 6.2), indicating that the t.l.c. systems were adequate for the purification of [^{14}C]β-carotene.

TABLE 6.2 PURIFICATION OF β-CAROTENE

Samples were extracted from mycelia grown in the presence of [2-^{14}C]MVA, and were then saponified and separated as described in the text. Specific activities (S.A.) are d.p.s./μg.

Purification stage	β-CAROTENE		
	μg	d.p.s.	S.A.
SAMPLE A:			
1. Separation by t.l.c. (System 1)	472	883	1.87
2. Purification by t.l.c. (System 3)	267	455	1.70
3. Crystallisation: Crystals	129	35	0.27
Mother liquor	456	128	0.28
SAMPLE B:			
1. Separation by column chromatography (grade III alumina)	409	651	1.59
2. Purification by t.l.c. (System 3)	345	543	1.57
3. Crystallisation: Crystals	117	41	0.35
Mother liquor	516	175	0.34

Subcellular Distribution of Radioactivity from [2-^{14}C]MVA

Mycelia were grown for 40h in the presence of DL-[2-^{14}C] MVA (50nCi/ml; specific activity 13mCi/mmole). After harvesting the mycelia the medium was found to contain 4.88μCi of radioactivity. The mycelia were homogenised in buffered sorbitol (70% w/v), and the vacuoles were separated by floating homogenate (17ml) through a linear sorbitol gradient (16ml) and overlay (7.5ml; Table 4.1).

Fractions (3ml) were collected, and aliquots (200μl) were removed from each and counted in Triton X-100/PPO scintillant (see p. 97). Fractions representing the sample zone and the vacuole fraction were combined, diluted, and centrifuged (100 000g_{av}, 1h). The radioactivity in the subsequent soluble and sedimentable fractions was then estimated. Lipid droplets were collected and resuspended in water, but no attempt was made to ensure quantitative recovery. Carrier terpenoids (200μg β-carotene, 150μg ergosterol, 50μg lanosterol) were added to each fraction, and the lipids were extracted by sonication with ethanol/diethyl ether (3:1 v/v), and radioassayed.

Only 21% of the radioactivity remaining in the sample zone was sedimentable, and more than 90% of the remainder was in the form of hydrophilic compounds, presumably [2-^{14}C]MVA and ^{14}C-labelled terpenyl pyrophosphates (Fig. 6.1). However, most of the sedimentable radioactivity in both the sample zone and the vacuole zone was in the form of lipids rather than hydrophilic compounds, indicating that these particulate fractions are not involved in the early stages of carotene and sterol biosynthesis. This is in agreement with the results of Shimizu *et al.* (1973) and Kawaguchi *et al.* (1973b), both of whom found that the conversion of MVA into FPP could occur in the supernatant fraction of fungal cells (see p. 76).

Whole mycelia were also extracted, using acetone and diethyl ether. The carotenes and sterols from this extract, together with those from the lipid globule and vacuole fractions, were purified by t.l.c. as described previously (pp.172-173 ; Table 6.3).

In both whole mycelia and in vacuoles about 25% of the radioactivity incorporated into lipids was found in β-carotene, but relatively more [^{14}C]β-carotene was present in the lipid globules

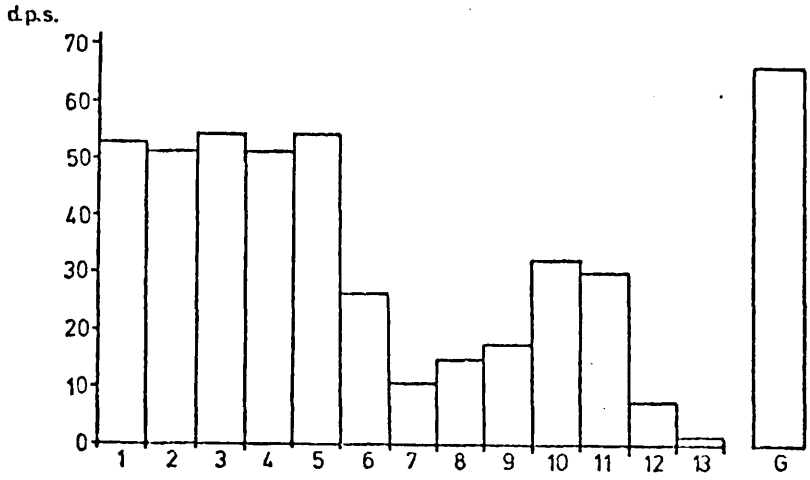


Fig. 6.1a

Distribution of Total Radioactivity

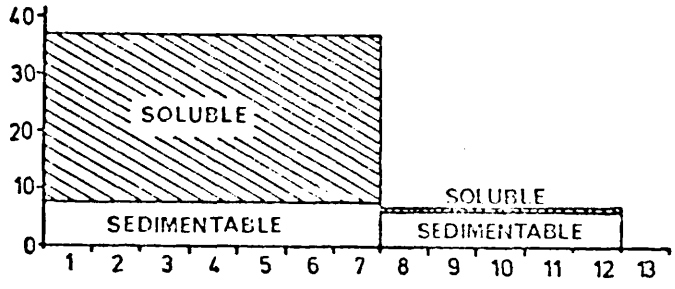


Fig. 6.1b

Sedimentability of Radioactivity

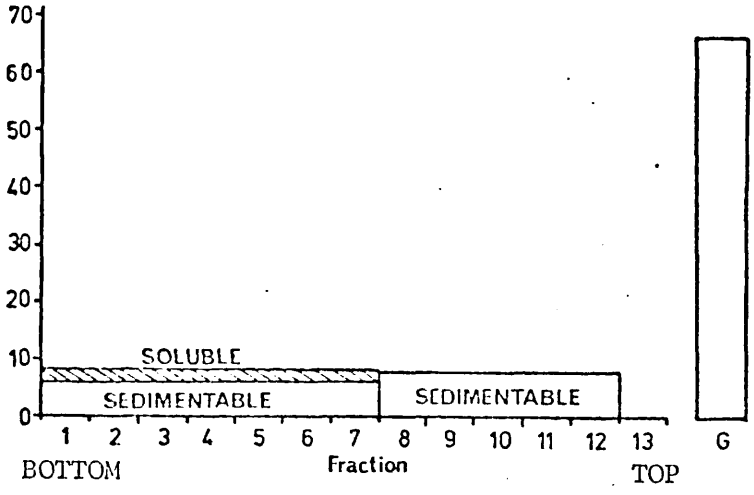


Fig. 6.1c

Incorporation into Lipids

Fig. 6.1 Distribution of Radioactivity from [2-^{14}C]MVA in Separated Cell Fractions

For details see text. G = lipid globule fraction

(Table 6.4). This distribution of radioactivity probably reflects the distribution of these compounds in the cell; sterols being present in all organelles, while most of the β-carotene is confined to the lipid droplets.

Time Course of Incorporation of [2-^{14}C]MVA into Lipids

In order to verify that the uptake of MVA by mycelia was linear with time DL-[2-^{14}C]MVA (10μCi) was added to a culture which had been growing for 32h. At 2h intervals aliquots were removed, weighed, and extracted with acetone and diethyl ether. The extracted lipids were then radioassayed. The incorporation of [2-^{14}C] MVA into lipids was linear for at least 8 to 10h (Fig. 6.2).

In a study on the intracellular distribution of sterol-synthesising enzymes, Knapp et al. (1969) incubated cut stems of pea seedlings in [2-^{14}C]MVA for 20 min to 24h, and then radioassayed the sterols in fractions prepared by differential centrifugation. Radioactivity was incorporated into squalene very quickly in the cytosolic fraction, while [^{14}C]sterols were present in the supernatant and microsomal fractions after only 20 min. Radioactive sterols, however, were not detected in the mitochondria until 2h after the commencement of the experiment.

Because of the success of this approach in elucidating the subcellular site of plant sterol biosynthesis the incorporation of [2-^{14}C]MVA in various fractions of P.blakesleeanus was investigated. Mycelia were grown for 34h after inoculation, and DL [2-^{14}C]MVA (5μCi; 13mCi/mmole) was added to each of three flasks containing 100ml of medium. The mycelia were harvested 1, 3 and 5h later, and were homogenised in buffered sorbitol (70% w/v). Vacuoles and lipid droplets were separated by the usual flotation method (Table 4.1), and the lipid droplets were collected quantitatively on muslin pads, while the vacuoles were collected by centrifugation (40 000g; 15 min). The sample zone was diluted to approximately 10% (w/v) sorbitol, and membranes were sedimented from this fraction (140 000g_{av}; 45 min).

Lipids were extracted from the globules by soaking the pads in ether, while the other fractions were extracted with ethanol:ether (3:1 v/v). Carrier β-carotene (100mg) and sterols (200mg) were added to the "membrane" and "supernatant" fractions before extraction. The vacuole and globule extracts were then saponified and separated by

TABLE 6.3 PURIFICATION OF β-CAROTENE AND STEROLS FROM MYCELIA GROWN IN THE PRESENCE OF [2-^{14}C]MVA

Mycelia (0.5g wet wt.) were homogenised in acetone and diethyl ether. The extracts were saponified and separated. β-Carotene and ergosterol were estimated spectrophotometrically. Specific activities (S.A.) are d.p.s./μg.

SAMPLE	RADIOACTIVE CONTENTS		
	μg	d.p.s.	S.A.
Unsaponified:			
β-Carotene	139	471*	-
Ergosterol	517		
T.l.c. - System 1:			
β-Carotene	88	82	0.93
Ergosterol	316	209*	-
T.l.c. - System 3:			
β-Carotene	57	49	0.87
T.l.c. - System 2:			
Ergosterol	233	170	0.73
Lanosterol	-	4.5	-

* includes radioactivity incorporated into lanosterol

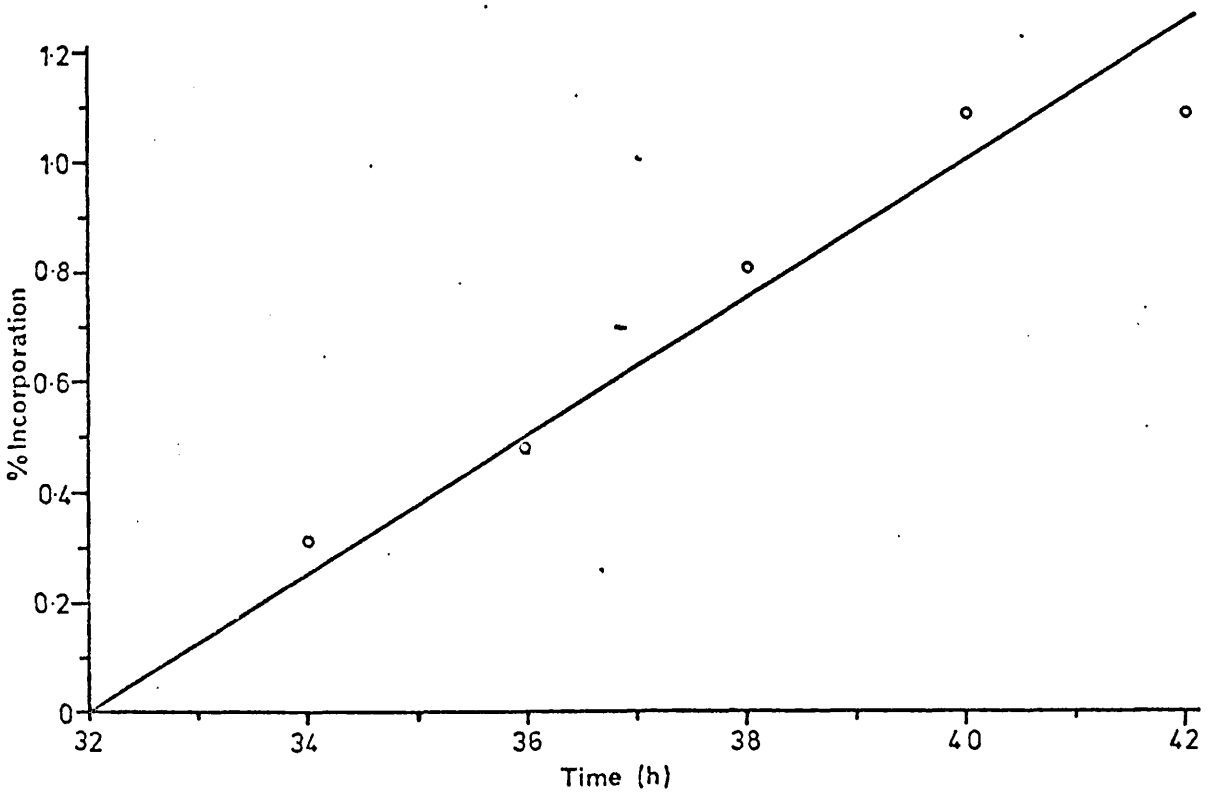


Fig. 6.2 Time Course of Incorporation of [2-^{14}C]MVA into Lipids

A mycelial culture, grown for 32h, was inoculated with DL[2-^{14}C]MVA (100nCi/ml). Aliquots were removed every 2h, and the lipids were extracted and radioassayed. Incorporations are based on the percentage of the biologically active isomer incorporated

TABLE 6.4 PURIFICATION OF β-CAROTENE AND STEROLS FROM ORGANELLES ISOLATED FROM MYCELIA GROWN WITH [2-^{14}C]MVA

Carrier β-carotene and sterols were added to the organelles before extraction. The incorporation into lanosterol was calculated assuming identical recoveries of lanosterol and ergosterol.

SAMPLE	RADIOACTIVITY (d.p.s.)				RECOVERY (%)
	Total Lipid	β-Carotene	Ergosterol	Lanosterol	
Lipid Droplets	67	19	40	1.5	90
Vacuoles	32	6	23	2.0	97
Mycelia (see Table 6.3)	471	121	377	10.0	108

t.l.c. (System 1; Table 2.2). Since insufficient radioactivity was present in some cases the extracts were not further purified.

The incorporation of [2-^{14}C]MVA into lipids was linear with time in each fraction (Fig. 6.3a). Since no lag was observed any transfer of lipids between organelles must have occurred very rapidly. The incorporation of radioactivity into the β-carotene and sterols of both vacuoles and globules was also linear with time (Fig. 6.3b,c). The specific activities of β-carotene and sterols in the vacuoles isolated after 1h were approximately double those in the lipid droplets, but they were similar in both organelles in mycelia incubated for 3 and 5h. However, this is probably a reflection of the smaller amount of β-carotene present in the vacuoles at the start of the experiment. The similarity of the specific activities after 3 and 5h suggests that lipids are able to move between the two fractions.

The linearity of [2-^{14}C]MVA incorporation with time suggests that β-carotene may be synthesized in both lipid droplets and vacuoles, or possibly that it is synthesized at a third location and then transported to these organelles simultaneously. However, since radioactive material apparently moves from one site to the other, the most likely explanation for these results is that β-carotene is synthesized at one site, and rapidly transported to the other.

If this is the case then repetition of the experiment using larger amounts of radioactive material and shorter incubation periods might have produced results as conclusive as those of Knapp et al. (1969; see p. 178). Unfortunately this was financially impracticable.

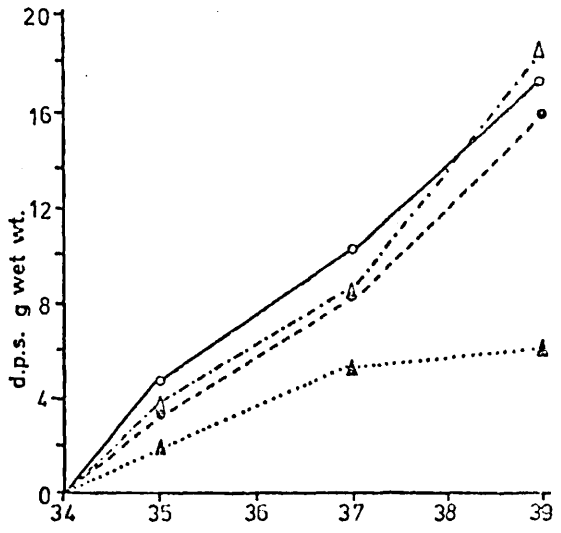


Fig. 6.3a

Incorporation into Total Lipids

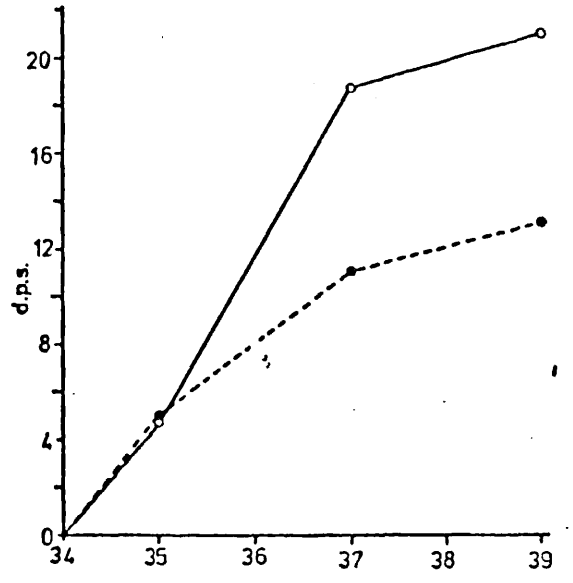


Fig. 6.3b

Incorporation into β-carotene

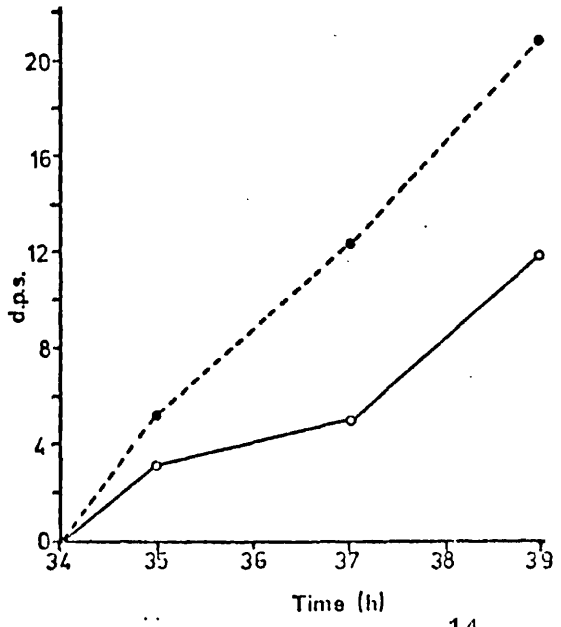


Fig. 6.3c

Incorporation into Sterols

Fig. 6.3 Incorporation of [2-^{14}C]MVA into Lipids of Isolated Cell Fractions

o = lipid globules

● = vacuoles

Δ = "membranes" (microsomes and mitochondria)

▲ = 100 000g supernatant

INCORPORATION OF [2-^{14}C] MEVALONIC ACID IN ISOLATED ORGANELLES

Because attempts to deduce the site of carotenogenesis from the incorporation of [^{14}C]MVA *in vivo* were unsuccessful, incorporation in cell-free extracts was studied. Since 1 molecule of β-carotene is synthesized from 8 molecules of MVA, its specific activity should be eight times that of the precursor, i.e. approximately 7180 d.p.s./μg in the experiments described in Chapter 6. This is 10^4 times greater than the actual specific activities obtained. *In vivo* incorporation experiments are complicated by the slow transport of the labelled compound into the cell, and its subsequent dilution by endogenously-produced precursors. However cell-free extracts do not have a permeability barrier, so the substrate is supplied in high concentration to the enzymes involved in its metabolism. Because of this the specific activity of β-carotene synthesized *in vitro* should be closer to the theoretical maximum, and consequently it should be easier to deduce the true site of biosynthesis.

Incorporation of [2-^{14}C]MVA in Cell Homogenates of the C115 Mutant of *Phycomyces blakesleeanus*

Mycelia were homogenised in 50mM Hepes, pH 7.4, containing 0.4M mannitol, and aliquots (0.88ml) were added to tubes containing DL-[2-^{14}C]MVA (0.1μCi; 7.7nmoles), together with the cofactors used by Bramley and Davies (1975; see p.90). After incubation for 2h in the dark at 25°C in a shaking water bath, ethanol/ether (3:1 v/v, 3 vol.) was added, together with carrier terpenoids (p. 91), and the lipids were extracted, saponified, and radioassayed, as described previously.

Incorporation of approximately 0.03% into unsaponifiable lipids were obtained using this procedure, so the experiment was repeated using 50mM tris-HCl, pH 8.0, containing 0.4M mannitol, since this pH is optimal for carotenoid synthesis in extracts of lyophilized mycelia of *P.blakesleeanus* (Bramley, 1973). Under these conditions the maximum incorporation was only 0.33%.

With both these buffers the incubations became cloudy as soon as the enzyme was added, suggesting that the buffer might not have sufficient capacity to prevent protein from being denatured. When 0.2M tris-HCl containing 0.4M mannitol was employed incorporations of 40 to 50% were obtained.

Mycelia were grown for 40h after inoculation, and then homogenised in 0.2M tris-HCl, pH 8.0, containing 0.4M mannitol (4 vol., w/v). After removal of cell debris (800g, 3 min), aliquots (0.82ml) were added to tubes containing cofactors (0.18ml) and DL-[2-^{14}C]MVA (0.5 µCi; 13mCi/mmole). Duplicate incubations were carried out both aerobically and anaerobically for 2h at 25°C, after which the lipids were extracted, saponified, and separated by t.l.c. (System 4, Table 2.2). Sterols, β-carotene, and squalene and phytoene were further purified by t.l.c. (Systems 2, 3, and 6 respectively).

Slightly more radioactivity was incorporated in the anaerobic incubations, but these contained more [^{14}C]squalene, and less [^{14}C] sterols (Table 7.1). This result is predictable on theoretical grounds since the cyclisation of squalene to lanosterol is an aerobic process (Tchen and Bloch, 1957; p.20).

The specific activities of the β-carotene isolated from the anaerobic incubations were 14.3 and 1.03 d.p.s./µg after the first t.l.c. separation but these converged to similar values (1.39 and 1.23 respectively) after purification (t.l.c., System 3, Table 2.2), indicating that under anaerobic conditions the conversion of phytoene to β-carotene is inhibited.

Since oxygen has previously been shown to be unnecessary for β-carotene biosynthesis (Bramley and Davies, 1975), this experiment was repeated, using the same purification procedures as Bramley and Davies (1975), i.e. column chromatography rather than t.l.c. for the preliminary separation of unsaponifiable lipids. Although this method produced a better recovery of radioactivity, the results (Table 7.2) were similar to those obtained in the previous experiment. The specific activities of β-carotene after the preliminary column chromatography separation were 1.67 and 0.98 d.p.s./µg in the anaerobic incubations, declining to 0.17 and 0.12 d.p.s./µg respectively when the carotene was purified by t.l.c. (System 3), indicating that β-carotene is not synthesized in the absence of oxygen.

These observations are difficult to explain, since oxygen is not thought to be directly involved in β-carotene biosynthesis, and is not required for β-carotene production in cell extracts prepared from lyophilized mycelia (Bramley and Davies, 1975). However a similar contradiction exists with regard to carotene biosynthesis in tomato fruits; oxygen is required for lycopene production by cell homogenates

TABLE 7.1 - INCORPORATION OF [2-^{14}C]MVA INTO MYCELIAL HOMOGENATES - EXPERIMENT 1

Mycelia were homogenised in 0.2M tris-HCl, pH 8.0, containing 0.4M mannitol, to give a final protein concentration of 2.7 mg/ml in the incubations. Figures are the averages of duplicates. Incorporations are the percentage of the active isomer incorporated. The control was boiled for 2 min.

INCUBATION CONDITIONS	INCORPORATION INTO UNSAPONIFIABLE LIPIDS					
	TOTAL	PHYTOENE	β-CAROTENE	SQUALENE	LANOSTEROL	ERGOSTEROL
CONTROL						
D.p.s.	25					
% Incorporation	0.3					
AEROBIC						
D.p.s.	3843	68	401	1215	1142	114
% Incorporation	41.5	0.7	4.3	13.1	12.3	1.2
ANAEROBIC						
D.p.s.	4355	81	59	1977	60	54
% Incorporation	47.1	0.9	0.6	21.4	0.6	0.6

TABLE 7.2 INCOPORATION OF [2-^{14}C]MVA INTO MYCELIAL HOMOGENATES - EXPERIMENT 2

Experimental details are the same as for Table 7.1, except that the protein concentration was 3.6 mg/ml; and the lipids were separated by chromatography on grade III alumina and then purified by t.l.c.

	INCORPORATION INTO UNSAPONIFIABLE LIPIDS					
	TOTAL	PHYTOENE	β-CAROTENE	SQUALENE	LANOSTEROL	ERGOSTEROL
CONTROL						
D.p.s.	10					
% Incorporation	0.1					
AEROBIC						
D.p.s.	2854	142	294	1264	675	23
% Incorporation	30.9	1.5	3.2	13.7	7.3	0.2
ANAEROBIC						
D.p.s.	2714	548	6	2192	120	18
% Incorporation	29.3	5.9	0.1	23.7	1.3	0.2

(Varma and Chichester, 1962), but not by purified enzymes (Jungalwala and Porter, 1967; Kushwaha et al., 1970).

The reduction in the apparent specific activity of anaerobically-biosynthesized β-carotene observed on t.l.c. in System 3 suggests that it is contaminated with another carotenoid, which has a similar mobility on t.l.c. in System 4. This could be an intermediate between phytoene and β-carotene which accumulates under anaerobic conditions, and its behaviour on column chromatography suggests that it could be phytofluene, ζ-carotene, or β-zeacarotene. However no attempt was made to confirm this suspicion.

Purification of Terpenoids Synthesized from [2-^{14}C]MVA

When unsaponifiable lipids were separated by chromatography on neutral alumina (activity grade III), squalene and phytoene were eluted from a 10g column by light petroleum (b.p. 40-60°C; 30ml); β-Carotene was eluted by diethyl ether in light petroleum (0.25% v/v, 30ml); and other lipids (mainly sterols) were eluted with diethyl ether (30ml). In order to ensure that this procedure yielded distinct fractions terpenoids synthesized in vitro from [2-^{14}C]MVA were separated by column chromatography, and then re-chromatographed. Duplicate aliquots of each fraction were chromatographed, and in every case 90 to 97% of the applied radioactivity was recovered in the expected fraction, with less than 1.5% being found in the other fractions.

Since large amounts of u.v.-absorbing material were eluted from the alumina and silica gel used in these separations it was not possible to determined directly the radiochemical purity of phytoene by purifying it to constant specific activity. Instead mixtures of phytoene and squalene, synthesized in vitro and separated by column chromatography, were subjected to t.l.c. in Systems 5 and 6 (Table 2.2). In each case a similar proportion (11.2 to 13.6%) of the total radioactivity was found in the zone corresponding to phytoene. Since separation in System 6 is based on polarity, while argentation t.l.c. (System 5) separates compounds on the basis of the number of double bands present, this suggests that phytoene purified by t.l.c. System 6 is radiochemically pure.

Only one other radioactive band (R_F 0.25) was present when

these compounds were separated by t.l.c. in System 6; this contained 86% of the total radioactivity, and co-chromatographed with squalene. However argentation t.l.c. (System 5) resolved this into two bands (R_F values 0.2 and 0.55), which contained respectively 33 and 54% of the recovered radioactivity. The latter band corresponded to squalene, but the former did not stain with Rhodamine 6G, and is thus unlikely to be a degradation product of any of the major lipids present.

In order to ascertain whether this separation was reproducible a sample of squalene, synthesized in another experiment, was purified by chromatography on a column of grade II alumina, from which it was eluted with light petroleum (b.p. 40-60°C; 50ml). This too separated when chromatographed in System 5, 7.% of the recovered radioactivity being found in the zone at R_F 0.2.

Since the R_F of this unknown compound is between that of squalene (6 double bonds) and phytoene (9 double bonds) it could be lycopersene. This has been detected in other cell-free carotenogenic systems when they are supplied with NADPH (pp. 30-31), and is probably produced by squalene synthetase (Qureshi et al., 1973b), but lycopersene should have an R_F intermediate between squalene and phytoene on t.l.c. in System 6 (Mercer et al., 1963). Alternatively this compound could be dihydro or tetrahydrosqualene, both of which have been identified in Halobacterium cutirubrum (Tornabene et al., 1969), but these would be expected to have an R_F greater than that of squalene on argentation t.l.c. Dehydrosqualene, which would have an R_F between squalene and phytoene in System 5, and which has been detected in Staphylococcus aureus (Suzue et al., 1968), would also have an intermediate R_F in System 6 (Taylor and Davies, 1974). Because of these contradictions the identity of this radioactive material remains unclear.

Effect of Protein Concentration on Incorporation of MVA by Cell Homogenates

Mycelia, homogenised in 0.2M Tris-HCl, pH 8.0, containing 0.4M mannitol, were centrifuged at 800g (3 min) to produce a supernatant which contained 3.4mg protein/ml. A series of incubations (1ml) containing DL-[2-^{14}C]MVA (0.5μCi; 38.5nmoles), cofactors, buffer, and protein (0.35 to 2.80mg) were incubated for 15 min, and then extracted in the usual manner.

Approximately 10% of the added radioactivity was incorporated into unsaponifiable lipids by incubations which contained over 2mg of protein, while those containing below 1mg incorporated only about 0.2% of the radioactivity (Fig. 7.1). The distribution of radioactivity amongst the various terpenoids was similar in each case.

These results indicate that some cooperation between membranes and supernatant proteins may be required for terpenoid synthesis. The supernatant could be involved in the synthesis of precursors, or in the transfer of intermediates from one organelle to another. In very dilute solutions the interaction between supernatant and organelles is greatly reduced, and no incorporation occurs. Since even [^{14}C]squalene is not synthesized under these conditions it seems likely that the conversion of MVA to squalene is not catalysed entirely by supernatant enzymes, a conclusion which is in agreement with results obtained from cell fractionation experiments on Cochliobolus heterostrophus (Kawaguchi et al., 1973b; p.76).

Incorporation of [2-^{14}C]MVA in Isolated Cell Fractions

Mycelia were homogenised in buffered sorbitol (70%w/v), and organelles were separated by flotation (Table 4.1). Vacuoles were collected by sedimentation (40 000g, 15 min), the sample zone was diluted, and membranes were collected from it by centrifugation (140 000g_{av}, 45 min). Supernatant and lipid droplet fractions were prepared by homogenising mycelia in 0.2M Tris-HCl, pH 8.0, containing 0.4M mannitol, and centrifuging the homogenate at 140 000g_{av} (45 min).

Portions of each fraction were resuspended in tris-mannitol buffer or supernatant, and aliquots (0.82ml) were added to tubes containing DL-[2-^{14}C]MVA (0.5µCi) and cofactors. Incubations were carried out aerobically, in the dark, at 25°C for 2h. Lipid extraction and separation procedures were the same as those used previously.

No incorporation of [2-^{14}C]MVA occurred in organelles incubated in the absence of supernatant enzymes (Table 7.3). This is in agreement with the results of other workers who have shown that the enzymes involved in the conversion of MVA to FPP are cytosolic (Kawaguchi et al., 1973b; Shimizu et al., 1973; p.76). However the supernatant was able to incorporate 20% of the MVA into unsaponifiable lipids when it was incubated alone. Of this incorporation 28% was in β-carotene and phytoene and 28% in sterols. No increase in incorporation

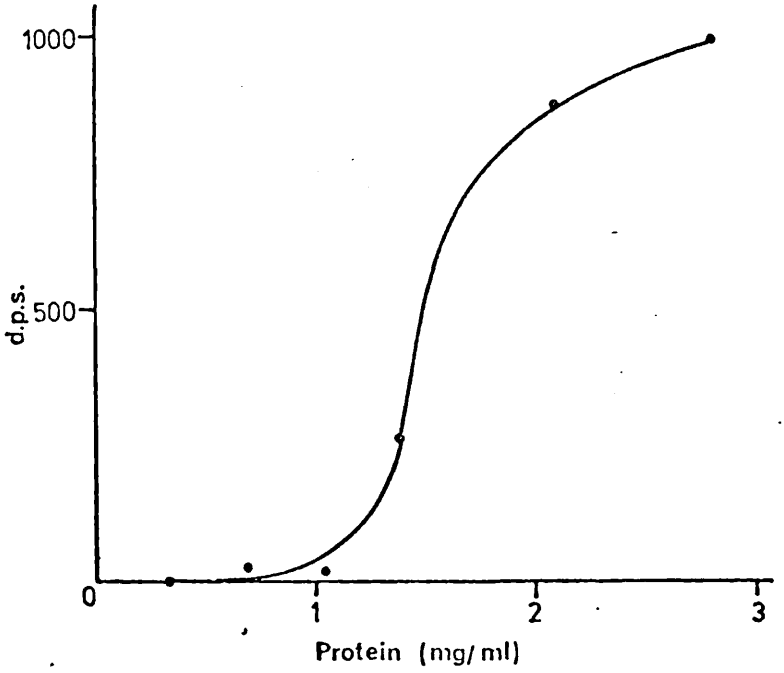


Fig. 7.1 Effect of Protein Concentration on Incorporation of [2-^{14}C]MVA by a Mycelial Homogenate

TABLE 7.3 INCORPORATION OF [2-^{14}C]MVA IN ISOLATED ORGANELLE FRACTIONS - EXPERIMENT 1

Organelles were suspended in 0.2M tris buffer or in 0.2M Tris buffer containing supernatant enzymes. Incubation volumes were 1.0ml. Incorporations (%I) are the percentage of the active isomer incorporated. N.A. means not assayed.

SAMPLE	PROTEIN (mg/ml)	INCORPORATION INTO UNSAPONIFIABLE LIPIDS					
		TOTAL	PHYTOENE	β-CAROTENE	SQUALENE	LANOSTEROL	ERGOSTEROL
MEMBRANES:	2.00						
D.p.s.		23					
%I		0.2					
VACUOLES:	0.15						
D.p.s.		5					
%I		0.1					
GLOBULES:	N.A.						
D.p.s.		9					
%I		0.1					
SUPERNATANT:	1.40						
D.p.s.		1812	140	363	839	262	251
%I		19.6	1.5	3.9	9.1	2.8	2.7
SUPERNATANT & MEMBRANES:	3.20						
D.p.s.		1234	26	95	880	227	26
%I		13.3	0.3	1.0	9.5	2.5	0.3
SUPERNATANT & VACUOLES:	1.72						
D.p.s.		1285	47	138	948	96	41
%I		13.9	0.5	1.5	10.2	1.0	0.4
SUPERNATANT & GLOBULES:	1.42						
D.p.s.		2090	141	229	374	318	84
%I		22.6	1.5	2.5	4.0	3.4	0.9

into either carotenes or sterols was observed when organelles were incubated in the presence of the supernatant.

Since data from the previous experiment (p.188) suggested that the supernatant alone is unable to synthesise carotenes or sterols, it is probable that the 140 000g_{av} supernatant used in this experiment contained membranous material. This is in agreement with the distributions of marker enzymes observed in earlier studies (pp. 116 and 146).

Because the presence of this material makes it impossible to observe any carotenogenic activity in other organelles, an attempt was made to remove any remaining membranes by centrifuging the homogenate at 260 000g_{av} (1h). Clear supernatant was collected from between the pellet and the floating lipid droplets using a syringe, and aliquots (0.82ml, 1.05mg protein) were incubated with [2-^{14}C]MVA and cofactors. Incorporation into unsaponifiable lipids was 5.5% in 2h.

The experiment described in Table 7.3 was repeated, using identical procedures, except that lipid droplets and supernatant were collected from a homogenate which had been centrifuged at 260 000g_{av} (45 min). In addition a sample of crude homogenate (800g supernatant) was stored at 6-8^{o}C while the organelles were separated from the remainder of the homogenate. This acted as a control to ensure that carotenogenic activity was not completely lost during the course of the experiment.

Centrifugation at this higher speed reduced incorporation by the supernatant of MVA into carotenes (6 fold) and sterols (3.5 fold), but the addition of lipid droplets to the supernatant increased the production of [^{14}C]carotenes (Table 7.4). The addition of either vacuoles or lipid droplets increased incorporation into squalene, but not sterols.

Although these results suggest that lipid droplets possess some carotenogenic activity, the evidence is not conclusive, since the addition of either globules or vacuoles stimulated total incorporation more than it increased incorporation into carotenes. The reason for the increased incorporation of MVA into squalene in the presence of organelles is unclear, but the addition of membranous material (sedimented from the sample zone of the gradient used to isolate vacuoles) to a supernatant /lipid globule mixture further increased incorporation into squalene (Table 7.4).

TABLE 7.4 INCORPORATION OF [2-^{14}C]MVA IN ISOLATED ORGANELLE FRACTIONS - EXPERIMENT 2

Organelles, isolated by density gradient centrifugation, were suspended in 260 000g supernatant and incubated for 2h in a total vol. of 1.0ml. Controls were boiled for 2 min. All figures are the averages of two experiments, both in duplicate, except for those marked '*' which are the results of one experiment only. Incorporations (%I) are the percentage of the active isomer incorporated.

SAMPLE	PROTEIN (mg/ml)	INCORPORATION INTO UNSAPONIFIABLE LIPIDS				
		TOTAL	PHYTOENE	β-CAROTENE	SQUALENE	STEROLS
HOMOGENATE:*	1.41					
D.p.s.		1765	59	17	1726	90
%I		19.1	0.6	0.2	18.7	1.0
BOILED HOMOGENATE:*	1.41					
D.p.s.		13		2		
%I		0.1				
BOILED SUPERNATANT:*	0.97					
D.p.s.		6				
%I		0.1				
SUPERNATANT:	0.97					
D.p.s.		410	52	27	190	150
%I		4.4	0.6	0.3	2.0	1.6
SUPERNATANT & VACUOLES:	1.48					
D.p.s.		1006	57	13	905	50
%I		10.9	0.6	0.1	9.8	0.5
SUPERNATANT & GLOBULES:	1.01					
D.p.s.		827	122	33	593	73
%I		8.9	1.3	0.4	6.4	0.8
SUPERNATANT & GLOBULES & MEMBRANES:*	1.71					
D.p.s.		1407	36	52	1241	119
%I		15.2	0.4	0.6	13.4	1.3

The high levels of incorporation of [2-^{14}C]MVA into β-carotene, sterols and squalene by the supernatant could indicate that it contains membranous material, or that the enzymes involved are soluble. Klein (1960) observed that a 100 000g supernatant from a yeast homogenate was able to incorporate [1-^{14}C]acetate into non-saponifiable lipids (mainly squalene). He later showed that this activity resided in small particles which resembled those involved in fatty acid synthesis (Klein, 1965). Since these results are at variance with the apparently particulate location of squalene synthesis in other organisms (see p. 76) it is likely that under certain conditions enzymes involved in terpenoid biosynthesis may become detached from membranes and released into the supernatant fraction. If such a solubilization of enzymes is occuring in these experiments then a more thorough investigation of the subcellular location of carotene synthesis must await the development of an alternative homogenisation procedure, or the use of substrates which may be converted to carotenes by isolated particles without any requirement for supernatant enzymes.

Incorporation of MVA into Terpenols

Since the supernatant is required for the conversion of MVA into terpenoids the nature of the pyrophosphate intermediates synthesized by this fraction was investigated.

A homogenate was centrifuged at 260 000g_{av} (45 min), and supernatant (0.8ml) was incubated with DL-[2-^{14}C]MVA (0.5μCi) and cofactors at 25°C for 1h. The reaction was terminated by the addition of ethanol (4 ml), and terpenyl pyrophosphates were extracted with three portions of butan-1-ol (prewashed with water) and one portion of benzene (Islam et al., 1977). The extract was concentrated by evaporation under reduced pressure at 35°C, and redissolved in butan-1-ol containing a drop of ammonia.

Aliquots of this solution were applied to Whatman No. 1 chromatography paper, which was developed in propan-1-ol: ammonium hydroxide (sp.gr. 0.88): water (3:1:1 by vol.), or propan-2-ol:2-methylpropan-1-ol: ammonium hydroxide:water (40:20:1:39 by vol.). Radioactive spots were detected by gas ionisation and liquid scintillation counting, and were tentatively identified by reference to published R_F values (Dugan et al., 1968; Davies et al., 1975).

Chromatography in propanol:NH_3:H_2O showed radioactive zones corresponding to IPP, FPP and GGPP, isopentenyl phosphate (IP), farnesyl phosphate (FP), geranylgeranyl phosphate (GGP), and unidentified material (R_F 0.50 to 0.75; Fig. 7.2). Chromatography in the second system revealed radioactive spots corresponding to FP and FPP, and IP and IPP. Unidentified material chromatographing with an R_F of 0.55 to 0.75 was also present (Fig. 7.2). In each case the bulk of the radioactivity (90 to 95%) was in the unidentified material.

The remainder of the extract was evaporated to dryness under N_2, and redissolved in 0.1M tris-HCl, pH 8.0 (0.5ml). To this was added alkaline phosphatase (60 Sigma units) and $MgCl_2$ (0.6 µmoles). The terpenyl pyrophosphates were hydrolysed by incubating this mixture at 37°C for 4h, followed by incubation at room temperature (19°C) for 14h (Jungalwala and Porter, 1969). The hydrolysis was terminated by the addition of ethanol (2ml), and the liberated terpenols were extracted with light petroleum (b.p. 40-60°C) and separated by reversed-phase t.l.c. in acetone:H_2O (70:30 v/v, saturated with liquid paraffin; (Porter, 1969). Standard geraniol, farnesol and squalene were also chromatographed. The plate was stained with Rhodamine 6G and the bands corresponding to the markers were scraped off, eluted with ether, and radioassayed. The remaining zones were also eluted.

Radioactive bands were present corresponding to squalene (R_F 0.0) and farnesol (0.15 - 0.25). In addition a large amount of radioactive material was present in a zone of R_F 0.35 to 0.55 (Fig. 7.3). This probably contained geraniol and isopentenol. A small band (R_F about 0.05) probably contained radioactivity due to geranylgeraniol (Porter, 1969).

This experiment showed that the supernatant fraction was capable of incorporating [2-^{14}C]MVA into terpenyl pyrophosphates, an observation which agrees with results obtained for other fungi (Shimizu et al., 1973; Kawaguchi et al., 1973b). If this is one of its functions in vivo then it is probably a prerequisite for carotene biosynthesis by isolated organelles in vitro in order to convert MVA into prenyl pyrophosphates, which can then be converted to carotenes by particulate enzymes. If this is so then incubation of isolated

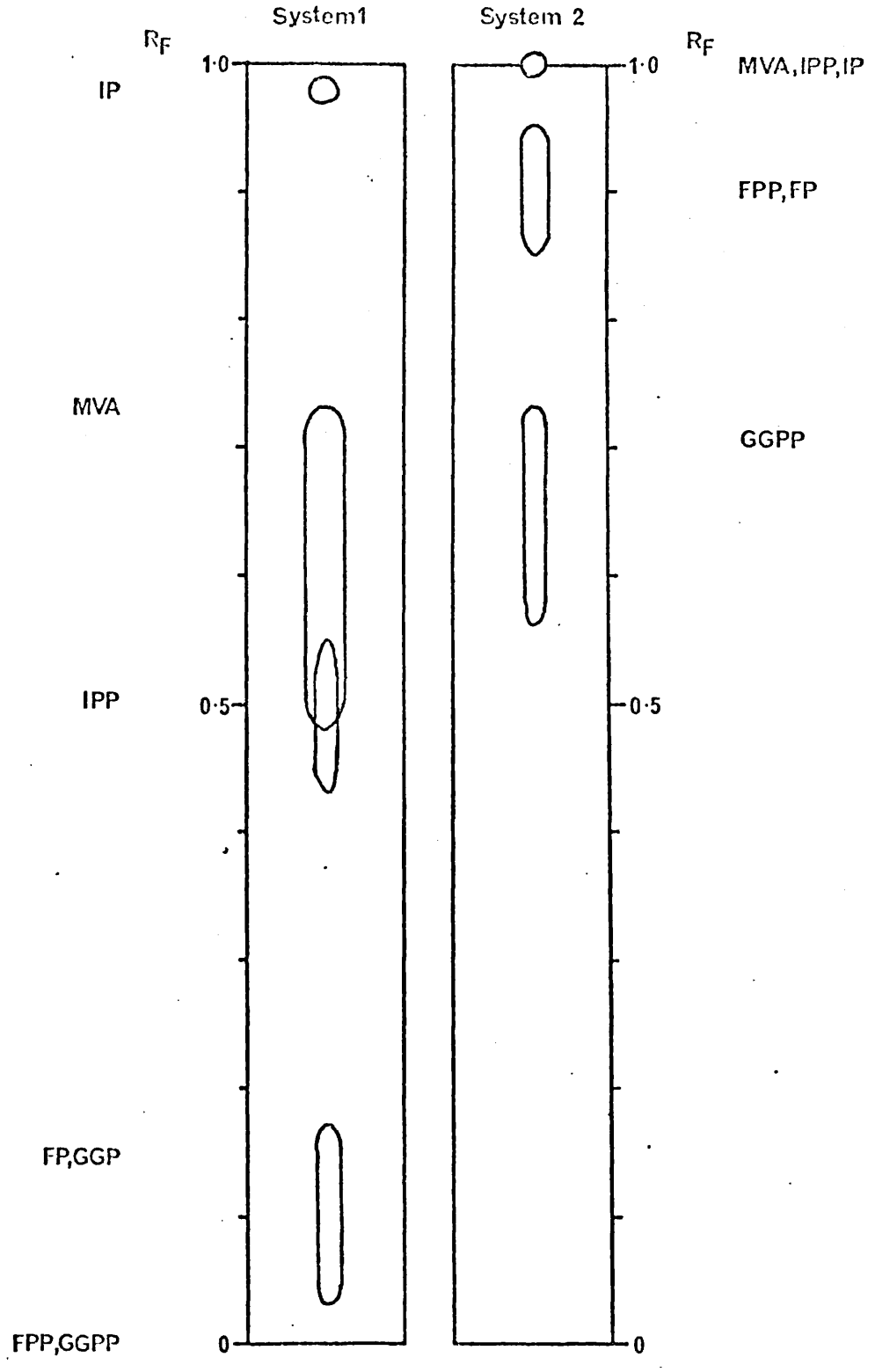


Fig. 7.2 Separation of [^{14}C]Terpenyl Pyrophosphates by Paper Chromatography

A 260 000g_{av} (45 min) supernatant (0.8ml) was incubated with [2-^{14}C]MVA for 1h. Terpenyl pyrophosphates were extracted and separated by paper chromatography in propan-1-ol: ammonium hydroxide : water (3:1:1 by vol.; System 1), or propan-2-ol:2-methylpropan-1-ol:ammonium hydroxide:water (40:20:1:39 by vol.; System 2).

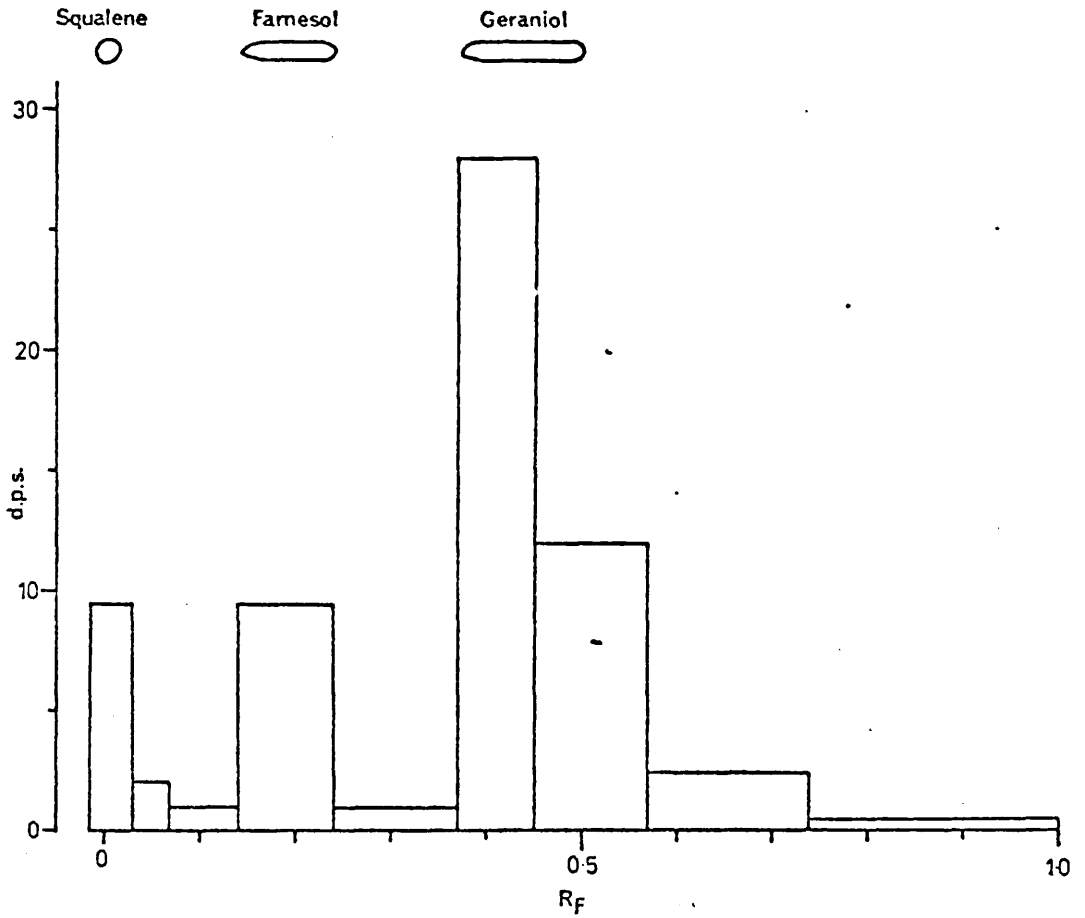


Fig. 7.3 Separation of [^{14}C]Terpenols by Reversed Phase Thin-Layer Chromatography

Terpenyl pyrophosphates, prepared as described in Fig. 7.2, were hydrolysed and chromatographed on Kieselgel G impregnated with light paraffin. This was developed in acetone/H_2O (70:30 v/v).

organelles with [^{14}C]GGPP, or possibly even [^{14}C]IPP, might result in an unambiguous demonstration of carotenogenic activity.

C O N C L U S I O N S

The isolation of organelles by density gradient centrifugation from mycelia of P.blakesleeanus C115 carS42 mad-107(-) has shown that nuclei, mitochondria and endoplasmic reticulum do not contain β-carotene, and that this pigment is confined to a floating, lipid droplet fraction and a sedimentable organelle (ρ=1.10 g/ml) which does not possess enzymes characteristic of either mitochondria or microsomes.

This result confirms a previous study, which suggested a dual location for β-carotene in mycelia of the Mucorales (Cederberg and Neujahr, 1970), but conflicts with previous reports of carotenoids in the mitochondria of N.crassa and P.blakesleeanus (Neupert and Ludwig, 1971; Keyhani et al., 1972). Both Keyhani et al. (1972) and Herber (1974) stated that the β-carotene associated with mitochondria isolated from P.blakesleeanus and M.hiemalis, respectively, could have been due to contamination with other organelles, and the results of these experiments (pp. 121, 136 and 140) have confirmed their conclusions. However, Neupert and Ludwig (1971) separated the inner and outer membranes of mitochondria isolated from N.crassa, and reported that only the outer membranes contained neurosporaxanthin. The outer membranes of mitochondria prepared from P.blakesleeanus by density gradient centrifugation are colourless, but they have a similar density to the pigmented fraction (p. 130). This suggests that aggregation of mitochondria and pigmented organelles could also have occurred in the work of Neupert and Ludwig (1971).

Although the initial objective of this project was to determine the subcellular location of β-carotene, the sterol and phospholipid contents of the purified organelles were also estimated, since these compounds are known constituents of membranes. These lipids were found in the same ratio in each fraction (1:10, p. 148), suggesting that they are components of membranes in Phycomyces, but the distributions of β-carotene and sterols were widely dissimilar indicating that β-carotene does not have a function in membranes of the type which has been proposed previously (see p. 49). Since β-carotene accumulates in the fat globules, it is possible that it is formed as a sink for excess carbon atoms (Attwood, 1971), although it could also exert a photo-protective effect of the type suggested by Goldstrohm and Lilly (1965, p. 48).

The identity of the sedimentable pigmented organelle fraction was not established, but the large degree of light scattering caused by the particles (p. 121) suggests that they are fairly large, probably about the same order of size as mitochondria. Although it was not the only fraction which possessed hydrolytic enzyme activity, the presence of some hydrolases, together with the absence of enzymes characteristic of other organelles, and also the low density of this fraction, suggest that it may contain vacuoles. The heterogeneity of distribution of hydrolytic enzymes observed in these experiments probably reflects in part an in vivo heterogeneity, as well as damage to the vacuoles during homogenisation and isolation. This problem could probably be mitigated by the use of density gradient solutes which exert a lower osmotic pressure than sorbitol (e.g. Ficoll and Metrizamide), but although some α-mannosidase activity remained in the sample zone during the flotation of vacuoles (p. 154) this was not accompanied by any β-carotene, so no attempt was made to improve the isolation procedure. Probably the α-mannosidase in the sample zone was due to the presence of fragments of vacuole membranes, rather than intact vacuoles, since no β-carotene remained in the sample zone. Furthermore, homogenisation did not appear to release carotene from the sedimentable fraction into the floating fraction, since the relative distribution of β-carotene between these fractions was unaffected by the degree of homogenisation (p. 164). This suggests that these two fractions are distinct from each other in vivo. However, the similarity in their amino acid compositions, and the presence of alkaline p-nitrophenylphosphatase activity, as well as β-carotene in both fractions, suggests that they may be related. Furthermore, the similarities in the specific activities of β-carotene in vacuoles and lipid globules isolated from mycelia incubated for 3 to 5h in [2-^{14}C]MVA (p. 181) suggest that material is able to move from one site to the other.

In higher plants lipid globules may develop from spherosomes, which are sedimentable and may contain hydrolytic enzymes (see pp. 69-72). If this process occurs in fungi then the fraction identified as "vacuoles" may also contain spherosomes, and these could contain the β-carotene observed in this fraction. However this development has not been unambiguously demonstrated in any organism (p. 72), and when the "vacuole" fraction was subjected to isopycnic centrifugation

on a shallow gradient all the material banded at the same density (p. 142), indicating that no transitional forms between spherosomes and lipid globules were present. However, vacuoles have been implicated in the digestion of lipids by fungi (p. 72), and lipid droplets have been observed in vacuoles in Rhodotorula gracilis (Ruinen et al., 1968) and S.cerevisiae (Matile et al., 1969; Bauer and Sigarlakie, 1975). Osmiophilic globules were also visible inside vacuoles in electron micrographs of mycelia of the C115 mutant of P.blakesleeanus (Figs. 3.5, 3.7). Their dimensions (approx. 0.3 to 0.8μm diameter) were similar to those of isolated lipid droplets (p. 169), suggesting that the pigmentation of the vacuole fraction may be due to the presence of lipid droplets which have been absorbed, and are undergoing digestion.

If this is the correct interpretation of the experimental data then lipid globules will probably possess carotenogenic activity, although carotenoids could be synthesized at another subcellular site, and then transported to the globules.

Previous work on the subcellular distribution of terpenoid-synthesizing activity in fungi has been confined to investigations on sterol and sesquiterpene biosynthesis (pp. 76-77). These have established that the conversion of MVA to FPP is catalyzed by supernatant enzymes, whereas the later reactions of sterol biosynthesis occur on the E.R. When mycelia of the C115 strain of P.blakesleeanus were grown in medium containing [2-^{14}C]MVA, most of the radioactivity in the supernatant was in the form of hydrophilic compounds, presumably MVA and prenyl pyrophosphates, whereas the sedimentable radioactivity was mostly incorporated into lipids (p. 175). This indicates that the distribution of terpenoid-synthesizing activity in this organism is similar to that reported for other fungi. This conclusion was reinforced by the low rate of incorporation of [2-^{14}C]-MVA by isolated organelles and dilute suspensions (pp. 188-192), and by chromatography of the radioactive compounds synthesized by the supernatant fraction (p. 195).

Because of this requirement for supernatant enzymes to carry out the initial conversions of MVA to prenyl pyrophosphates no isolated fractions showed any carotenogenic activity when incubated with [2-^{14}C]-MVA. Unfortunately the supernatant was able to synthesize significant

amounts of phytoene, β-carotene and sterols when it was incubated alone, and this endogenous activity may have masked any carotenogenic activity of other organelles when they were incubated with it. When supernatant enzymes were incubated together with lipid droplets the incorporation of MVA into both phytoene and β-carotene was stimulated, but so was incorporation into squalene. Hence no firm conclusion can be reached about carotenogenic activity in the lipid droplets. Incorporation into squalene was also stimulated by the addition of either vacuoles or a membrane fraction, suggesting that either the enzymes involved in squalene synthesis are distributed ubiquitously on the membranes of the cell, or possibly that they have been solubilized during cell fractionation, but require a membranous interface for maximal activity. The latter possibility is reasonable, since FPP is water-soluble, whereas squalene itself is hydrophobic. If the enzymes of squalene biosynthesis have become soluble during the isolation of organelles then it is also possible that the carotenogenic enzymes have been solubilized in a similar manner. If this is so then further investigations on the subcellular site of carotene biosynthesis will have to employ a different method of cell fractionation, or else the isolated organelles will have to be incubated with other substrates, such as [^{14}C]GGPP, which can be converted directly into carotenes without requiring initial metabolism by supernatant enzymes.

Although it was not possible to demonstrate directly whether lipid droplets possessed carotenogenic activity, some circumstantial evidence suggests that they are the site of β-carotene biosynthesis. Since the mean diameter of the lipid droplets does not alter with age (p. 169), increases in the triglyceride content of the cell must require the production of more droplets, rather than the enlargement of older globules. However the proportion of β-carotene in the lipid globules increased linearly with the age of the mycelium (p. 166). This increase could be due to the presence within the globules themselves of carotenogenic enzymes which remain active throughout growth, or to the continuous activity of extra-globular enzymes, the products of which accumulate in the droplets as the mycelium ages. However, the increase in β-carotene concentration in each globule, appears to be linear with the age of the mycelium, and since the number of globules

also increases with time this linear increase in β-carotene content per droplet would require an accelerating rate of biosynthesis at an extra-globular site, but only a constant rate of biosynthesis if the enzymes were present within each droplet. Although the former explanation is possible, the linear increase of β-carotene concentration with time makes the latter hypothesis more likely.

Although lipid droplets contain very little protein (Table 1.2 and p. 170), globules from maize seedlings and castor bean seeds have been shown to possess enzymes for lipid biosynthesis (Semadeni, 1967; Harwood et al., 1971, p. 68). Harwood et al. (1971) suggested that the surface of the globule could provide an interface at which hydrophilic enzymes could be in contact with water-soluble substrates and coenzymes, while remaining able to release hydrophobic products into a lipophilic environment. Since the lipid droplets of Phycomyces are probably surrounded by a layer of protein (pp. 160 and 170) this could serve the same function, since the prenyl pyrophosphate precursors of carotenes are water-soluble, whereas the products of GGPP condensation are hydrophobic.

An ultimate aim of this project was the isolation of an organelle(s) able to synthesize carotenoids in vitro, so that studies on the enzymology of these processes could be facilitated. Although the C115 mutant of P.blakesleeanus synthesizes significantly greater amounts of β-carotene than most other carotenogenic fungi, the total amount synthesized (approx. 0.3% of total dry weight after 96h; Figs. 3.1 and 3.2) is still so low that the specific activities of carotenogenic enzymes in a mycelial homogenate would also be extremely low. However, if lipid droplets do contain the enzymes of carotenoid biosynthesis then the preparation of a crude lipid droplet fraction from e.g. a carB mutant of Phycomyces would represent an approximately 200-fold purification of phytoene synthetase. Because such a simple and highly effective method for the initial purification of this enzyme is probably feasible it should now be possible for studies on the enzymology of carotene biosynthesis in microorganisms to begin to approach the same degree of sophistication as recently reported investigations on phytoene synthesis in tomato fruits (Maudinas et al., 1975, 1977; Islam et al., 1977).

B I B L I O G R A P H Y

Aebi, H. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), 2nd edn., pp.673-684, Academic Press, London and New York

Agardh, C.A. (1817) Synopsis algarum Scandinaviae (Lund), p. 46 (citation from Cerdá-Olmedo, 1974)

Agogbua, S.I. and Wynn, C.H. (1976) Biochem.J. 153, 415-421

Agranoff, B.W., Eggerer, H., Henning, U. and Lynen, F. (1960) J.Biol.Chem. 235, 326-332

Ainsworth, G.C. (1971) Ainsworth and Bisby's Dictionary of the Fungi, 6th edn., Commonwealth Mycological Institute, Kew, Surrey

Akhtar, M., Hunt, P.F. and Parvez, M.A. (1967) Biochem.J. 103, 616-622

Allen, C.F., Good, P., Mollenhauer, H.H. and Totten, C. (1971) J.Cell Biol. 48, 542-546

Allen, C.M., Alworth, W., Macrae, A. and Bloch, K. (1967) J.Biol.Chem. 242, 1895-1902

Allfrey, V. (1959) in The Cell (Brachet, J. and Mirsky, A.E., eds), Vol. 1, pp. 193-290, Academic Press, London and New York

Alexander, G.J., Gold, A.M. and Schwenk, E. (1957) J.Amer.Chem.Soc. 79, 2967

Alexander, K.T.W., Akhtar, M., Boar, R.B., McGhie, J.R. and Barton, D.H.R. (1972) Chem.Commun. 7, 383-385

Alexander, K.T.W., Mitropoulos, K.A. and Gibbons, G.F. (1974) Biochem.Biophys.Res.Commun. 60, 460-467

Altman, L.J., Ash, L., Kowerski, R.C., Epstein, W.W., Larsen, B.R., Rilling, H.C., Muscio, F. and Gregonis, D.E. (1972) J. Amer.Chem.Soc. 94, 3257-3259

Anderson, D.G. and Porter, J.W. (1962) Arch.Biochem.Biophys. 97, 509-519

Anderson, D.G., Rice, M.S. and Porter, J.W. (1960) Biochem.Biophys Res.Commun. 3, 591-595

Anderson, N.G., Harris, W.W., Barber, A.A., Rankin, C.T. and Chandler, E.L. (1966) Nat.Cancer Inst.Monogr. 21, 256

Anderson, S.M. and Krinsky, N.I. (1973) Photochem.Photobiol. 13, 403-408

Arlinghaus, R.B. and Ascione, R. (1972) in Methods in Molecular Biology (Last, J.A. and Laskin, A.I. eds.), vol. 2, pp. 29-57, Marcel Dekker, New York

Aragón, C.M.G., Murillo, F.J., de la Guardia, M.D. and Cerdá-Olmedo, E. (1976) Eur.J.Biochem. 63, 71-75

Armentrout, V.N., Smith, G.G. and Wilson, C.L. (1968) Amer.J.Bot. 55, 1062-1067

Armentrout, V.N., Hünssler, G. and Maxwell, D.P. (1976) Arch.Microbiol. 107, 7-14

Ashwell, M. and Work, T.S. (1970) Ann.Rev.Biochem. 39, 251-290

Attwood, M.M. (1971) Antonie van Leeuwenhoek J.Microbiol.Serol. 37, 369-378

Aung Than, Bramley, P.M., Davies, B.H. and Rees, A.F. (1972) Phytochem. 11, 3187-3192

Austin, D.G., Bu'Lock, J.D. and Winstanley, D.J. (1969) Biochem.J. 113, 34P

Austin, D.G., Bu'Lock, J.D. and Drake, D. (1970) Experientia 26, 348-349

Avers, C.J. (1971) Sub-Cell. Biochem. 1, 25-37

Balz, J-P (1966) Planta 70, 207-236

Bandlow, W. (1972) Biochim.Biophys.Acta 282, 105-122

Barnes, F.J., Qureshi, A.A., Semmler, E.J. and Porter, J.W. (1973) J.Biol.Chem. 248, 2768-2773

Bartlett, G.R. (1959) J.Biol.Chem. 234, 466-468

Bartlett, K. and Mercer, E.I. (1974) Phytochem. 13, 1115-1121

Bartlett, K., Keat, M.J. and Mercer, E.I. (1974) Phytochem. 13, 1107-1113

Bartnicki-Garcia, S. (1968) Ann.Rev.Microbiol. 22, 87-105

Barton, D.H.R., Corrie, J.E.T., Widdowson, D.A., Bard, M. and Woods, R.A. (1974) J.Chem.Soc.Perkin I 1326-1333

Batra, P.M., Gleason, R.M. and Louda, J.W. (1973) Phytochem. 12, 1309-1313

Bauer, H. and Sigarlakie, E. (1975) J.Ultra.Res. 50, 208-215

Bauer, H., Sigarlakie, E. and Bracco, U. (1975) J.Ultra.Res. 51, 32-39

Beckett, A., Heath, I.B. and McCaughlin, D.J. (1975) An Atlas of Fungal Ultrastructure, Longmans, London

Bednarz-Prashad, A.J. and Mize, C.E. (1974) Biochemistry 13, 4237-4242

Bergman, K., Burke, P.V., Cerdá-Olmedo, E., David, C.N. Delbrück, M., Foster, K.W., Goodell, E.W., Heisenberg, M., Meissner, G., Zalokar, M., Dennison, D.S.and Shropshire, W. (1969) Bacteriol.Rev. 33, 99-157

Berjak, P. (1972) Ann.Bot. 36, 73-81

de Bernard, L. (1958) Bull.Soc.Chim.Biol. 40, 161-164

Beytía, E.D. and Porter, J.W. (1976) Ann.Rev.Biochem. 45, 113-142

Beytía, E., Qureshi, A.A. and Porter, J.W. (1973) J.Biol.Chem. 248, 1856-1867

Bimpson, T., Goad, L.J. and Goodwin, T.W. (1969) Chem.Commun. 297-298

Bligh, E.G. & Dyer, W.J. (1959) Can.J.Biochem.Physiol. 37, 911-917

Blobel, G. and Potter, V.R. (1968) Biochim.Biophys.Acta. 166, 48-57

Bloch, K., Chaykin, S., Phillips, A.H. and de Waard, A. (1959) J.Biol.Chem. 234, 2595-2604

Boesewinkel, H.J. (1976) Trans.Brit.Mycol.Soc. 66, 183-185

Boller, T., DUrr, M. and Wiemken, A. (1975) Eur.J.Biochem. 54, 81-91

Bowden, L. and Lord, J.M. (1976) Biochem.J. 154, 491-499, 501-506

Bracker, C.E. (1967) Ann.Rev.Phytopathol. 5, 343-374

Bracker, C.E. (1974) Proc. 8th Intern.Congr.Electron Microsc. Canberra 2, 558-559

Brady, D.R. and Gaylor, J.L. (1971) J.Lip.Res. 12, 270-276

Bramley, P.M. (1973) Ph.D. Thesis, University of Wales

Bramley, P.M. and Davies, B.H. (1975) Phytochem. 14, 463-469

Bramley, P.M. and Davies, B.H. (1976) Phytochem. 15, 1913-1916

Bramley, P.M., Aung Than and Davies, B.H. (1977) Phytochem. 16, 235-238

Brenner, D.M. and Carroll, G.C. (1968) J.Bacteriol. 95, 658-671

Briedenbach, R.W. and Beevers, H. (1967) Biochem.Biophys.Res.Commun. 27, 462-469

Brierley, G.P. and Jurkowitz, M. (1976) Biochem.Biophys.Res.Commun. 68, 82-88

Britten, R.J. and Roberts, R.B. (1960) Science 131, 32-33

Britton, G. (1971) in Aspects of Terpenoid Chemistry and Biochemistry (Goodwin, T.W., ed.), pp. 255-289, Academic Press, London and New York

Britton, G. (1976) in Chemistry and Biochemistry of Plant Pigments (Goodwin, T.W. ed.), 2nd edn., vol.1, pp. 262-327, Academic Press, London and New York

Brooker, J.D. and Russell, D.W. (1975) Arch.Biochem.Biophys. 167, 730-737

Brown, D.J., Britton, G. and Goodwin, T.W. (1975) Biochem.Soc.Trans. 3, 741-742

Brunswik, H. (1924) Bot.Abh.K.Goebel 5, 1-152

Bucher, N.L.R., Overath, P. and Lynen, F. (1960) Biochim.Biophys. Acta 40, 491-501

Buggy, M.J., Vose, J.R., Britton, G. and Goodwin, T.W. (1969) cited by Britton (1971)

Bull, H.B. (1947) Adv.Protein Chem. 3, 95-121

Bu'Lock, J.D., Hamilton, D., Hulme, M.A., Powell, A.J., Smalley, H.M., Shepherd, D. and Smith, G.N. (1965) Can.J.Microbiol. 11, 765-778

Burchard, R.P., Gordon, S.A. and Dworkin, M. (1966) J.Bacteriol. 91, 896-897

Burgeff, H. (1925) Flora(Jena) 118/119, 40-46

Burnett, J.H. (1968) Fundamentals of Mycology, Edward Arnold, London

Burton, K. (1968) Methods Enzymol. 12B, 163-166

Cabib, E., Ulane, R. and Bowers, B. (1973) J.Biol.Chem. 248, 1451-1458

Cagliotti, L., Cainelli, G., Camerino, B., Mondelli, R., Prieto, A., Quilico, A., Salvatori, T. and Selva, A. (1966) Tetrahedron Supp. 7, 175-187

Carafoli, E. (1975) Mol.Cell Biochem. 8, 133-140

Cartledge, T.G. and Lloyd, D. (1972) Biochem.J. 126, 755-757

Cassady, W.E. and Wagner, R.P. (1971) J.Cell Biol. 49, 536-541

Casselton, P.J. (1976) in The Filamentous Fungi (Smith, J.E. and Berry, D.R., eds.), vol.2, pp. 121-136, Edward Arnold, London

Casselton, P.J. Fawole, M.O. and Casselton, L.A. (1969) Can.J. Microbiol. 15, 637-640

Cederberg, E. and Neujahr, H.Y. (1970) Experientia 26, 366-367

Cerdá-Olmedo, E. (1974) in Handbook of Genetics (King, R.C., ed.), vol. 1, pp. 343-357, Plenum Press, New York

Charlton, J.M., Treharne, K.J. and Goodwin, T.W. (1967) Biochem. J, 105, 205-212

Chesterton, C.J. (1968) J.Biol.Chem. 243, 1147-1151

Ching, T.M. (1968) Lipids 3, 482-488

Claude, A. (1946) J.Exp.Med. 84, 51-59, 61-89

Clausen, M.K., Christiansen, K., Jensen, P.K. and Behnke, O. (1974) FEBS Lett. 43, 176-179

Cobon, G.S., Crowfoot, P.D. and Linnane, A.W. (1974) Biochem.J. 144, 265-275

Cobon, G.S., Crowfoot, P.D., Murphy, M. and Linnane, A.W. (1976) Biochem.Biophys.Acta. 441, 255-259

Cochrane, V.W. (1958) Physiology of Fungi, pp. 131-148, John Wiley, New York

Cook, G.M.W. (1973) in Lysosomes in Biology and Pathology (Dingle, J.T., ed.), vol 3, pp. 237-277, North Holland, Amsterdam

Corey, E.J., Russey, W.E. and Ortiz de Montellano, P.R. (1966) J.Amer.Chem.Soc. 88, 4750-4751

Cornforth, J.W. (1973) Chem.Soc.Rev. 2, 1-20

Cornforth, J.W. and Popják, G. (1959) Tetrahedron Lett. 19, 29-35

Curry, G.M. and Gruen, H.E. (1959) Proc.Nat.Acad.Sci.U.S. 45, 797-804

Dallner, G. (1974) Methods Enzymol. 31, 191-201

Dallner, G. and Ernster, L. (1968) J.Histochem.Cytochem. 16, 611-632

Dallner, G. and Nilsson, R. (1966) J.Cell Biol. 31, 181-193

Dangeard, P.A. (1922) C.R.Acad.Sci. 175, 7-12

Davies, B.H. (1965) in Chemistry and Biochemistry of Plant Pigments (Goodwin, T.W., ed.), pp. 489-532, Academic Press, London and New York

Davies, B.H. (1970) Biochem.J. 116, 93-99

Davies, B.H. (1973) Pure Appl.Chem. 35, 1-28

Davies, B.H. (1975) Ber.Deutsche Bot.Ges. 88, 7-25

Davies, B.H. and Rees, A.F. (1973) Phytochem. 12, 2745-2750

Davies, B.H., Goodwin, T.W. and Mercer, E.I. (1961) Biochem.J. 81, 40P

Davies, B.H., Jones, D. and Goodwin, T.W. (1963) Biochem.J. 87, 326-329

Davies, B.H., Holmes, E.A., Loeber, D.E., Toube, T.P. and Weedon, B.C.L. (1969) J.Chem.Soc.(C) 1266-1268

Davies, B.H., Hallett, C.J., London, R.A. and Rees, A.F. (1974) Phytochem. 13, 1209-1217

Davies, B.H., Rees, A.F. and Taylor, R.F. (1975) Phytochem. 14, 717-722

Davies, W.E., Goodwin, T.W. and Mercer, E.I. (1964) in The Determination of Sterols, Monograph No. 2, Society for Analytical Chemists, pp. 38-50

Davis, J.B., Jackman, L.M., Siddons, P.T. and Weedon, B.C.L. (1961) Proc.Chem.Soc. 261-263

Davis, J.B., Jackman, L.M., Siddons, P.T. and Weedon, B.C.L. (1966) J.Chem.Soc.(C) 2154-2165

Dauwalder, M., Whaley, W.G. and Kephart, J.E. (1972) Sub-Cell.Biochem. 1, 225-275

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1959) Data for Biochemical Research, p. 259, Clarendon Press, Oxford

Dean, P.B.G., Ortiz de Montellano, P.R., Bloch, K. and Corey, E.J. (1967) J.Biol.Chem. 242, 3-14-3015

Dean, R.T. and Barrett, A.J. (1976) in Essays in Biochemistry (Campbell, P.N. and Aldridge, W.N., eds.), vol 12, pp. 1-40, Academic Press, London

Decker, K. and Uehleke, H. (1961) Z.Physiol.Chem. 323, 61-76

Delbrück, M. (1973) Phycomyces Genetics Nomenclature, California Institute of Technology

Delbrück, M. and Shropshire, W. (1960) Plant Physiol. 35, 194-204

Delbrück, M., Katzir, A. and Presti, D. (1976) Proc.Nat.Acad.Sci.U.S. 73, 1969-1973

Demel, R.A. and de Kruyff B. (1976) Biochim.Biophys.Acta. 457, 109-132

Demel, R.A., Kinsky, S.C., Kinksky, C.B. and van Deenen, L.L.M. (1968) Biochim.Biophys.Acta 150, 655-665

Dempsey, M.E. (1969) Methods Enzymol. 15, 501-514

Dobrota, M. (1972) in Separations with Zonal Rotors (Reid, E., ed.), p.Z-6.1, University of Surrey, Guildford

Donaldson, R.P., Tolbert, N.E. and Shnarrenberger, C. (1972) Arch.Biochem.Biophys. 152, 199-215

Douce, R., Christensen, E.L. and Bonner, W.D. (1972) Biochim.Biophys. Acta 275, 148-160

Drawert, H. (1953) Ber.Deutsche Bot.Ges. 66, 134-150

Drochmans, P. (1962) J.Ultra.Res. 6, 141-163

Duffus, J.H. (1969) Biochim.Biophys.Acta 195, 230-233

Dugan, R.E., Rasson, E. and Porter, J.W. (1968) Anal.Biochem. 22, 249-259

Dusenbery, R.L. (1975) Biochim.Biophys.Acta 378, 363-377

de Duve, C. (1969) Ann.N.Y.Acad.Sci. 168, 369-381

de Duve, C., Wattiaux, R. and Baudhuin, P. (1962) Adv.Enzymol. 24, 291-358

Earnshaw, M.J. (1975) FEBS. Lett. 59, 109-112

Eberhardt, N.L. and Rilling, H.C. (1975) J.Biol.Chem. 250, 863-866

Edmond, J., Popják, G., Wong, S. and Williams, V.P. (1971) J.Biol.Chem. 246, 6254-6271

Epstein, W.W. and Rilling, H.C. (1970) J.Biol.Chem. 245, 4597-4605

Esleva, A.P. and Cerdá-Olmedo (1974) Plant Sci.Lett. 2, 9-14

Eymé, J. and Parriaud, H. (1970) C.R. Acad.Sci. 270D, 1890-1892

de Fabo, E.C., Harding, R.W. and Shropshire, W. (1976) Plant Physiol. 57, 440-445

Faires, R.A. and Parks, B.H. (1973) Radioisotope Laboratory Techniques 3rd edn., Butterworths, London

Ferguson, J.J., Durr, I.F. and Rudney, H. (1958) Fed.Proc. 17, 219

Fernley, H.N. (1971) in The Enzymes (Boyer, P.D., ed.), 3rd edn., vol. 4, pp. 421-422, Academic Press, London and New York

Fiske, C.H. and Subbarow, Y. (1925) J.Biol.Chem. 66, 375-400

Fonken, G.J. (1967) in Organic Photochemistry (Chapman, D.L., ed.), vol. 1, pp. 197-246, Edward Arnold, London

Foote, C.S. and Denny, R.W. (1968) J.Amer.Chem.Soc. 90, 6233-6235

Foote, C.S., Chang, Y-C. and Denny, R.W. (1970) J.Amer.Chem.Soc. 92, 5218-5219

Frantz, I.D., Davison, A.G., Dulit, E. and Mobberly, M.L. (1959) J.Biol.Chem. 234, 2290-2294

Frey-Wyssling, A., Grieshaber, E. and Mühlethaler, K. (1963) J.Ultra.Res. 8, 506-516

Fryberg, M., Oehlschlager, A.C. and Unrau, A.M. (1973a) J.Amer.Chem.Soc. 95, 5745-5757

Fryberg, M., Oehlschlager, A.C. and Unrau, A.M. (1973b) Biochem. Biophys. Res.Commun. 51, 219-222

Fujimori, E. and Livingston, R. (1957) Nature 180, 1036-1038

Gahan, P.B. (1967) Intern.Rev.Cytol. 21, 1-63

Garber, E.D., Baird, M.L. and Chapman, D.J. (1975) Bot.Gaz. 136, 341-346

Garrett, R.H. and Nason, A. (1969) J.Biol.Chem. 244, 2870-2882

Garton, G.A., Goodwin, T.W. and Lijinsky, W. (1951) Biochem.J. 48, 154-163

Gaylor, J.L. (1974) in Biochemistry of Lipids MTP International Review of Science, Biochemistry Series I (Kormberg, H.L. and Phillips, D.C., eds.), vol.4, pp. 1-37, Butterworths, London

Gerlach, U. and Hilby, W. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 2nd edn., vol. 2, pp. 871-875, Academic Press, London and New York

Gibbons, G.F., Goad, L.J. and Goodwin, T.W. (1968) Chem.Commun. 1458-1460

Glover, J. (1964) in The Determination of Sterols, Monograph No. 2, Society for Analytical Chemists, pp. 10-17

Glomset, J.A. (1968) J.Lip.Res. 9, 155-167

Goldstrohm, D.D. and Lilly, V.G. (1965) Mycologia 57, 612-623

Gooday, G.W., Fawcett, P., Green, D. and Shaw, G. (1973) J.Gen. Microbiol. 74, 233-239

Gooday, G.W., Green, D., Fawcett, P. and Shaw, G. (1974) Arch.Microbiol. 101, 145-151

Goodwin, T.W. (1952) Biochem.J. 50, 550-558

Goodwin, T.W. (1971a) in Carotenoids (Isler, O., ed.), pp. 577-636, Birkhäuser Verlag, Basel

Goodwin, T.W. (1971b) Biochem.J. 123, 293-329

Goodwin, T.W. (1972) in Progress in Industrial Microbiology (Hockenhull, D.J.P. ed.), vol. 11, pp. 29-88, Churchill Livingstone, Edinburgh and London

Goodwin, T.W. (1976) in The Filamentous Fungi (Smith, J.E. and Berry, D.R., eds.), vol. 2, pp. 423-444, Edward Arnold, London

Goodwin, T.W. and Lijinsky, W. (1951) Biochem.J. 50, 268-273

Goodwin, T.W. and Williams, R.J.H. (1965) Biochem.J. 94, 5-7C

Goodwin, T.W. and Willmer, J.S. (1952) Biochem.J. 51, 213-217

Goulston, G. and Mercer, E.I. (1969) Phytochem. 8, 1945-1948

Goulston, G., Mercer, E.I. and Goad, L.J. (1975) Phytochem. 14, 457-462

Graebe, J.E. (1968) Phytochem. 7, 2003-2020

Gregonis, D.E. and Rilling, H.C. (1973) Biochem.Biophys.Res.Commun. 54, 449-454

Gregonis, D.E. and Rilling, H.C. (1974) Biochemistry 13, 1538-1542

Griffiths, M., Sistrom, W.R., Cohen-Bazire, G. and Stanier, R.Y. (1955) Nature 176, 1211-1214

Grob, E.C. (1959) in The Biosynthesis of Terpenes and Sterols, Ciba Foundation Symp. (Wolstenholme, G.E.W. and O'Connor, C.M., eds.), p. 267, Little, Brown and Co., Boston

Grob, E.C. and Boschetti, A. (1962) Chimia 16, 15-16

Grob, E.C. and Bütler, R. (1955) Helv.Chim.Acta 38, 1313-1316

Grob, E.C. and Bütler, R. (1956) Helv.Chim.Acta 39, 1975-1980

Grob, E.C., Kirschner, K. and Lynen, F. (1961) Chimia 15, 308-310

Grove, S.N. and Bracker, C.E. (1970) J.Bacteriol. 104, 989-1009

Grove, S.N., Bracker, C.E. and Morré, D.J. (1968) Science 161, 171-173

Grove, S.N., Bracker, C.E. and Morré, D.J. (1970) Amer.J.Bot. 57 245-266

Grunwald, C. (1974) Plant Physiol. 54, 624-628

Grunwald, C. (1975) Ann.Rev.Plant Physiol. 26, 209-236

de la Guardia, M.D., Aragòn, C.M.G., Murillo, F.J. and Cerdá-Olmedo, E. (1971) Proc.Nat.Acad.Sci.U.S. 68, 2012-2015

Guillermond, A. (1921) C.R.Acad.Sci. 172, 1676-1678

Guillermond, A., Mangenot, G. and Plantefol, L. (1933) Traité de Cytolgie Végétale, Paris

Gurr, M.I., Blades, J., Appleby, R.S., Smith, C.G., Robinson, M.P. and Nichols, B.W. (1974) Eur.J.Biochem. 43, 281-290

Haber, J.E. and Koshland, D.E. (1970) J.Mol.Biol. 50, 617-639

Hageman, R.H. and Flesher, D. (1960) Plant Physiol. 35, 700-708

Hallermayer, G. and Neupert, W. (1974) Hoppe-Seyler's Z.Physiol.Chem. 355, 279-288

Hänssler, G., Maxwell, D.P. and Maxwell, M.P. (1975) J.Bacteriol. 124, 997-1006

Hanstein, J. (1880) Bot.Abh.Morph.Physiol.(Bonn) 4, H.2. (citation from Frey-Wyssling et al., 1963)

Harwood, J.L. and Stumpf, P.K. (1972) Lipids 7, 8-19

Harwood, J.L., Sodja, A., Stumpf, P.K. and Spurr, A.R. (1971) Lipids 6,851-854

Hasilik, A., Müller, H. and Holzer, H. (1974) Eur.J.Biochem. 48, 111-117

Hawker, L.E. (1965) Biol.Rev. 40, 52-92

Hawker, L.E. and Abbott, P.McV. (1963) J.Gen.Microbiol. 30, 401-408

Hayman, E.P., Chichester, C.O. and Simpson, K.L. (1974a) Phytochem. 13, 1123-1127

Hayman, E.P., Yokoyama, H., Chichester, C.O. and Simpson, K.L. (1974b) J.Bacteriol. 120, 1339-1343

Hazen, G.G. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U. ed.), 2nd edn., pp. 1000-1005, Academic Press, London and New York

Headon, D.R. and Duggan, P.F. (1971) in Separations with Zonal Rotors (Reid, E., ed.), pp. V-2.1 to V-2.12,University of Surrey, Guildford.

Heath, I.B. and Greenwood, A.D. (1970) J.Gen.Microbiol. 62, 129-137

Heath, I.B., Gay, J.L. and Greenwood, A.D. (1971) J.Gen.Microbiol. 65, 225-232

Heim, P. (1946a) C.R. Acad.Sci. 222, 1354-1355

Heim, P. (1946b) C.R. Acad.Sci. 223, 1170-1172

Heim, P. (1947) Rev. de Mycol. 12, 104-125

Heim, P. (1949) Le Botaniste 34, 231-241

Heim, R. (1949) Rev. de Mycol. 14, 113-120

Heiniger, H. and Matile, Ph. (1974) Biochem.Biophys.Res.Commun. 60, 1425-1432

Henderson-Smith, J. (1923) Ann.Bot. 37, 341-343

Herber, R. (1974) D. ès S. Thesis, Université de Nancy I

Herber, R., Maudinas, B., Villoutreix, J. and Granger, P. (1972) Biochim.Biophys.Acta 280, 194-202

Hill, H.M., Calderwood, S.K. and Rogers, L.J. (1971) Phytochem. 10, 2051-2058

Hislop, E.C., Barnaby, V.M., Shellis, C. and Laborda, F. (1974) J.Gen.Microbiol. 81, 79-99

Hoch, H.C. and Maxwell, D.P. (1974) Can.J.Microbiol. 20, 1029-1035

Hoffman, H-P., Szabo, A. and Avers, C.J. (1970) J.Bacteriol. 104, 581-584

Holley, R.A. and Kidby, D.K. (1973) Can.J.Microbiol 19, 113-117

Hsu, W-J, Allion, D.C. and Delbrück, M. (1974) Phytochem. 13, 1463-1468

Huang, A.H.C. and Beevers, H. (1971) Plant Physiol. 48, 637-641

Huang, L. and Haug, A. (1974) Biochim.Biophys.Acta 352, 361-370

Hübscher, G. and West, G.R. (1965) Nature 205, 799-800

Hunsley, D. and Burnett, J.H. (1970) J.Gen.Microbiol. 62, 203-218

Imblum, R.L. and Rodwell, V.W. (1974) J.Lip.Res. 15, 211-222

Indge, K.J. (1968) J.Gen.Microbiol. 51, 425-432

Islam, M.A., Lyrene, S.A., Miller, E.M. and Porter, J.W. (1977) J.Biol.Chem. 252, 1523-1525

Iten, W. and Matile, Ph. (1970) J.Gen.Microbiol. 61, 301-309

Jacks, T.J., Yatsu, L.Y. and Altschul, A.M. (1967) Plant Physiol. 42, 585-597

Jan, Y.N. (1974) J.Biol.Chem. 249, 1973-1979

Jauréguiberry, G., Law, J.H., McCloskey, J.A., and Lederer, E. (1965) Biochemistry 4, 347-353

Jelsema, C.L., Morré, D.J. and Ruddat, M. (1975) Proc.Indiana Acad.Sci. 84, 166-178

Jerebzoff-Quintin, S., Jerebzoff, S. and Jacques, R. (1975) Physiol.Végétale 13, 55-67

Johnson, J.H., Reed, B.C. and Rilling, H.C. (1974) J.Biol.Chem. 249, 402-406

Jungalwala, F.B. and Porter, J.W. (1965) Arch.Biochem.Biophys. 110, 291-299

Jungalwala, F.B. and Porter, J.W. (1967) Arch.Biochem.Biophys. 119, 209-219

Jungalwala, F.B. and Porter, J.W. (1969) Methods Enzymol. 15, 454-460

Kadenbach, B. (1966) Biochim.Biophys.Acta 134, 430-432

Kandutsch, A.A., Paulus, H., Levin, E. and Bloch, K. (1964) J.Biol.Chem. 239, 2507-2515

Katz, A.M., Repke, D.I., Upshaw, J.E. and Polascik, M.A. (1970) Biochim.Biophys.Acta 205, 473-490

Kawaguchi, A., Kobayashi, H. and Okuda, S. (1973a) Chem.Pharm. Bull. 21, 577-583

Kawaguchi, A., Nozoe, S. and Okuda, S. (1973b) Biochim.Biophys Acta 296, 615-623

Keith, A.D., Wisnieski, B.J., Henry, S. and Williams, J.C. (1973) in Lipids and Biomembranes of Eukaryotic Microorganisms (Erwin, J.A. ed.), pp. 259-321, Academic Press, London and New York

Keyhani, J., Keyhani, E. and Goodgal, S.H. (1972) Eur.J.Biochem. 27, 527-534

King, T.E. (1967) Methods Enzymol. 10, 216-225

Kinsky, S.C. (1967) in Antibiotics (Gottlieb, D. and Shae, P.D. eds.), vol. 1, p.122, Springer, New York

Klein, H.P. (1960) J.Bacteriol. 80, 665-672

Klein, H.P. (1965) J.Bacteriol. 90, 227-234

Klein, H.P., Volkmann, C. and Weibel, J. (1967) J.Bacteriol. 94, 475-481

Knapp, F.F., Aexel, R.T. and Nichols, H.J. (1969) Plant Physiol. 44, 442-446

Kobr, M.J. and Vanderhaeghe, F. (1973) Experientia 29, 1221-1223

Kobr, M.J., Vanderhaeghe, F. and Combépine, G. (1969) Biochem. Biophys.Res.Commun. 37, 640-645

Koch, A.L. (1975) J.Gen.Microbiol. 89, 209-216

Kotyk, A. (1967) cited by Matile et al. (1969)

Kotyk, A. and Matile, Ph. (1969) cited by Matile et al. (1969)

Kreger, D.R. (1954) Biochim.Biophys.Acta 13, 1-9

Krinsky, N.I. (1971) in Carotenoids (Isler, O., ed.), pp. 669-716, Birkhäuser, Verlag, Basel.

de Kruyff, B., Demel, R.A. and van Deenen, L.L.M. (1972) Biochim. Biophys.Acta 255, 331-347

Kunisawa, R. and Stanier, R.Y. (1958) Arch.Mikiobiol. 31, 146-156

Kunze, G. (1823) in Mykologische Hefte (Kunze, G. and Schmidt, J.C. eds.), vol. 2, pp. 113-116, Voss, Leipzig

Kushwaha, S.C., Suzue, G., Subbarayan, C. and Porter, J.W. (1970) J.Biol.Chem. 245, 4708-4717

Kushwaha, S.C., Kates, M. and Porter, J.W. (1976) Can.J.Biochem. 54, 816-823

Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys.Acta 150, 333-340

Land, E.J. (1975) Photochem.Photobiol. 22, 286-288

Langdon, R.G. (1966) Methods Enzymol. 9, 126-131

Lee, A., Chance, K., Weeks, C. and Weeks, G. (1975) Arch.Biochem Biophys. 171, 407-417

Lee, T-C and Chichester, C.O. (1969) Phytochem. 8, 603-609

Lee, T-C., Lee, T.H. and Chichester, C.O. (1972) Phytochem. 11, 681-687

Leighton, T.J., Dill, B.C., Stock, J.J. and Phillips, C. (1971) Proc.Nat.Acad.Sci.U.S. 68, 677-680

Lowry, L.K. (1968) Diss.Abs. 29, 2291-2292B

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol.Chem. 193, 265-275

Luck, D.J.L. (1963) J.Cell Biol. 16, 483-499

Lucy, J.A. and Dingle, J.T. (1962) Biochem.J. 84, 76-77P

Luft, J.H. (1961) J.Biochem.Biophys.Cytol. 9, 409-414

Lynen, F., Eggerer, H., Henning, U. and Kessel, I. (1958) Angew.Chem. 70, 738-742

Mackenzie, C.G., Mackenzie, J.B., Reiss, O.K. and Philpott, D.E.(1966) Biochemistry 5, 1454-1461

Mahadevan, P.R. and Tatum, E.L. (1965) J.Bacteriol. 90, 1073-1081

Mathews, M.M. (1963) Photochem.Photobiol. 2, 1-8

Matile, Ph. (1965) Z.Zellforschung 65, 884-896

Matile, Ph. (1969) in Lysosomes in Biology and Pathology (Dingle, J.T. and Fell, H.B., eds.), vol. 1, pp. 406-430, North-Holland, Amsterdam.

Matile, Ph. (1971) Cytobiologie 3, 324-330

Matile, Ph. (1975) The Lytic Compartment of Plant Cells, Cell Biology Monographs , vol. 1, Springer Verlag, Wien and New York

Matile, Ph. and Moor, H. (1968) Planta 80, 159-175

Matile, Ph. and Spichiger, H. (1968) Z.Pflanzenphysiol. 58, 277-280

Matile, Ph. and Wiemken, A. (1967) Arch.Mikrobiol. 56, 148-155

Matile, Ph. and Wiemken, A. (1974) Methods Enzymol. 31, 572-578

Matile, Ph. Balz, J-P, Semadeni, E. and Jost, M. (1965) Z.Naturforschung 20b, 693-698

Matile, Ph., Moor, H. and Robinson, C.F. (1969) in The Yeasts(Rose, A.H. and Harrison, J.S., eds.). vol.1, pp. 219-302, Academic Press, London and New York

Maudinas, B., Bucholtz, M.L., Papastephanou, C., Katiyar, S.S., Briedis, A.V. and Porter, J.W. (1975) Biochem.Biophys. Res.Commun. 66, 430-436

Maudinas, B., Bucholtz, M.L., Papastephanou, C., Katiyar, S.S., Briedis, A.V. and Porter, J.W. (1977) Arch.Biochem.Biophys 180, 354-362

Maxwell, D.P., Williams, P.H. and Maxwell, M.D. (1970) Can.J.Bot. 48, 1689-1691

Maxwell, D.P., Maxwell, M.D., Hünssler, G., Armentrout, V.N., Murray, G.M. and Hoch, H.C. (1975) Planta 124, 109-123

McClure, W.K., Park, D. and Robinson, P.M. (1968) J.Gen.Microbiol 50, 177-182

McCorkindale, N.J. (1976) in The Filamentous Fungi (Smith, J.E. and Berry, D.R., eds.), vol. 2, pp. 369-422, Edward Arnold, London

McDermott, J.C.B., Britton, G. and Goodwin, T.W. (1973) Biochem.J. 134, 1115-1117

McDermott, J.C.B., Brown, D.J., Britton, G. and Goodwin, T.W. (1974) Biochem.J. 144, 231-243

Meissel, M.N., Medvedeva, G.A., Kozlova, T.M., Pomoshnikova, N.A., Zaikina, A.I. and Fedoseeva, G.E. (1973) in Proc.3rd Intern. Spec.Symp. on Yeasts (Suomalainen, H. and Waller, C., eds.), part 2, pp. 149-168, Symposium Secretariate, Helsinki

Meissner, G. and Delbrück, M. (1968) Plant Physiol. 43, 1279-1283

Mendgen, K. (1973) Protoplasma 78, 477-482

Mercer, E.I. and Bartlett, K. (1974) Phytochem. 13, 1099-1105

Mercer, E I. and Johnson, M.W. (1969) Phytochem. 8, 2329-2331

Mercer, E.I. and Russell, S.M. (1975) Phytochem. 14, 445-449

Mercer, E.I., Davies, B.H. and Goodwin, T.W. (1963) Biochem.J. 87, 317-325

Metzger, H., Shapiro, M.B., Mosiman, J.E. and Vinton, J.E. (1968) Nature 219, 1166-1168

Meyer, R., Parish, R.W. and Hohl, H.R. (1976) Arch.Microbiol. 110, 215-224

Millonig, G. (1961) J.Appl.Phys. 32, 1637

Mills, G.L. and Cantino, E.C. (1975a) Cell Differentiation 4, 35-43

Mills, G.L. and Cantino, E.C. (1975b) Biochem.Biophys.Ress.Commun. 67, 1256-1263

Mills, G.L. and Cantino, E.C. (1977) Arch.Microbiol. 112, 195-200

Miura, K-I. (1967) Methods Enzymol. 12A, 543-545

Mollenhauer, H.H. and Morré, D.J. (1966) Ann.Rev.Plant.Physiol. 17, 27-46

Mollenhauer, H.H., Whaley, W.G. and Leech, J.H. (1961) J.Ultra.Res. 5, 193-200

Moore, J.T. and Gaylor, J.L (1969) J.Biol.Chem. 244, 6334-6340

Moore, P.R. and Baumann, C.A. (1952) J.Biol.Chem. 195, 615-621

Moore, R.T. and McAlear, J.H. (1961) Mycologia 53, 194-200

Moore, S. and Stein, W.H. (1963) Methods Enzymol. 6, 819-831

Morré, D.J. (1971) Methods Enzymol. 22, 130-148

Muller, L.L. and Jacks, T.J. (1975) J.Histochem.Cytochem. 23, 107-110

Munro, H.N. and Fleck, A. (1966) Analyst 91, 78-88

Murillo, F.J. and Cerdá-Olmedo, E. (1976) Mol.Gen.Genet. 148, 19-24

Murray, G.M. and Maxwell, D.P. (1974) Can.J.Bot. 52, 2335-2340

Muscio, F. Carlson, J.P., Kuehl, L.R. and Rilling, H.C. (1974) J.Biol.Chem. 249, 3746-3749

Muto, S. and Beevers, H. (1974) Plant Physiol. 54, 23-28

Nakamura, K.D. and Schlenk, F. (1974) J.Bacteriol. 118, 314-316

Neupert, W. and Ludwig, G.D. (1971) Eur.J.Biochem. 19, 523-532

de Nollin, S., Thoné, F. and Borgers, M. (1975) J.Histochem. Cytochem. 23, 758-765

North, M.J. (1973) J.Gen.Microbiol. 77, xvi-xvii

Northcote, D.H. and Pickett-Heaps, J.D. (1965) Biochem.J. 98, 159-167

Nunn, R.E. (1970) Electron Microscopy: Preparation of Biological Specimens, Butterworths, London

Nurminen, T., Taskinen, L. and Suomalainen, H. (1976) Biochem.J. 154, 751-763

Ogur, M. and Rosen, G. (1950) Arch.Biochem. 25, 262-276

Ogura, K., Koyama, T. and Seto, S. (1969) Biochem.Biophys.Res. Commun. 35, 875-879

Ogura, K., Saito, A. and Seto, S. (1974) J.Amer.Chem.Soc. 96, 4037-4038

Olsen, R.A. (1973) Physiol.Plant. 28, 507-515

Olson, J.A. and Knizley, H. (1962) Arch.Biochem.Biophys. 97, 138-145

Olson, J.A., Lindberg, M. and Bloch, K. (1957) J.Biol.Chem. 226, 941-956

Ootaki, T. and Wolken, J.J. (1973) J.Cell.Biol. 57, 278-288

Ootaki, T., Lightly, A.C., Delbrück, M. and Hsu, W.J. (1973) Molec.Gen.Genet. 121, 57-70

Ory, R.L., Yatsu, L.Y. and Kircher, H.W. (1968) Arch.Biochem. Biophys. 123, 255-264

O'Sullivan, J. and Casselton, P.J. (1973) J.Gen.Microbiol. 75, 333-337

Ottolenghi, P. (1967) C.R. Lab.Carlsberg 36, 95-111

Overath, P., Schairer, H.U. and Stoffer, W. (1970) Proc.Nat. Acad.Sci.U.S. 67, 606-612

Palczewska, I. and Jagodzka, G. (1972) Acta Soc.Bot.Pol. 41, 265-282

Papastephanou, C., Barnes, F.J., Briedis, A.V. and Porter, J.W. (1973) Arch.Biochem.Biophys. 157, 415-425

Parish, R.W. (1975a) Arch.Microbiol. 105, 187-192

Parish, R.W. (1975b) Planta 123, 1-13

Parish, R.W. (1975c) Planta 123, 15-31

Park, D. and Robinson, P.M (1967) Symp.Soc.Exp.Biol. 21, 323-335

Parks, L.W. (1968) J.Amer.Chem.Soc. 80, 2023-2024

Peat, A. and Banbury, G.H. (1967) New Phytol. 66, 475-484

Perlman, P.S. and Mahler, H.R. (1970) Arch.Biochem.Biophys. 136, 245-249

Pfeiffer, H.H. (1963) Protoplasma 57, 636-642

Pierce, C.H., Dubos, R.J. and Schaeffer, W.B. (1953) J.Exp.Med. 97, 189-206

Pitt, D. (1968) J.Gen.Microbiol. 52, 67-75

Pitt, D. and Walker, P.J. (1967) Nature 215, 783-784

Plaut, G.W.E. (1963) Methods Enzymol. 6, 231-236

Popják, G.J. and Cornforth, J.W. (1960) Adv.Enzymol. 22, 281-335

Popják, G., Goodman, de W.S., Cornforth, J.W., Cornforth, R.H. and Ryhage, R. (1961) J.Biol.Chem. 236, 1934-1947

Porter, J.W. (1969) Pure Appl.Chem. 20, 449-481

Porter, J.W. and Lincoln, R.E. (1950) Arch.Biochem.Biophys. 27, 390-403

Prevost, F. (1968) J.Microscopie 7, 52a

Price, C.A. (1974) in Subcellular Particles, Structures and Organelles (Laskin, A.I. and Last, J A., eds.), pp. 155-230, Marcel Dekker, New York

Price, R.G. and Dance, N. (1972) Biochim.Biophys.Acta 271, 145-153

Qureshi, A.A., Barnes, F.J. and Porter, J.W. (1972) J.Biol.Chem. 247, 6730-6732

Qureshi, A.A., Beytía, E.D. and Porter, J.W. (1973a) J.Biol.Chem. 248, 1848-1855

Qureshi, A.A., Barnes, F.J., Semmler, E.J. and Porter, J.W. (1973b) J.Biol.Chem. 248, 2755-2767

Qureshi, A.A., Andrewes, A.G., Qureshi, N. and Porter, J.W. (1974) Arch.Biochem.Biophys. 162, 93-107

Rahman, R., Sharpless, K.B., Spencer, T.A. and Clayton, R.B. (1970) J.Biol.Chem. 245, 2667-2671

Rambourg, A., Hernandez, W. and Leblond, C.P. (1969) J.Cell.Biol. 40, 395-414

Reed, B.C. and Rilling, H.C. (1975) Biochemistry 14, 50-54

Reid, E. (1969) in Subcellular Components:Preparation and Fractionation (Birnie, G.D. and Fox, S.M., eds.), 1st edn., p.41, Plenum. New York

Reinhardt, M.O. (1892) Jahrbuch Wiss.Bot. 23, 479-566

Rest, J.A. and Vaughan, J.G. (1972) Planta 105, 245-262

Rilling, H.C. (1965) Arch.Biochem.Biophys. 110, 39-46

Rilling, H.C. (1966) J.Biol.Chem. 241, 3233-3236

Rilling, H.C. (1972) Biochem.Biophys.Res.Commun. 46, 470-475

Ritter, M.C. and Dempsey, M.E. (1970) Biochem.Biophys.Res.Commun. 38, 921-929

Ritter, M.C. and Dempsey, M.E. (1973) Proc.Nat.Acad.Sci.U.S. 70, 265-269

Rose, A.H. (1976) in The Filamentous Fungi (Smith, J.E. and Berry, D.R., eds.), vol. 2, pp. 308-327, Edward Arnold, London

Rosenberger, R.F. (1976) in The Filamentous Fungi (Smith, J.E. and Berry, D.R., eds.), vol. 2, pp. 328-344, Edward Arnold, London

Rottem, S., Cirillo, V.P., de Kruyff, B., Shinitzky, M. and Razin, S. (1973) Biochim.Biophys.Acta 323, 509-519

Rowley, B.I. and Pirt, S.J. (1972) J.Gen.Microbiol. 72, 553-563

Ruinen, J., Deinema, M.H. and van der Scheer, C. (1968) Can.J. Microbiol. 14, 1133-1137

Russell, P.T., van Aller, R.T. and Nes, W.R. (1967) J.Biol.Chem. 242, 5802-5806

Ruzicka, L. (1953) Experentia 9, 357-367

Ruzicka, L. (1959) Proc.Chem.Soc. 341-360

Scallen, T.J., Schuster, M.W. and Dhar, A.K (1971) J.Biol.Chem. 246, 224-230

Scallen, T.J., Sprikantaiah, M.V., Skrdlant, H.B. and Hansbury, E. (1972) FEBS Lett. 25, 227-233

Scallen, T.J., Seetharam, B., Spikantaiah, M.V., Hansbury, E. and Lewis, M.K. (1975) Life Sci. 16, 853-874

Scarpa, A. and Azzi, A. (1968) Biochim.Biophys.Acta 150, 473-481

Schatz, G. and Klima, J. (1964) Biochim.Biophys.Acta 81, 448-461

Schechter, I., Sweat, F.W. and Bloch, K. (1970) Biochim.Biophys.Acta 220, 463-468

Schenck, G.O. (1954) Naturwiss. 41, 452-453

Schlenk, F. and Dainko, J.L. (1965) J.Bacteriol. 89, 428-436

Schneider, E.F. and Seaman, W.L. (1977) Can.J.Microbiol. 23, 190-196

Schneider, W.C. (1957) Methods Enzymol. 3, 680-684

Schopfer, P., Bajracharya, D., Bergfeld, R. and Falk, H. (1976) Planta 133, 73-80

Schopfer, W.H. (1928) Bull.Soc.Bot.Genève Ser.II 20, 149-323

Schrantz, J.P. (1965) Revue Cytol.Biol.Végétale 28, 31-37

Schwarzenbach, A.M. (1971a) Cytobiologie 4, 145-147

Schwarzenbach, A.M. (1971b) Ber.Schweiz.Bot.Ges. 81, 70-90

Schwenk, E. and Alexander, G.J. (1958) Arch.Biochem.Biophys. 76, 65-74

Scott, W.A., Munkries, K.D. and Metzenberg, R.L. (1971) Arch. Biochem.Biophys. 142, 623-632

Semadeni, E.G. (1967) Planta 72, 91-118

Shah, D.V., Feldbruegge, D.H., Houser, A.R. and Porter, J.W. (1968) Arch.Biochem.Biophys. 127, 124-131

Sharpless, K.B., Snyder, T.E., Spencer, T.A., Makeshwari, K.K., Guhn, G. and Clayton, R.B. (1968) J.Amer.Chem.Soc. 90, 6874-6875

Shatkin, A.J. and Tatum, E.L. (1959) J.Biophys.Biochem.Cytol. 6, 423-426

Shaw, G. (1971) in Sporopollenin (Brooks, J., Grant, P.R., Muir, M.D., van Gitzel, P. and Shaw, G., eds.), pp. 305-350, Academic Press, London and New York

Shimizu, I., Nagai, J., Hatanaka, H., Saito, E. and Katsuki, H. (1971) J.Biochem.(Tokyo) 70, 175-177

Shimizu, I., Nagai, J., Hatanaka, H. and Katsuki, H. (1973) Biochim.Biophys.Acta 296, 310-320

Sistrom, W.R., Griffiths, M. and Stanier, R.Y. (1956) J.Cell.Comp. Physiol. 48, 473-515

Slayman, C.L. and Gradman., D. (1975) Biophys.J. 15, 968-971

Smith, C.G. (1974) Planta 119, 125-142

Smith, P.F. (1963) J.Gen.Microbiol. 32, 307-319

Smith, P.F. (1969) Lipids 4, 331-336

Sorokin, H.P. (1967) Amer.J.Bot. 54, 1008-1016

Sorokin, H.P. and Sorokin, S. (1966) Protoplasma 62, 216-236

Srikantaiah, M.V.,Hansbury, E., Loughran, E.D. and Scallen, T.J. (1976) J.Biol.Chem. 251, 5496-5504

Subbarayan, C., Kushwaha, S.C., Suzue, G. and Porter, J.W. (1970) Arch.Biochem.Biophys. 137, 547-557

Subden, R.E. and Goldie, A.H. (1973) Genetica (The Hague) 44, 615-620

Suberkropp, K.F. and Cantino, E.C. (1973) Arch.Microbiol. 89, 205-221

Suerth, C. (1974) cited by Price (1974)

Susani, M., Zimniak, P., Fessl, F. and Ruis, H. (1976) Hoppe-Seyler's Z.Physiol.Chem. 357, 961-970

Sutter, R.D. and Rafelson, M.E. (1968) J.Bacteriol. 95, 426-432

Suzue, G., Tsukada, K. and Tanaka, S. (1968) Biochim.Biophys Acta 164, 88-93

Swanson, M.A. (1955) Methods Enzymol. 2, 541-543

Szabo, A.S. and Avers, C.J. (1969) Ann.N.Y.Acad.Sci. 168, 302-312

Tai, H.H. and Bloch, K. (1972) J.Biol.Chem. 247, 3767-3773

Tavormina, P.A., Gibbs, M.H. and Huff, J.W. (1956) J.Amer.Chem. Soc. 78, 4498-4499

Taylor, R.F. and Davies, B.H. (1974) Biochem.J. 139, 751-760

Tchen, T.T. and Bloch, K. (1957) J.Biol.Chem. 226, 921-930

Theimer, R.R. (1973) in Yeast, Mould and Plant Protoplasts (Villanueva, J.R., García-Acha, J., Gascón, J. and Uruburu, F. eds.), pp. 247-254, Academic Press, London and New York

Thomas, D.M. and Goodwin, T.W. (1967) Phytochem. 6, 355-360

Thompson, E.D., Bailey, R.B. and Parks, L.W. (1974) Biochim. Biophys.Acta 334, 116-126

Throneberry, G.O. (1970) Phytopathol. 60, 143-147

Tolbert, N.E. (1971) Ann.Rev.Plant Physiol. 22, 45-74

Tornabene, T.G., Kates, M., Gelpi, E. and Oro, J. (1969) J.Lip.Res. 10, 294-303

Torriani, A. (1960) Biochim.Biophys.Acta 38, 460-479

Trocha, P.J. and Sprinson, D.B. (1976) Arch.Biochem.Biophys. 174, 45-51

Trocha, P.J., Jasne, S.J. and Sprinson, D.B. (1974) Biochem. Biophys.Res.Commun. 59, 666-671

Tsuda, S. and Tatum, E.L. (1961) J.Biophys.Biochem.Cytol. 11, 171-177

Uzaka, Y., Kanamori, T., Koga, T., Tanaka, K. and Naganuma, T. (1975) J.Gen.Appl.Microbiol. 21, 157-168

Vail, W.J. and Lilly, V.G. (1968) Mycologia 60, 902-907

Valadon, L.R.G., Herber, R., Villoutreix, J. and Maudinas, B. (1973) Phytochem. 12, 161-163

Varma, T.N.R. and Chichester, C.O. (1962) Arch.Biochem.Biophys. 96, 265-269

Vaskovsky, V.E. and Kostetsky, E.Y. (1968) J.Lip.Res. 9, 396

Vaskovsky, V.E., Kostetsky, E.Y. and Vasendin, I.M. (1975) J.Chromatog. 114, 129-141

Vigil, E.L. (1973) Sub-Cell.Biochem. 2, 237-285

Watson, K. (1976) in The Filamentous Fungi (Smith, J.E. and Berry, D.R. eds.), vol.2, pp. 92-120, Edward Arnold, London

Watson, K., Bertoli, E. and Griffiths, D.E. (1975) Biochem.J. 146, 401-407

Wattiaux, R. (1974) Mol.Cell.Biochem. 4, 21-29

Weedon, B.C.L. (1971) in Carotenoids (Isler, O., ed.), pp. 29-59, Birkhäuser Verlag, Basel

Weeks, O.B. (1971) in Aspects of Terpenoid Chemistry and Biochemistry (Goodwin, T.W., ed.), pp. 291-313, Academic Press, London

Weete, J.D. (1973) Phytochem. 12, 1843-1864

Wergin, W.P., Dunkle, L.P., van Etten, J.L., St. Julian, G. and Bulla, L.A. (1973) Developmental Biol. 32, 1-14

Whaley, W.G., Mollenhauer, H.H. and Leech, J.H. (1960) Amer.J.Bot. 47, 401-449

Wicksell, S.D. (1925) Biometrika 17, 84-99

Wiemken, A. (1975) in Methods in Cell Biology (Prescott, D.M., ed.), vol. 12, pp. 99-109, Academic Press, London and New York

Wiemken, A. and Nurse, P. (1973) in Proc.3rd Intern.Spec.Symp. on Yeasts (Suomalainen, H. and Waller, C., eds.), part 2, pp. 331-347, Symposium Secretariate, Helsinki

Wiener, E. and Ashworth, J.M. (1970) Biochem.J. 118, 505-512

van der Wilden, W., Matile, Ph., Schellenburg, M., Mayer, J. and Wiemken, A. (1973) Z.Naturforsch. 28c, 416-421

Williams, C.H. and Kamin, H. (1962) J.Biol.Chem. 237, 587-595

Williams, R.J.H., Davies, B.H. and Goodwin, T.W. (1965) Phytochem. 4, 759-760

Williams, R.J.H., Britton, G., Charlton, J.M. and Goodwin, T.W. (1967a) Biochem.J. 104, 767-777

Williams, R.J.H., Britton, G. and Goodwin, T.W. (1967b) Biochem.J. 105, 99-105

Wilson, C.I., Stiers, D.L. and Smith, G.G (1970) Phytopathol. 60, 216-227

Wolken, J.J. (1972) Intern.J.Neuroscience 3, 135-146

Woodward, D.O. and Munkres, K.D. (1966) Proc.Nat.Acad.Sci.U.S. 55, 872-880

Wüersch, J., Huang, R.L. and Bloch, K. (1952) J.Biol.Chem. 195, 439-446

Yamamoto, H.Y., Yokoyama, H., Simpson, K.L., Nakayama, T.O.M. and Chichester, C.O. (1961) Nature 191, 1299-1300

Yatsu, L.Y. and Jacks, T.J. (1972) Plant Physiol. 49, 937-943

Yatsu, L.Y., Jacks, T.J. and Hensarling, T.P. (1971) Plant Physiol. 48, 675-682

Yokoyama, H., Chichester, C.O. and Mackinney, G. (1960) Nature 185, 687-688

Yokoyama, H., Nakayama, T.O.M. and Chichester, C.O. (1962) J.Biol.Chem. 237, 681-686

Young, A.H. and Whiteley, H.R. (1975) J.Biol.Chem. 250, 479-487

Zalokar, M. (1954) Arch.Biochem.Biophys. 50, 71-80

Zalokar, M. (1959a) Amer.J.Bot. 46, 602-610

Zalokar, M. (1959b) Amer.J.Bot. 46, 555-559

Zalokar, M. (1960) Exp.Cell Res. 19, 114-132

Zalokar, M. (1961) J.Biophys.Biochem.Cytol. 9, 609-617

Zalokar, M. (1969) J.Cell Biol. 41, 494-509

Zahler, W.L., Saito, A. and Fleischer, S. (1968) Biochem. Biophys.Res.Commun. 32, 512-518

Zechmeister, L. and Koe, B.K. (1954) J.Amer.Chem.Soc. 76, 2923-2926

Zopf (1892) cited by Schopfer (1928)

Amdur, H., Rilling, H. and Bloch, K. (1957) J.Amer.Chem.Soc. 79, 2646-2647

Viglietti, C., Scannerini, S., Panzica, G. and Giunta, C. (1973) Allionia (Turin) 19, 77-87

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THE SUBCELLULAR DISTRIBUTION OF CAROTENOIDS IN *PHYCOMYCES BLAKESLEEANUS* C115 *car-42 mad-107(—)*

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Summary

1. Subcellular fractions of the C115 *car-42 mad-107(—)* strain of *Phycomyces blakesleeanus*, prepared by differential centrifugation, were irreversibly associated with extraneous lipids. Such contamination was avoided by the use of density gradient centrifugation.

2. The two β-carotene-containing fractions, a particulate fraction and lipid globules, also contained sterol, phospholipid and alkaline *p*-nitrophenylphosphatase activity. Both fractions revealed close similarities on amino acid analysis.

Introduction

Although the pathways of carotenoid biosynthesis have been elucidated in a number of fungi [1,2], there have been relatively few investigations on the intracellular distribution of these pigments in such tissues. The majority of these studies, using subcellular fractions isolated by differential centrifugation, have yielded conflicting results. Carotenoids have been found either exclusively in a single organelle, e.g. mitochondria [3,4], or in a combination of organelles [5—7].

In an attempt to clarify these conflicting reports, we have investigated the subcellular distribution of β-carotene (β,β-carotene) in the C115 *car-42 mad-107(—)* mutant of *Phycomyces blakesleeanus*, using organelles which were isolated by density gradient centrifugation and were assessed for homogeneity by marker enzyme activities.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

Materials

The C115 *car-42 mad-107(—)* mutant of *P. blakesleeanus* contains only one carotenoid, β-carotene [8] and was kindly provided by Dr. Max Delbrück, Division of Biology, California Institute of Technology, Pasadena, Calif. U.S.A.

All the solvents were of analar grade and purified in the usual manner [9]. Antimycin A, L-kynurenine sulphate, 2,6-dichlorophenol-indophenol, NADPH, NADH, NADP, cytochrome *c*, glucose 6-phosphate, *p*-nitrophenyl phosphate (ditris salt), bovine serum albumin and HEPES were purchased from the Sigma Chemical Company, Kingston-upon-Thames, Surrey. Kieselgel G was purchased from Merck Laboratory Chemicals, Darmstadt, Germany, while Woelm neutral alumina and 4-methyl-umbelliferylglucosaminide were from Koch Light Laboratories Ltd., Colnbrook, Bucks. Oxoid Ltd., London, provided the yeast extract. Other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset.

Methods

Culture conditions. The mould was maintained either as a spore suspension in deionised water at 4°C or on 2% (w/v) agar slants containing glucose, 30 g; L-asparagine, 2 g; $MgSo_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1.5 g; yeast extract, 1 g and thiamine hydrochloride, 0.25 mg/l. The liquid medium and cultural conditions were the same as those used previously [9], except that the spore suspension was heat shocked at 45°C for 10 min, prior to inoculation. Larger quantities of mycelia were grown in a fermenter (LH Engineering Co. Ltd., Stoke Poges, Bucks, U.K.) under the same cultural conditions. Mycelia were harvested as described previously [9].

Preparation of cell extracts. Washed mycelia were cut into small pieces and suspended in 8 vols. of ice-cold homogenisation buffer, which contained mannitol, 0.4 M; HEPES, 50 mM; KCl, 10 mM and $MgCl_2 \cdot 6H_2O$, 2 mM, adjusted to pH 7.4 with KOH. Subsequent studies showed that the carotenoid-containing particulate fraction could be isolated more easily from a homogenisation buffer which contained 70% (w/v) sorbitol in place of 0.4 M mannitol. All subsequent procedures were carried out at 4°C.

The suspension was homogenised with a teflon-glass homogeniser (clearance 0.4 mm) for 15 passes and at 1500 rev./min. The resultant homogenate was strained through cheesecloth prior to centrifugation.

Centrifugation procedures. Differential centrifugation of the cell extract at 800 × *g* for 10 min, then at 12 000 × *g* for 15 min and finally at 100 000 × *g* for 1 h sedimented the nuclear, mitochondrial and microsomal fractions, respectively.

All sorbitol solutions contained KCl, 10 mM; $MgCl_2 \cdot 6H_2O$, 2 mM and HEPES, 50 mM, pH 7.4, and are quoted as w/v. Organelles prepared by density gradient centrifugation were isolated from a 800 × g_{max} supernatant fraction, which did not contain cell debris or nuclei and is referred to as cell extract in the following sections.

(a) The mitochondrial fraction was isolated by centrifugation of the cell

extract (15 ml) at 12 500 × g_{max} for 1 h through a discontinuous gradient of 70% sorbitol, 5 ml; 57.5% sorbitol, 5 ml and 50% sorbitol, 5 ml (3 × 43 ml swing-out rotor). Intact mitochondria, located at the 70—57.5% sorbitol interface, were purified by flotation through a 70—25% linear sorbitol gradient for 3 h at 100 000 × g_{max} (6 × 16.5 swing-out rotor).

(b) The microsomal fraction was prepared by a three-stage process. Cell extract (15 ml) was centrifuged on the same discontinuous gradient as that used for the preparation of mitochondria. The sample —50% sorbitol interface was removed, and sorbitol solution added to a final concentration of 70%. Flotation through a linear gradient (15 ml) of 20—60% sorbitol with a 5% overlay of sorbitol at 60 000 × g_{max} for 1 h (3 × 43 ml swing-out rotor) separated the microsomes from fraction (c). Removal of the original sample layer, followed by dilution and centrifugation at 100 000 × g for 1 h sedimented the microsomal fraction.

(c) The carotenoid-containing particulate fraction was isolated from the cell extract (15 ml, containing 70% sorbitol in place of 0.4 M mannitol) by flotation through a continuous gradient of sorbitol (20—57.5%, 15 ml) overlayed with 5% sorbitol (5 ml). Centrifugation at 60 000 × g_{max} for 1 h (3 × 43 ml swing-out rotor) caused this subcellular fraction to collect at the 5—20% interface. It was separated from any contaminating lipid globules by sedimentation at 17 000 × g for 15 min (8 × 50 ml angle rotor). Isopycnic gradient centrifugation on a continuous gradient of sorbitol (20—40%) was used to obtain a density value for this particulate fraction.

(d) Lipid particles floated to the top of the gradients and were collected on a circular metal spatula. Purification was achieved by repated flotation of the particles, suspended in 10% sorbitol, through a solution of 5% sorbitol for 1 h at 100 000 × g (8 × 50 ml angle rotor).

(e) The cytosol was the supernatant obtained after the sedimentation of fractions (b) and (c).

(f) Cell walls were prepared by the method of Mahadevan and Tatum [10].

Enzyme assays. All assays were performed at room temperature, except for protease, arylsulphatase and hexosaminidase activities, which were assayed at 37°C. Succinate:cytochrome *c* oxidoreductase and NADH:cytochrome *c* oxidoreductase were assayed by the method of Douce et al. [11]; kynurenine 3-hydroxylase and antimycin-insensitive NADH:cytochrome *c* reductase by the method of Bandlow [12], while succinate dehydrogenase, malate dehydrogenase, catalase, glucose 6-phosphate dehydrogenase, NADPH:cytochrome *c* reductase, alkaline phosphatase, protease, arylsulphatase and *N*-acetyl-β-D-hexosaminidase were assayed using documented procedures (refs. 13—21, respectively). Samples were treated with 0.05% (w/v) Triton-X-100 prior to the assays for catalase, protease, arylsulphatase, alkaline phosphatase and hexosaminidase.

Lipid analysis. Lipids were extracted from subcellular fractions by the method of Bligh and Dyer [22]. Phospholipids were estimated by the method of Bartlett [23] using phosphatidylcholine as a standard. Unsaponifiable lipids were subjected to chromatography on columns of aluminium oxide (Woelm neutral alumina, Brockmann activity grade III). β-Carotene, eluted with 0.25% (v/v) diethyl ether in light petroleum (b.p. 40—60°C), was estimated using an

$E^{1\%}_{1\,cm}$ value of 2500 in light petroleum (b.p. 40—60°C) [24]. Sterols were eluted with 10—15% (v/v) diethyl ether in light petroleum (b.p. 40—60°) and were assayed by the Liebermann-Burchard reaction [25], using ergosterol as a standard.

Thin layer chromatograms of silica gel G with a developing solvent of light petroleum (b.p. 60—80°C)/diethyl ether/acetic acid (82 : 18 : 1, by vol.) were used to separate the various classes of lipids. Colourless compounds were visualised with iodine vapour.

Amino acid analysis. Samples were analysed on a Jeol model JAC-6AH amino acid analyser using the method of Moore and Stein [26].

Other determinations. Protein was measured by the method of Lowry et al. [27] with bovine serum albumin as a standard. DNA and RNA were extracted by the technique of Ogur and Rosen [28] and assayed spectrophotometrically [29]. Sorbitol concentrations were measured with a refractometer and densities calculated from the data of Price [30].

TABLE I

DISTRIBUTION OF MARKER ENZYMES IN FRACTIONS OBTAINED BY DENSITY GRADIENT CENTRIFUGATION OF C115 *car-42 mad-107*(—) HOMOGENATES

Fractions were isolated by the methods described in the text. All figures are from duplicates of 3 (*), 4 (**), or 5 (†) separate experiments and are quoted ± S.D. Units of enzyme activities are nmols substrate converted per min and activities (S.A.) are units per mg protein. RNA is quoted as μg/mg protein. H is the homogenate (800 × *g* supernatant), M the mitochondrial fraction, P the microsomal fraction, V the particulate fraction, ρ = 1.10 g/ml, S the cytosol and G the lipid particles. S.U. (%) is the percentage of sedimentable enzyme activity, S.Q. (%) the percentage of sedimentable RNA. N.A. means not assayed. DCPIP is 2,6-dichlorophenolindophenol.

Fraction	Protein (*)		RNA (†)			Succinate : DCPIP reductase (**)		
	Total (μg)	Total (%)	Total (μg)	Specific quantity	S.Q. (%)	Units	S.A.	S.U. (%)
H	83.4 ± 4.0		10 661 ± 1943	127.8		1030 ± 140	12.4	
M	4.1 ± 0.40	4.9	190 ± 40	46.9	8.9	120 ± 30	29.6	60.0
P	8.6 ± 2.3	10.3	1890 ± 220	220.5	88.5	70 ± 10	8.2	35.0
V	1.1 ± 0.30	1.3	55 ± 25	52.4	2.6	10 ± 10	9.5	5.0
S	63.6 ± 0.90	76.3	4640 ± 2640	73.0		760 ± 60	11.9	
G	0.47 ± 0.17	0.56	N.A.			10 ± 10	21.3	
Recovery (%)		93.4		63.5			94.2	

Results

The choice of homogenisation and centrifugation procedures

Maximum protein and β-carotene yields (3.5 mg/ml and 48 μg/ml, respectively) from the mycelia were obtained with 40 passes of the teflon homogeniser, but 15 strokes were used in these investigations as this figure represented the best compromise between an adequate protein yield (2.3 mg/ml) and minimal breakage of cell organelles.

Subcellular fractions isolated by differential centrifugation all contained β-carotene and showed satisfactory distribution of succinate: 2,6-dichlorophenolindophenol and glucose 6-phosphate dehydrogenase enzyme activities in the nuclear, mitochondrial, microsomal and cytosol fractions. On subsequent linear gradient centrifugation of the separate fractions, however, the organelles banded at variable densities, all of which were lower than expected (e.g. 1.150 −1.165 g/ml for mitochondria). The organelles had aggregated non-uniformly during differential centrifugation and were also associated with differing amounts of contaminating lipids, including β-carotene, which could not be completely removed by subsequent centrifugation procedures. Consequently, all subsequent studies were carried out on organelles obtained exclusively by density gradient centrifugation of a 800 × g_{max} supernatant fraction.

Fractionation of homogenate by density gradient centrifugation and analysis of cell organelles

The presence of particulate enzymes in the cytosol fraction (Table I) indicates that organelles had been broken during the disruption of the mycelia, but it was concluded on the basis of the distribution of sedimentable enzyme

TABLE I (continued)

Catalase (†)			NADPH : DCPIP reductase (*)			Glucose 6-phosphate dehydrogenase (†)		
Units	S.A.	S.U. (%)	Units	S.A.	S.U. (%)	Units	S.A.	Total (%)
128 ± 60	1.5		9200 ± 1000	110.3		2350 ± 550	28.2	
20 ± 2	4.9	62.5	120 ± 60	29.3	12.5	80 ± 7	19.5	3.4
10 ± 1.0	1.2	31.3	780 ± 130	90.7	81.3	590 ± 30	2.9	25.1
2 ± 0.2	1.9	6.8	60 ± 80	54.5	6.3	0	0	0
105 ± 18	1.7		3930 ± 90	61.8		1900 ± 75	29.9	80.9
0	0	0	70 ± 7	149.0		20 ± 20	1.8	0.85
	107.0			54.0			110.0	

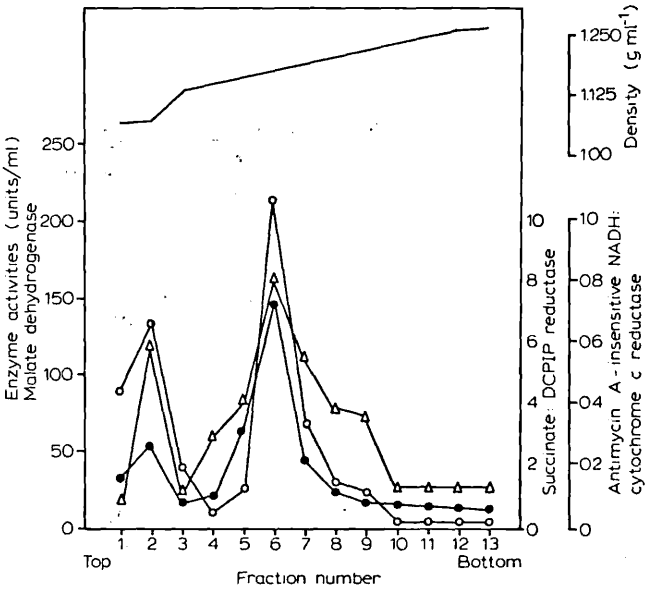

Fig. 1. Isolation of mitochondria by density gradient centrifugation. Homogenate (15 ml of 800 X g supernatant) was layered over 18 ml of a discontinuous sorbitol gradient (70%, 6 ml; 57.5%, 6 ml; 50%, 6 ml; all w/v) and centrifuged at 12500 X g_{max} for 1 h. The 70—57.5% sorbitol interface was collected, diluted slowly with buffer to 15% (w/w) with respect to sorbitol and pelleted at 35 000 X g. The pellet was resuspended in homogenisation buffer (1 ml) and centrifuged through a linear gradient of 75—30% (w/v) buffered sorbitol (12 ml) at 100000 X g_{max} for 1.5 h. Fractions (1 ml) were assayed (nmol substrate converted per ml) for malate dehydrogenase (○), succinate:2,6-dichlorophenolindophenol reductase (△) and antimycin A-insensitive NADH:cytochrome c reductase (●). Fraction 2 represents the interface between the sample volume and the linear gradient.

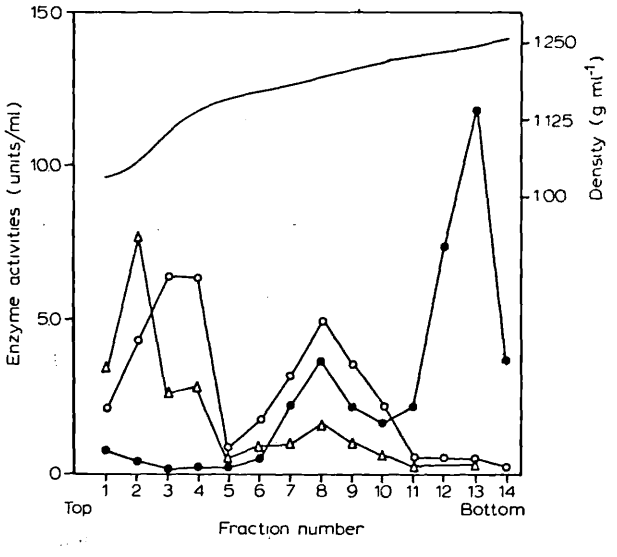

Fig. 2. Separation of outer and inner membranes of mitochondria. Mitochondria were broken by the methods described in the text, and the membranes collected by centrifugation at 100000 X g for 45 min. The pellet, suspended in homogenisation buffer (1 ml), was layered on to a linear sorbitol gradient (75—30% w/v, 13 ml) and centrifuged for 1.5 h at 100000 X g_{max}. Fractions (1 ml) were assayed for succinate:2,6-dichlorophenolindophenol reductase (●), antimycin A - insensitive NADH:cytochrome c reductase (△) and kynurenine hydroxylase (○). Units are the same as for Fig. 1.

TABLE II

β-CAROTENE, PHOSPHOLIPID AND STEROL COMPOSITION OF FRACTIONS OBTAINED BY DENSITY GRADIENT CENTRIFUGATION OF C115 *car-42 mad-107*(—) HOMOGENATES

Subcellular fraction and cell walls were isolated and their lipid content estimated by techniques described in the text. Lipid values are given as μg ± S.D. from 5 experiments. Abbreviation are the same as those listed in Table I.

Fraction	Lipid content (μg)					
	β-Carotene		Phospholipids		Sterols	
	Total	Total (%)	Total	Total (%)	Total	Total (%)
H	280 ± 100		3755 ± 125		1725 ± 636	
M	0.1	0	345 ± 100	9.2	60 ± 25	3.5
P	0.1	0	630 ± 185	16.8	110 ± 50	6.4
V	4.0 ± 1.4	1.4	165 ± 65	4.4	30 ± 5	1.7
S	0.1	0	3667 ± 2975	97.6	1100 ± 495	63.8
G	142 ± 35	50.7	220 ± 95	5.9	425 ± 225	24.6
Cell wall	0.1	0	N.A.		N.A.	
Recovery (%)	52.1		133.7		100.0	

TABLE III

DISTRIBUTION OF HYDROLYTIC ENZYMES IN FRACTIONS OBTAINED BY DENSITY GRADIENT CENTRIFUGATION OF C115 *car-42 mad-107*(—) HOMOGENATES

Fractions were isolated and enzymes assayed by the methods described in the text. Enzyme units and specific activities are the same as in Table I except for protease which is quoted as ng azocasein hydrolysed per min and units per mg protein, respectively. Figures for alkaline *p*-nitrophenyl phosphatase and protease activities are qouted ± S.D. of 3 experiments, while those of arylsulphatase and *N*-acetyl-β-D-hexosaminidase are the mean of duplicates from a single experiment. Abbreviations are the same as those in Table I.

Fraction	Enzyme in fraction									
	Alkaline *p*-nitrophenyl phosphatase			Protease			Arylsulphatase		N-Acetyl-β-D-hexosaminidase	
	Units	S.A.	S.U. (%)	Units	S.A.	S.U. (%)	Units	S.U. (%)	Units	S.U. (%)
H	11 000 ± 190	130		3320 ± 113	39.8		84.4		150.0	
M	1900 ± 100	470	31.6	260 ± 66	64.2	29.9	7.3	47.0	2.3	12.5
P	4100 ± 130	480	68.2	450 ± 21	52.3	51.7	4.6	29.0	13.3	83.3
V	410 ± 36	390	6.8	160 ± 22	145.5	18.4	3.7	24.0	0.4	2.5
S	5500 ± 750	90		1230 ± 340	19.3		69.7		70.0	
G	440 ± 25	940		260 ± 34	553.2		5.0		0	
Recovery (%)		112.0			71.0		107.0		59.0	

TABLE IV

PURIFICATION OF LIPID PARTICLES BY REPEATED FLOTATION

Lipid particles were repeatedly floated through 5% (w/v) buffered sorbitol as described in the Methods section. Enzyme activities are qouted as nmol substrate converted. $min^{-1} \cdot mg^{-1}$ protein and lipid:protein ratios as μg/μg. The β-carotene:protein ratio in the homogenate is 0.015. N.A., not assayed.

	Number of flotations				
	1	2	3	4	5
Alkaline p-nitrophenyl phosphatase	15.2	10.7	19.0	19.9	19.9
Succinate:2,6-dichlorophenolindophenol reductase	21.3	0	0	N.A.	N.A.
Glucose 6-phosphate dehydrogenase	1.8	0	0	N.A.	N.A.
β-Carotene/protein ratio	0.049	0.066	0.132	0.133	0.130
Phospholipid/protein ratio	0.447	0.135	0.190	N.A.	0.180

activities that there were sufficient intact organelles for the determination of β-carotene after final purification of the fractions.

The mitochondrial fraction, which also contained microbodies as shown by the presence of catalase, banded at a density of 1.175—1.180 g/ml on a linear sorbitol gradient (Fig. 1). No β-carotene was detected in this organelle fraction, although both sterols and phospholipids were present (Table II). Mitochondria were broken by the method of Neupert and Ludwig [4] and the inner and

TABLE V

A COMPARISON OF THE AMINO ACID COMPOSITION OF PROTEINS FROM LIPID PARTICLES AND THE CAROTENOID-CONTAINING PARTICULATE FRACTION OF C115 *car-42 mad-107*(—) WITH THOSE FROM LIPID GLOBULES OF *MUCOR HIEMALIS* AND *LIPOMYCES STARKEYI*

Lipid particles were isolated as described in the text and purified by reflotation four times. The particulate fraction, prepared by density gradient centrifugation, was washed twice before hydrolysis. Figures are averages of duplicate hydrolyses.

Amino acid	Mol per cent of amino acid			
	Lipid particles	Particulate fraction (ρ = 1.10 g · ml^{-1})	*Mucor hiemalis* [7]	*Lipomyces starkeyi* [31]
Lys	6.8	7.8	8.4	7.8
His	2.5	2.5	3.4	2.5
Arg	3.3	4.0	4.5	4.3
Asx	8.5	8.8	12.1	10.1
Thr	5.5	5.9	4.7	5.5
Ser	11.2	5.3	5.6	6.7
Glx	9.0	9.4	13.0	12.6
Pro	4.0	4.4	0	trace
Gly	7.0	8.6	10.9	11.2
Ala	8.3	8.8	10.0	9.5
Cys	2.3	2.4	3.1	trace
Val	7.6	8.4	2.2	7.7
Met	2.0	1.6	1.7	3.7
Ile	6.7	6.7	2.3	5.6
Leu	9.1	9.1	8.1	7.0
Tyr	1.8	1.6	3.5	2.4
Phe	4.3	4.7	4.5	3.4

outer membranes isolated (Fig. 2). No β-carotene was associated with either membrane, in contrast to the outer membrane of *Neurospora crassa* mitochondria [4].

The pigmented sub-microsomal fraction (density 1.10 g/ml) contained negligible amounts of RNA and NADPH:2,6-dichlorophenolindophenol reductase activity (Table I), but did contain phospholipids, sterols and β-carotene (Table II), and had a β-carotene:protein ratio (μg/μg) of 0.025 : 1. Several hydrolytic enzymes were associated with this particulate fraction (Table III).

The only other pigmented subcellular fraction, the lipid particles, contained 97.3% of the recovered β-carotene, as well as sterols and phospholipids (Table II). After repeated flotation the ratios of β-carotene to protein, phospholipid to protein and the specific activity of *p*-nitrophenylphosphatase reached constant values (Table IV).

Amino acid analyses of the two β-carotene-containing fractions were similar, but were considerably different from those of proteins found in the lipid particles of *Mucor hiemalis* [7] and *Lipomyces starkeyi* [31] (Table V).

Discussion

This publication, the first complete study on the subcellular distribution of carotenoids in cell extracts of *P. blakesleeanus*, differs from previous investigations on other fungi since all the organelles have been isolated by density gradient centrifugation. On the basis of our observation that the sedimentation of organelles by differential centrifugation causes the irreversible binding of lipids to particulate fractions, it is probable that previous reports of the presence of carotenoids in mitochondria of *Blakeslea trispora* [6], *N. crassa* [4] and *Phycomyces* [3] are a consequence of using this technique. No β-carotene was detected in mitochondria of the C115 mutant isolated by density gradient centrifugation (Table II).

The lipid globules, which contained the overwhelming majority of β-carotene (Table II) are probably similar to those isolated from both plant and fungal material under a variety of names [7,31—33]. The presence of both phospholipids and protein (Tables II and IV) indicates that the globules are surrounded by a membrane resembling that around other lipid particles [7, 31], although the proteins vary in amino acid content from organism to organism (Table V).

It is tempting to identify the particulate fraction which contains the remainder of the β-carotene as a vacuole on account of its density (1.10 g/ml) and the presence of several hydrolytic enzymes (Table III), since both properties are similar to those of vacuoles from *N. crassa* [34]. The multilocational distribution of the hydrolytic enzymes, however, (Table III) implies that it may be only a part of the lysosomal system in *Phycomyces* [35].

The close similarity between the amino acids of the proteins of the lipid globules and the pigmented particulate fraction (Table V) and the presence of some hydrolytic enzymes in both fractions suggests that these two organelles may have a similar subcellular origin.

The role of these two pigmented fractions in the biosynthesis of β-carotene

has yet to be investigated. It is possible that the multi-enzyme complex thought to be essential for carotenoid formation [36,37] is located in the particulate fraction, and once the β-carotene has been synthesised it subsequently enters the lipid globules. Alternatively, the lipid particles themselves may have carotenogenic activity, analogous to the formation of fatty acids and triacylglycerols in globules from castor bean seeds [33]. Investigations aimed at elucidating these possibilities are now in progress.

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References

1 Goodwin, T.W. (1971) in Carotenoids (Isler, O., ed.), pp. 577–636, Birkhäuser, Basel
2 Davies, B.H. (1973) in Carotenoids other than Vitamin A, Vol. III, pp. 1–28, Butterworths, London
3 Keyhani, J., Keyhani, E. and Goodgal, S.H. (1972) Eur. J. Biochem. 27, 527–534
4 Neupert, W. and Ludwig, G.D. (1971) Eur. J. Biochem. 19, 523–532
5 Zalokar, M. (1969) J. Cell Biol. 41, 494–509
6 Cederberg, E. and Neujahr, H.Y. (1970) Experientia 26, 366–367
7 Herber, R. (1974) D. ès Sc. Thesis, University of Nancy, France
8 Meissner, G. and Delbrück, M. (1968) Plant Physiol. 43, 1279–1283
9 Aung Than, Bramley, P.M., Davies, B.H. and Rees, A.F. (1972) Phytochemistry 11, 3187–3192
10 Mahadevan, P.R. and Tatum, E.L. (1965) J. Bacteriol. 90, 1073–1081
11 Douce, R., Christensen, E.L. and Bonner, W.D. (1972) Biochim. Biophys. Acta 275, 148–160
12 Bandlow, W. (1972) Biochim. Biophys. Acta 282, 105–122
13 King, T.E. (1967) Method Enzymol. 10, 216–225
14 Watson, K., Bertoli, E. and Griffiths, D.E. (1975) Biochem. J. 146, 401–407
15 Aebi, H. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) pp. 673–684, Academic Press, London
16 Langdon, R.G. (1966) Methods Enzymol. 9, 126–131
17 Dignam, J.D. and Strobel, H.W. (1975) Biochem. Biophys. Res. Commun. 63, 845–852
18 Torriani, A. (1960) Biochim. Biophys. Acta 38, 460–479
19 Hazen, G.G. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) pp. 1000–1005, Academic Press, London
20 Agogbua, S.I. and Wynn, C.H. (1976) Biochem. J. 153, 415–421
21 Price, R. and Dance, N. (1972) Biochim. Biophys. Acta 271, 145–153
22 Bligh, M.C. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–915
23 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
24 Davies, B.H. (1965) Chemistry and Biochemistry of Plant Pigments (Goodwin, T.W. ed.), pp. 489–532, Academic Press, London
25 Glover, J. (1964) in The Determination of Sterols, pp. 10–17, No. 2, Society for Analytical Chemistry Monograph
26 Moore, S. and Stein, W.H. (1963) Method Enzymol. 6, 819–831
27 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
28 Ogur, M. and Rosen, G. (1950) Arch. Biochem. 25, 262–276
29 Munro, H.N. and Fleck, A. (1966) Analyst 91, 78–88
30 Price, C.A. (1974) in Subcellular Particles, Structures and Organelles (Laskin, A.I. and Last, J.A., eds.), pp. 155–230, Marcel Dekker, New York
31 Uzuka, Y., Kanamori, T., Koga, T., Tanaka, K. and Naganuma, T. (1975) J. Gen. Appl. Microbiol. 21, 157–168
32 Yatsu, L.Y. and Jacks, T.J. (1972) Plant Physiol. 49, 937–947
33 Harwood, J.L., Sodja, A., Stumpf, P.K. and Spurr, A.R. (1971) Lipids 6, 851–854
34 Matile, Ph. (1971) Cytobiologie 3, 324–330

35 Matile, Ph. (1975) The Lytic Compartment of Plant Cells, Continuation of Protoplasmatologie, Vol. I, Springer-Verlag, Vienna
36 De la Guardia, M.D., Aragón, C.M.G., Murillo, F.J. and Cerdá-Olmedo, E. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2012—2015
37 Aragón, C.M.G., Murillo, F.J., de la Guardia, M.D. and Cerdá-Olmedo, E. (1976) Eur. J. Biochem. 63, 71—75