Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp.

Chapter 3: Metabolism and biochemistry of hyphal systems

In this chapter I present an account of the ways in which fungal hyphal systems obtain, absorb, metabolise, reprocess and redistribute nutrients. The description is relatively brief and more details can be found in texts on fungal physiology, such as Jennings (1995).

This material is relevant here for a number of reasons. First, these metabolic and biochemical activities of fungi provide the background and context within which their differentiation and morphogenesis occur. In discussing differentiation and morphogenesis there will be frequent need to refer to biochemical processes and it is useful to have those details readily to hand for reference. Second, fungi adapt the metabolism which normally serves their vegetative hyphal growth phases in specific ways to provide for and support their morphogenesis. Consequently, description of the basic metabolism is a valuable preparation for understanding the ways in which it is adapted. Third, description of basic metabolism provides another opportunity to make comparisons with the other eukaryotic kingdoms and to show, again, how kingdom Fungi make use of advanced and sophisticated mechanisms in the management of their cell biology. Almost all of the processes described in this chapter will appear in some guise again in discussions of fungal morphogenesis in later chapters.

3.1 Nutrients in nature

Texts on fungal physiology usually present lists of chemicals that fungi can utilise when added to culture media. Like attempting to study the nutrition of *Homo sapiens* by noting the range of fast food outlets on the local high street, such an approach merely illustrates the range of nutrients the organism can be forced to endure. It reveals little about the natural substrates which have formed part of the evolutionary biology of the organism.

There are three major fungal nutritional modes: probably the majority are ***saprotrophs*** for which the substrates are dead organic materials not killed by the fungus itself (Cooke and Rayner, 1984). ***Necrotrophs*** invade living tissues which they kill and then utilise, whereas ***biotrophs*** exploit host cells which remain alive. In the latter case one might expect that though local digestion of host tissue may be necessary for penetration or establishment of the pathogen, only simple nutrients would be removed from the host because of the damage which would be inflicted on the host by large-scale digestion of polymeric cell constituents. Biotrophs may be host-specific, but saprotrophs and necrotrophs generally have a very large range of habitats open to them, and in the majority of these polymeric sources of nutrients predominate.

This predominance of polymers as sources of nutrients is obviously true for such materials as herbaceous plant litter, wood and herbivore dung, but it also applies to the soil. The bulk of plant litter consists of plant cell walls and consequently contains large amounts of cellulose, hemicellulose and lignin, even though the cytoplasm of the dead plant cells will contribute lipids, proteins and organic phosphates to the remains. Wall components amount to 90% of the dry weight of wheat straw, for example (Chang, 1967). Wood, since it derives from secondary wall growth, is especially rich in wall polymers and correspondingly relatively poor in other potential nutrients, particularly nitrogen and phosphorus (Swift, 1977). Digested litter (a euphemism for herbivore dung) is, on the other hand, relatively enriched in nitrogen, vitamins, growth factors and minerals, since in passage through the intestine it accumulates the remains of bacteria, protozoa and other microorganisms (Lodha, 1974). The composition of animal tissue varies enormously according to the particular organ system considered, but in nature most animal remains will be eaten by animal scavengers too rapidly for any microbes to be able to compete, so the microorganisms will be left with the parts - ‘skin, gristle and bone’ - that other organisms cannot reach.

In soil, nitrogen exists largely in the form of organic compounds; the proportion of nitrogen occurring as ammonium, nitrate or nitrite rarely exceeds 2%, although there may be a higher proportion of clay-fixed ammonium in some soils (Bremner, 1967). Inorganic nitrogen compounds only predominate in agricultural soils which are repeatedly dosed with chemical fertilizers. Nitrite is not usually detectable and nitrate content is usually very low in natural soils because these salts are so readily leached out by rain, so in most cases exchangeable-ammonium and the organic nitrogen provide saprotrophs with the most readily-available sources of this element. In most surface soils, 20-50% of total nitrogen occurs in proteinaceous form, and 5-10% as combined and complexed amino sugars. The amino sugars also contribute, of course, to the carbohydrate component of the soil, which represents 5-16% of the total organic matter. Here, again, though most soil carbohydrate is in polymeric form. Monosaccharides represent less than 1% of the carbohydrate but cellulose can account for up to 14% of total carbohydrate and chitin must also be well represented in view of the occurrence of amino sugars. Ericoid mycorrhizas are able to use chitin as a sole source of nitrogen (Leake and Read, 1990). About 50-70% of total phosphorus in soil is organic, mostly as phosphate esters related to or derived from compounds like nucleic acids, inositol phosphates and phospholipids.

Although inorganic forms of sulfur may accumulate in some soils (e.g. as calcium and magnesium sulfates in arid regions; calcium sulfate co-crystallised with calcium carbonate in calcareous soils) there is little inorganic sulfur in the surface horizons of soils in humid regions. Organic sulfur occurs in the form of methionine and cystine (and derivatives), and sulfate esters, including sulfated polysaccharides and lipids.

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

That fungi can utilise the polymeric nutrient sources which are in most ready supply can be demonstrated indirectly by experimentally monitoring growth on, or degradation of, particular materials. For example, supplementation of mushroom compost with vegetable oils and linoleic and oleic acid esters causes growth stimulation of the cultivated mushroom *Agaricus bisporus* (Wardle and Schisler, 1969), which must mean that, at least in pure culture, the organism is able to utilise supplied lipids. Similarly, though lignin is considered to be highly resistant to degradation, about two-thirds of the lignin present in compost when first inoculated with *A. bisporus* has disappeared by the time the crop is picked. *Agaricus bisporus*, shares with two other litter-degrading mushrooms, *Coprinus cinereus* and *Volvariella volvacea*, the ability to use protein as efficiently as the sugar glucose as a sole source of carbon, and can use protein, additionally, as a source of nitrogen and sulfur (Kalisz *et al*., 1986). *A. bisporus* and a wide range of other filamentous fungi (Fermor and Wood, 1981; Grant *et al*., 1986) have been shown to be able to degrade dead bacteria and to utilise them as sole source of carbon, nitrogen and phosphorus.

For most fungi in most circumstances, therefore, the initial nutritional step is the excretion of enzymes able to convert polymers to the simple sugars, amino acids, carboxylic acids, purines, pyrimidines, etc, the cell can absorb. In doing this they obviously contribute to recycling and, mineralisation of nutrients.

3.2 Extracellular polymer-degrading enzymes

Besides lignin (see below), the bulk of plant cell biomass consists of the polysaccharides cellulose, hemicelluloses, and pectins in varying proportions depending on the type of cell and its age. Plant biomass does not consist of neatly isolated packets of polysaccharide, protein and lignin; these three (and other materials) are intimately mixed together, so that it is better to think of the degradation of lignocellulosic and/or lignoprotein complexes. A typical agricultural residue, like cereal straw or sugar cane bagasse, contains 30-40% cellulose, 20-30% hemicellulose and 15-35% lignin. Organisms may differ in their ability to degrade components of this mixture. On this sort of basis wood-decay fungi have been separated into white-rot, brown-rot and soft-rot species. The white-rot fungi (about 2000 species, mostly basidiomycetes) can metabolise lignin, on the other hand, brown-rot fungi (about 200 basidiomycete species) degrade the cellulose and hemicellulose components without much effect on the lignin. Soft-rot species (mostly soil-inhabiting ascomycetes and deuteromycetes) have rather intermediate capabilities, being able to degrade cellulose and hemicellulose rapidly, but lignin only slowly. These differences in behaviour are a reflection of the different enzymes produced by these organisms and serve to emphasise that the organisms must digest complexes of potential nutrient sources and assemble panels of different enzymes to do so. For ease of presentation here, however, I must consider degradation of specific compounds separately.

3.2.1 Polysaccharide degradation

Polysaccharides are polymers of monosaccharides in which the constituent sugars are connected with glycosidic bonds. There is a considerable variety of polysaccharides, both because of the number and variety of available sugars and because of the diversity of bonding possibilities between different carbon atoms of the adjacent sugar residues. There is a matching variety of enzymes, hydrolases or glucosidases, capable of hydrolysing this range of glycosidic links. Enzymes responsible for polymer degradation (any polymer, not just polysaccharide) may employ one of two strategies of attack. They may attack randomly, effectively fragmenting the polymer molecule into a number of oligomers, these are the endo-enzymes, or they may approach terminally, digesting away monomers or dimers, the exo-enzymes.

Cellulose is the most abundant organic compound on Earth and accounts for over 50% of organic carbon; about 10^{11} tons are synthesised each year. It is an unbranched polymer of glucose in which adjacent sugar molecules are joined by β-1→4 linkages (Fig. 3.1); there may be from a few hundred to a few thousand sugar residues in the polymer molecule, corresponding to molecular weights from about 50,000 to approaching 1 million. Breakdown of cellulose is chemically straightforward, but is complicated by its physical form, which is still not completely understood. Mild acid hydrolysis of cellulose releases soluble sugars, but does not go to completion; oligomers of 100-300 glucose residues remain. The fraction which is readily hydrolysed is called amorphous cellulose while that which is resistant to acid is called crystalline cellulose. Since it influences chemical breakdown, the conformation and three-dimensional structure of cellulose must influence cellulolytic enzyme activity.

The cellulolytic enzyme (cellulase) complex of white-rot fungi like *Phanerochaete chrysosporium* and deuteromycetes like *Trichoderma reesei* consists of a number of hydrolytic enzymes: endoglucanase, exoglucanase and cellobiase (which is a β-glucosidase) which work synergistically and are organised into an extracellular multienzyme complex called a cellulosome (Lemaire, 1996). Endoglucanase attacks cellulose at random, producing glucose, cellobiose (a disaccharide) and some cellotriose (a trisaccharide). Exoglucanase attacks from the non-reducing end of the cellulose molecule, removing glucose units; it may also include a cellobiohydrolase activity which produces cellobiose by attacking the non-reducing end of the polymer. Cellobiase is responsible for hydrolysing cellobiose to glucose. Glucose is, thus, the readily-metabolised end-product of cellulose breakdown by enzymatic hydrolysis.

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

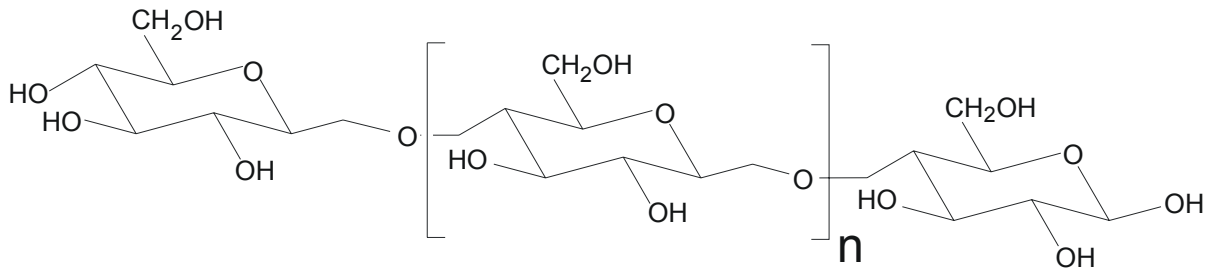


Fig. 3.1. Structural formula of cellulose.

In addition to catalytic regions, many cellulolytic enzymes contain domains not involved in catalysis, but participating in substrate binding, multi-enzyme complex formation, or attachment to the cell surface. These domains assist in the degradation of crystalline cellulose by securing the enzymes to the substrate, by focusing hydrolysis on restricted areas in which the substrate is synergistically destabilized by multiple cutting events, and by enabling recovery of degradation products by the producing organism (Beguin and Aubert, 1994; Radford *et al*., 1996).

When grown on cellulose, the white-rot fungi like *Phanerochaete chrysosporium* produce two cellobiose oxidoreductases; a cellobiose: quinone oxidoreductase (CBQ) and cellobiose oxidase (CBO). Cellobiose oxidase is able to oxidise cellobiose to the δ-lactone, which can then be converted to cellobionic acid and then glucose + gluconic acid; cellobiose δ-lactone can also be formed by the enzyme cellobiose: quinone oxidoreductase. Similar cellobiose-oxidizing enzymes, capable of utilizing a wide variety of electron acceptors, have been detected in many other fungi, though their role is uncertain. These enzymes are probably of most significance in regulating the level of cellobiose and glucose, the accumulation of which can inhibit endoglucanase activity. The role originally ascribed to CBQ was as a link between cellulose and lignin degradation. Cellobiose oxidase also reduces Fe(III) and together with hydrogen peroxide, generates hydroxyl radicals. These radicals can degrade both lignin and cellulose, possibly indicating that cellobiose oxidase has a central role in degradation of wood by wood-degrading fungi (Ander, 1994). However, most evidence available so far indicates that the presence of CBO/CBQ with lignin peroxidases and laccases actually reduces the rate of oxidation of lignin degradation products (Eriksson *et al*., 1993).

Brown-rot fungi use a rather different initial cellulolytic system to the hydrolytically-based one employed by the white-rots. Brown-rot fungi are able to depolymerise cellulose rapidly and virtually completely. Even cellulose deep within the walls and protected by lignin polymers is prone to attack. The process seems to depend on H_2O_2 (secreted by the fungus) and ferrous ions in the wood oxidising sugar molecules in the polymer, thereby fragmenting it and leaving it open to further attack by hydrolytic enzymes. Interestingly, it has been suggested that the oxalate crystals which coat so many fungal hyphae are responsible for reducing the ferric ions normally found in wood to ferrous ions, so potentiating oxidative cleavage of the cellulose. Although the white-rot fungi produce H_2O_2 for lignin degradation they do not secrete oxalate and therefore fail to depolymerise cellulose oxidatively.

Hemicellulose is a name which covers a variety of branched-chain polymers containing a mixture of various hexose and pentose sugars, which might also be substituted with uronic and acetic acids. The main hemicelluloses found in plants are xylans (1→4-linked polymers of the pentose sugar xylose), but arabans (polyarabinose), galactans (polygalactose), mannans and copolymers (e.g. glucomannans and galactoglucomannans) are also encountered. The major angiosperm hemicellulose is a xylan with up to 35% of the xylose residues acetylated, and it is also substituted with 4-*O*-methylglucuronic acid in dicotyledonous plants. Enzymes responsible for hemicellulose degradation are named according to their substrate specificity; for example, mannanases degrade mannans, xylanases degrade xylans, etc. As xylans predominate in plant walls, more is known about xylanases.

Xylanases can be induced by their substrate, the response being for the fungus to produce a complex of enzymes rather than a single one. The complex consists of at least two endoxylanases and a β-xylosidase. The endoxylanases degrade xylan to xylobiose and other oligosaccharides while the xylosidase degrades these smaller sugars to xylose. Some arabinose is also formed, showing that the xylanase complex is able to hydrolyse the branch points in xylan.

Pectins consist of chains of β-1→4 linked galacturonic acids, in which about 20 to 60% of the carboxyl groups are esterified with methanol. They occur primarily in the middle lamella between plant cells. As this represents only a small proportion of the plant wall they are correspondingly of little importance as a component of plant litter. However, extensive breakdown of the middle lamella of living plants is brought about by necrotrophic parasites. Pectinases, therefore, are of great importance during fungal invasion of plant tissue

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

(Byrde, 1982).

Polygalacturonases and pectin lyases attack the true pectins, while arabanases and galactanases degrade the neutral sugar polymers associated with them. These activities have drastic effects on the structural integrity of the tissues which may extend to death of the cell due to osmotic stresses imposed by damage to the wall. It seems likely that the products of pectinase activity will be absorbed as nutrient by the fungus, but these enzymes are better considered to be part of the machinery by which plant defences are breached than as being concerned primarily with nutrient supply.

Chitin, in which the repeating unit is the same as that in cellulose except that the hydroxyl group at C-2 is replaced by an acetamido group (Fig. 2.1), is the second most abundant polymer on Earth as it occurs in the exoskeletons of arthropods and, of course, in fungal cell walls. Polysaccharides which contain amino sugars or their derivatives are called mucopolysaccharides. Chitin is degraded by chitinase, a glucan hydrolase which attacks the β-1→4 glycosidic bonds, eventually producing the disaccharide chitobiose which is then converted to the monosaccharide *N*-acetylglucosamine by chitobiase. Chitinase may also be involved in fungal wall synthesis (see Chapter 2).

Starch, the major reserve polysaccharide of plants, contains glucose polymers with α-1→4 glycosidic bonds. Amylose is constituted of long unbranched chains, whereas amylopectins (comprising 75-85% of most starches) have branch points formed from α-1→6 glycosidic bonds (Fig. 3.2). Starch degrading enzymes include: α-amylases, which are endoamylases acting on 1→4 bonds and bypassing the 1→6 bonds; β-amylases, which are exoamylases producing the disaccharide maltose by splitting alternate 1,4 bonds until they reach a 1,6 branch point (which they cannot bypass); amyloglucosidases (or glucoamylases), which can act on both 1→4 and 1→6 bonds, seem to occur almost exclusively in fungi; debranching enzymes (e.g. pullulanase) which sever 1→6 bonds; α-glucosidases which hydrolyse 1→4 glycosidic linkages in disaccharides and oligosaccharides, producing glucose as the end-product of starch breakdown (Radford *et al*., 1996).

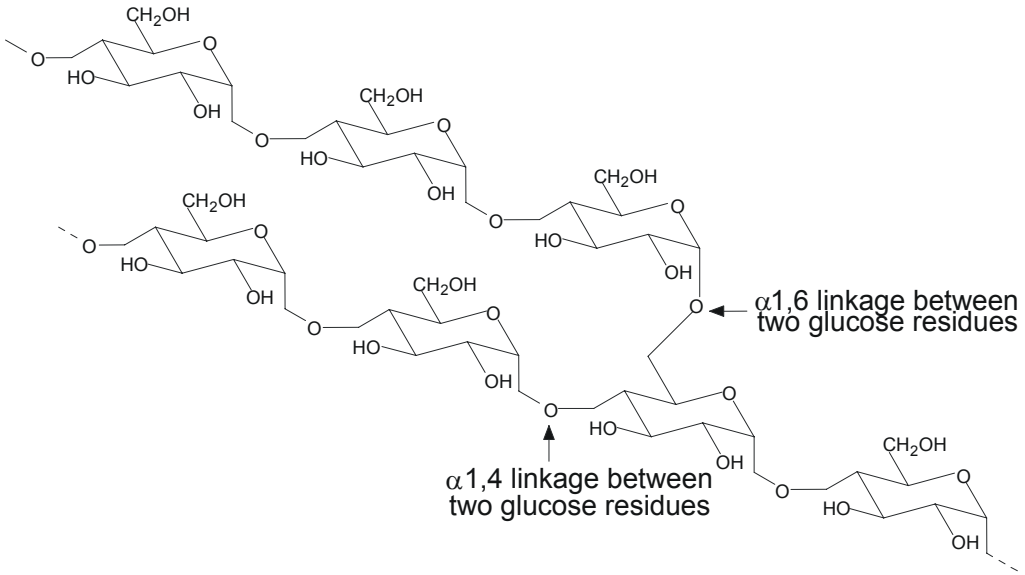


Fig. 3.2. Structural formula of amylopectin.

Glycogen is very similar to starch, being a branched polymer composed of glucose residues linked by α-1→4 glycosidic bonds; about every tenth residue is involved in a branch formed by α-1→6 glycosidic bonds. It is the polysaccharide reserve found in animal tissues, and in the fungi themselves. Most fungi are likely to encounter glycogen in their surroundings, as they are likely to be surrounded by dead and dying fungal cells. Intracellularly, glycogen is degraded by a phosphorylase which releases glucose 1-phosphate for metabolic use (Fig. 3.3), with the aid of a transferase and α-1→6-glucosidase (activities of a single polypeptide) to deal with the branches. Extracellularly, glycogen is probably degraded by components of the amylase enzyme complex.

3.2.2 Lignin degradation

Lignins are high-molecular weight, insoluble polymers which have complex and variable structures, being composed essentially of many methoxylated derivatives of benzene, especially coniferyl, sinapyl, and coumaryl alcohols, the proportions of these three differing between angiosperms and gymnosperms (Fig. 3.4). The ability to degrade lignin is limited to a very few microorganisms, including a range of basidiomycetes, some ascomycetes and a few bacteria. Consequently, lignin is extremely resistant to microbial degradation itself and can protect other polymers from attack. It has been suggested that lignin may be degraded non-enzymically, being oxidised by a chemically-produced 'activated oxygen'; at least part of the argument being that lignins are

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

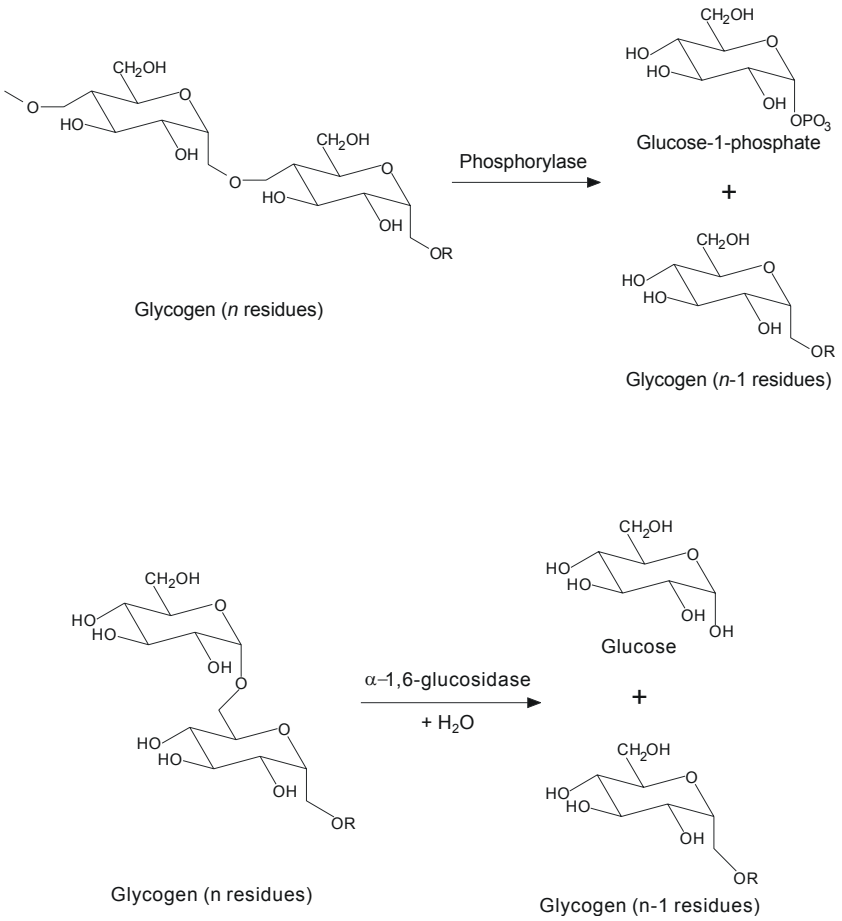


Fig. 3.3. Intracellular degradation of glycogen. The top panel shows glycogen phosphorylase activity severing one of the 1→4 linkages. The bottom panel shows the 'debranching activity' of the glucosidase hydrolysing a 1→6 linkage.

so variable in chemical structure that any enzymes concerned would have to be non-specific. However, even the information about enzymes seems to point towards something like a 'combustion' process (Reid, 1995).

Most research has been concentrated on two ligninolytic organisms; the white-rot basidiomycete fungus *Phanerochaete chrysosporium* (= *Sporotrichum pulverulentum*) and the actinomycete bacterium *Streptomyces viridosporus*. *P. chrysosporium*, in axenic culture, is able to mineralise lignin completely to CO_2 and water and it is now quite clear that both organisms do produce extracellular enzymes which are specifically and necessarily involved in lignin degradation, the best characterized of which are laccase, lignin peroxidases and manganese peroxidases.

Lignin peroxidase (ligninase) is the key lignin-degrading enzyme of white-rot fungi. *P. chrysosporium* produces a family of lignin peroxidases, which are extracellular glycosylated heme proteins, as major components of its lignin-degrading system. There can be as many as 15 lignin peroxidase isozymes, ranging in molecular mass from 38,000 to 43,000, the spectrum of isozymes produced depending on culture conditions and strains employed. Manganese-dependent peroxidases are a second family of extracellular heme proteins produced by *P. chrysosporium* that are also believed to be important in lignin degradation by this organism (Elisashvili, 1993; Reddy and Dsouza, 1994; Broda *et al*., 1996; Cullen and Kersten, 1996).

The lignin-degradative system of *P. chrysosporium* appears after cessation of primary growth (i.e. it is an aspect of the secondary metabolism of the organism) and can be induced by nitrogen starvation. Intracellular cAMP levels appear to be important in regulating the production of lignin peroxidases and manganese peroxidases, though production of the former is affected more than that of the latter. When the fungus is grown in low-nitrogen medium there is an increase in H_2O_2 production by cell extracts which correlates with the appearance of ligninolytic activity; experimental destruction of H_2O_2 by adding the enzyme catalase strongly inhibits lignin breakdown. Thus, evidence for involvement of H_2O_2 in lignin degradation by *P. chrysosporium* is conclusive. So it seems that activated oxygen is involved in degrading lignin, but is held in the active site of a specific extracellular enzyme, the lignin peroxidase. The initial step involves oxidation by one electron and produces unstable intermediates which can undergo a wide range of subsequent oxidative reactions (Kirk *et al*., 1990; Hatakka, 1994; Cullen and Kersten, 1996).

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

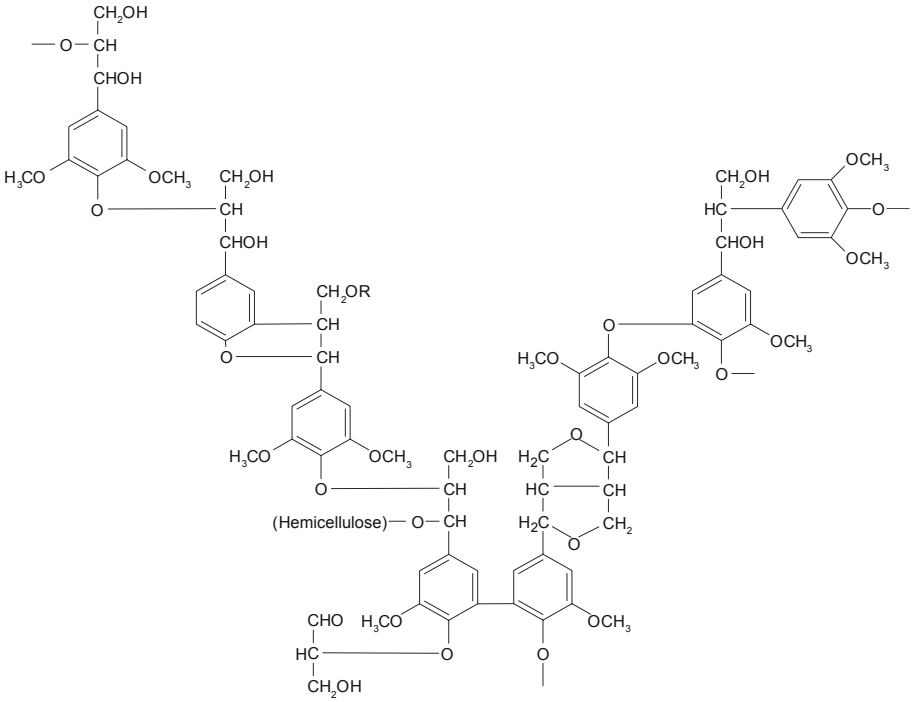


Fig. 3.4. Schematic formula of angiosperm lignin.

Although few fungi produce ligninolytic enzymes, a much wider range excrete laccases as extracellular enzymes. These are copper-containing oxygenases which are able to oxidise *o*- and *p*-phenols and are required for the metabolism of lignin degradation-products. They are particularly interesting as their appearance or disappearance in fungal cultures has been correlated with sexual and asexual reproduction in a number of cases. Thus, during mycelial growth of the cultivated mushroom, *Agaricus bisporus*, a large proportion of the compost lignin is degraded and correspondingly high activities of laccase are recorded. This one enzyme can amount to 2% of the total fungal protein (Wood, 1980a). Yet, as the culture forms fruit bodies laccase activity is rapidly lost, initially by inactivation and subsequently by proteolysis (Wood, 1980b). The activity of a manganese-dependent lignin-degrading peroxidase has also been monitored from the time of colonization of the compost through the development of fruit bodies and was found to be correlated with the laccase, suggesting that both enzymes have significant roles in lignin degradation by this fungus (Bonnen *et al*., 1994). The pattern of behaviour of these two enzymes illustrates both the changing nutritional demands of fungal mycelia as they process through successive developmental phases and the ability of the mycelium to act on its environment to satisfy those demands.

3.2.3 Protein degradation

Proteinases are peptide hydrolases; a group of enzymes which hydrolyse the peptide bonds of proteins and peptides, cleaving the substrate molecule into smaller fragments and, eventually, into amino acids. This is a complex group of enzymes, varying greatly in physicochemical and catalytic properties. Proteolytic enzymes are produced intra- and extracellularly, playing important roles in regulatory processes of the cell as well as contributing to nutrition through degradation of protein food sources. Intracellular proteolysis seems to be the responsibility of large multicatalytic complexes of proteinases which are called proteasomes. 20S proteasomes are cylindrical particles found in the cytoplasm and nucleoplasm of all eukaryotes. They are composed of a pool of 14 different subunits (M_r 22-25 kDa) arranged in a stack of 4 rings. 26S proteasomes are larger, comprising a 20S proteasome as core particle with additional subunits complexed at the ends of the 20S cylinder. Proteasomes are needed for stress-dependent and ubiquitin-mediated proteolysis. They are involved in the degradation of short-lived and regulatory proteins and are, therefore, important for cell differentiation, adaptation to environmental changes, and control of the cell cycle (Hilt and Wolf, 1995).

Extracellular proteinases are involved mainly in the hydrolysis of large polypeptide substrates into the smaller molecules which can be absorbed by the cell. Extracellular proteinases are produced by many species of fungi but most is known about protein utilisation and proteinase production by *Aspergillus* species and *Neurospora crassa*. The basidiomycetes *Agaricus*, *Coprinus*, and *Volvariella* have been shown to be able to use protein as a sole source of carbon about as efficiently as they can use the sugar glucose, and can also use protein as a source of nitrogen and sulfur (Kalisz *et al*., 1986, 1987, 1989). The real value of protein as a nutrient for fungi became evident only comparatively recently. Mycorrhizal fungi have been shown to use protein as a source of both nitrogen and carbon (Bajwa and Read, 1985; Spinner and Haselwandter, 1985) and some

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

ectomycorrhizas supply nitrogen derived from proteins in soil to their higher plant symbionts (Abuzinadah and Read, 1986a, 1986b; Abuzinadah *et al*., 1986; Read *et al*., 1989; Read, 1991). Protein is probably the most abundant nitrogen source available to plant-litter-degrading organisms in the form of plant protein, lignoprotein and microbial protein. Many pathogenic microorganisms secrete proteinases which are involved in the infection process and some, including the apple pathogenic fungus *Monilinia fructigena*, are known to utilise host proteins for nutrition. The virulence of a few pathogenic fungi is correlated with their extracellular proteinase activity. Several species release specific proteinases which can hydrolyse structural and other proteins resistant to attack by most other proteinases, such as insect cuticles (Samuels and Paterson, 1995: St Leger, 1995). The dermatophytes *Microsporum* and *Trichophyton* produce collagenases, elastases and keratinases.

Enzymes which degrade proteins form two major groups - peptidases and proteinases (Kalisz, 1988). Exopeptidases remove terminal amino acids or dipeptides and are subdivided according to whether they act at the carboxy terminal end of the substrate protein (carboxypeptidases); the amino terminal end (aminopeptidases), or on a dipeptide (dipeptidases). Proteinases cleave internal peptide bonds - they are endopeptidases. The proteinase catalytic mechanism can be determined indirectly by study of response to inhibitors which react with particular residues in the active site of the enzyme. This leads to subclassification into four groups:

- Serine proteinases are the most widely distributed group of proteolytic enzymes. They have a serine residue in the active site, are generally active at neutral and alkaline pH, and show broad substrate specificities.
- Cysteine proteinases occur in few fungi though extracellular cysteine proteinases have been reported in *Microsporum* sp., *Aspergillus oryzae*, and *Phanerochaete chrysogenum* (*Sporotrichum pulverulentum*).
- Aspartic proteinases show maximum activity at low pH values (pH 3 to 4) and are widely distributed in fungi.
- Metalloproteinases have pH optima between 5 and 9 and are inhibited by metal-chelating reagents,such asethylenediamine tetraacetic acid (EDTA). In many cases the EDTA-inhibited enzyme can be reactivated by zinc, calcium or cobalt ions. Metalloproteinases are widespread, but only a few have been reported in fungi and most of these are zinc-containing enzymes, with one atom of zinc per molecule of enzyme.

3.2.4 Lipases and esterases

Lipases and esterases catalyse the hydrolysis of esters made between alcohols and organic ('fatty') acids. They generally have low specificity and any lipase will hydrolyse virtually any organic ester, though different esters will be acted upon at different rates. The main factors influencing what specificity is expressed are the lengths and shapes of hydrocarbon chains either side of the ester link. The term esterase is generally applied to enzymes 'preferring' short carbon chains in the acyl group and these are the enzymes which have so often been examined for electrophoretic variants in studies of population genetics. The lipases 'proper' tend to favour long carbon chains in the acyl group. Their substrates include fats, the lipid components of lipoprotein and the ester bonds in phospholipids.

Extracellular lipase production has been detected in *Agaricus bisporus* during degradation of bacteria (Fermor and Wood, 1981). In fermenter cultures most of the lipase is produced in the stationary phase (i.e. is a secondary metabolic activity) and regulation of lipase production in *Rhizopus* is very much affected by carbon and nitrogen sources in the medium and by the oxygen concentration (Mukhamedzhanova and Bezborodov, 1982; Guiseppin, 1984).

3.2.5 Phosphatases and sulfatases

Phosphatases are also esterases, acting on esters of alcohols with phosphoric acid. They are enzymes of comparatively low specificity but fall into groups depending on their activity as phosphomonoesterases, phosphodiesterases and polyphosphatases. The phosphomonoesterases are further distinguished according to their pH optima as alkaline or acid phosphatases. For example, phosphatases are among the extracellular enzymes produced by *Agaricus bisporus* during growth on compost and must be important, therefore, in the nutrition of such litter degrading fungi. Sulfatases act on sulfate esters in the same way that phosphatases act on phosphate esters. They may be important in recovery of sulfate from the sulfated polysaccharides which are found in soils.

3.3 Production, location, regulation and use of degradative enzymes

3.3.1 Production

Extracellular enzymes are produced within the cell but act outside it. Consequently, they must be secreted across the plasmalemma. The indications are that the processes involved in protein translocation across membranes are very similar in all eukaryotes. Polypeptides destined for secretion are identified by short amino terminal transient 'signal' sequences which consist of uninterrupted stretches of at least six hydrophobic amino acid residues. The signal sequence is in the first part of the polypeptide to be synthesised on the ribosome and, as its hydrophobicity confers an affinity for the lipid environment of a membrane bilayer, the signal sequence 'targets'

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

the ribosome producing it onto the endoplasmic reticulum membrane. The translocation machinery includes a targeting system on the side of the membrane where the polypeptide is being synthesised, an oligomeric transmembrane channel, a translocation motor powered by the hydrolysis of nucleotide triphosphate, and a protein folding system on the far side of the membrane (Edwardson and Marciniak, 1995; Schatz and Dobberstein, 1996). The signal peptides are cleaved off by proteolysis during membrane passage, yielding active polypeptides which can be delivered to their site of action in membrane-bound vesicles.

Fungal compartments housing Golgi functions in secretory transport do not resemble the interphase Golgi apparatus of other eukaryotes, but are more fragmentary. Nevertheless, the overall process is similar to the mammalian secretion scheme and morphologically distinct populations of transport microvesicles are formed from endoplasmic reticulum and Golgi cisternae. Thus, transfer of proteins between different compartments in the cell and between the endoplasmic reticulum and the plasmalemma involves coated vesicles which bud off from the endoplasmic reticulum, migrate to the plasmalemma with which they fuse, externalising their contents. Components of the membrane-associated protein translocation machinery have been recognised in the yeast, *Saccharomyces cerevisiae*. Similarly, coated vesicles have been identified in *S. cerevisiae*, *Neurospora crassa* and the rust fungus *Uromyces phaseoli*. The protein clathrin, which has a major role in constructing a basket-like coat around these transport vesicles has been extracted from *S. cerevisiae*, and has been associated with vesicles containing secretable enzymes. Gene products involved in sorting vacuolar protein, which are responsible for the recognition, packaging, and vesicular transport of proteins to the vacuole of yeast are homologous with those of other eukaryotic cells. There is no doubt that the characteristic protein export mechanisms of eukaryotes operate in fungi, but with some modification, presumably to compensate for the poorly-developed Golgi complex. For example, in fungi there is evidence that the nuclear envelope lumen houses certain functions normally associated with the endoplasmic reticulum and some steps of outer-chain glycosylation may occur in microvesicles during transport.

3.2 Location

Some of the extracellular enzymes which are produced are soluble and are freely dispersed in fluid films surrounding the hyphae, but others are fixed in space by being bound to the hyphal wall, extracellular matrix or even to the substrate itself. The exo- and endoglucanases of the cellulase complex strongly absorb to native cellulose; cellobiase does not absorb at all. The fungus excretes the enzymes into the substratum where the glucanases bind to the insoluble native cellulose to produce cellobiose which is hydrolysed in the soluble phase by cellobiase. The producing organism must then absorb the glucose end-product. Some lipases act preferentially at a water/lipid interface. In contrast, other extracellular enzymes seem to remain associated with the envelope of the producing cell.

Many cell-wall bound enzymes have been reported in fungi, including glucosidase of *Trichoderma reesei*, trehalase, invertase and proteinases of *Neurospora*, and cellulases of *Volvariella volvacea*. Enzymes have also been found inside layers of extracellular polysaccharide-rich sheaths outside the cell wall.

This sort of natural immobilisation of enzymes offers advantages to fungi. Wall-located enzymes would only degrade substrate in the immediate vicinity of the cell, so ensuring that the organism producing the enzyme has some advantage in the competition with surrounding organisms for the nutrients produced by the enzyme activity. In this way the fungus can exert a degree of control over its immediate environment. For basidiomycetes there is evidence that wall-bound proteinases may be released as their substrate becomes depleted so that they can scavenge further afield in the substratum. Other extracellular enzymes produced by their parent organisms may be among the proteins they scavenge: proteolysis is one of the processes causing decline in extracellular laccase when fruit bodies form in cultures of *Agaricus bisporus* (Wood, 1980b).

3.3.3 Regulation

Production of most enzymes is regulated according to the need for the enzyme activity. The cellulase enzyme complex, for example, is inhibited and probably repressed by the end-products of its activity, i.e. cellobiose and, especially, glucose. On the other hand, enzyme synthesis can be induced, and known inducers of cellulases include cellulose, cellobiose, sophorose and lactose. Induction of cellulases *in vivo* appears to be due to soluble products generated from cellulose by cellulolytic enzymes synthesised constitutively at a low level. These products are thought to be converted into true inducers by transglycosylation reactions.

In *Aspergillus* species, proteinase production is controlled by derepression; the *Neurospora crassa* proteinase is controlled by induction and repression. In neither case is proteinase produced in the presence of ammonia; which appears, therefore, to be the primary source of nitrogen. In sharp contrast, production of extracellular proteinases in the basidiomycetes is regulated mainly by induction; as long as substrate protein is available the proteinases are produced, even in the presence of adequate alternative supplies of ammonia, glucose and sulfate (Kalisz *et al*., 1986). So in this case the protein might be presumed to be the ‘first choice’ substrate.

3.4 The menu of basic nutrients

With the prevalence of glucose polymers in the natural world it is inevitable that this monosaccharide proves to

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

be the most widely utilised carbon source. Other monosaccharides will serve too, since most can be readily incorporated into metabolism after minor isomeric transformation. Among nitrogen sources, nitrate will be rarely encountered, ammonium could be met with in quantity either as in its clay-bound form or as an 'excretion' product of microorganisms using protein as a carbon source. However, in most locations it seems likely that fungi will find amino sugars and, especially, amino acids to be quantitatively the most available source of nitrogen and to represent good return on any energy invested in uptake as they would serve as carbon sources also. This sort of consideration is likely to explain the fact that the biomass yields of certain basidiomycete fungi were 4-7 times greater from an ammonium-medium containing both glucose and protein as carbon sources than from media containing either one of these nutrients as sole carbon source (Kalisz *et al*., 1986).

3.5 The wall and membrane as barriers

3.5.1 Barriers to the extracellular environment

The plasma membrane is the boundary between what constitutes the cell and what constitutes the environment. In models of the origin of life, formation of such a membrane represents a crucial step. Only if the cell is completely separated from its environment can the fundamental chemistry of living things be made to work. The essential function of the plasma membrane is protection. Its purpose is to prevent leakage of cellular material to the environment and to prevent intrusion of environmental molecules into the cell.

The wall, too, serves a protective function, but a primarily mechanical one. However, it is essential to appreciate that wall and plasma membrane together form a physicochemical mechanism to regulate entry into the cell of that all-pervasive environmental molecule, water. Unlike other parts of the cell, for example, enzymes, mitochondria, even mitotic division spindles, isolated fungal walls have chemical and physical properties but exhibit no function similar to their natural one. Also, the other part of the mechanism, fungal protoplasts, which (in theory, at least) are intact fungal cells from which the wall has been removed (Peberdy and Ferenczy, 1985), are unable to regulate water flow and must be stabilised by suspension in abnormally high concentrations of osmotically active solutes. Evidently, the native function is lost when the two components of the mechanism are separated. The water relationships of fungal cells will be discussed towards the end of this section; prior to that, attention will be focused on the relationship of aqueous solutes with the wall and membrane.

3.5.2 Transfer across the plasma membrane

The plasma membrane is a lipoidal layer which effectively separates the aqueous 'bubble' of the cell from its aqueous surroundings. Obviously, this separation is not complete or absolute. The cell must exchange chemicals with the environment, extruding excretion products and absorbing nutrients. However, only molecules which dissolve readily in lipid are able to penetrate the membrane without assistance. Since the vast majority of molecules the cell needs to transfer across the membrane are hydrophilic rather than lipophilic, plasma membranes have evolved a range of associated transfer systems (see below) which permit selective communication between the two sides of the membrane. This selectivity permits the cell to exercise considerable control over its interaction with the environment.

3.5.3 Barriers within the cell: compartments

Prokaryotic cells are simply single compartments lacking internal membranous subdivision and bounded by the plasma membrane. In eukaryotic cells, on the other hand, membranous structures abound. What has been said above about the plasma membrane acting as a barrier to the free movement of molecules between the aqueous environment of the cell and the aqueous external environment applies equally to intracellular membranes. These, too, separate a volume of an aqueous solution (in this case 'cytoplasm') from its surroundings (also 'cytoplasm') so that the compartment they delimit can, again through the selective transfer of molecules, undertake chemical reactions which are not possible elsewhere in the cell. Even the membrane itself may be a compartment, in the sense that it can serve as a structural support for enzyme trains whose successive reactions have to be coordinated by being spatially fixed relative to one another.

Some of these intracellular compartments (mitochondria, vacuoles, nuclei) are visible with the light microscope, others require electron microscopy (endoplasmic reticulum, Golgi-like cisternae, cytoplasmic vesicles) and still others can only be inferred from indirect experimentation. They all permit selective localisation of enzymes, substrates and/or products; providing the means for attaining reaction conditions (high metabolite concentration, unusual pH or ionic concentrations, for example) which cannot be maintained, or perhaps cannot be permitted, through the whole volume of the cell. Such compartmentalisation also has implications for control of metabolism as, for example, two processes with a common substrate requirement may be regulated independently by having their enzyme systems in different membrane-bound compartments so that transfer of the substrate into one or other of the compartments determines which process is carried out (Jennings, 1995).

Much of the metabolic integration which is responsible for the rich variety of eukaryotic biochemistry is dependent on intracellular compartmentalisation. Unfortunately, because it does occur within the structure of

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

the living cell it is extremely difficult to investigate and very little is known about it.

3.6 The flow of solutes

3.6.1 Solute behaviour in solutions

In an unbounded solution molecules of solute can move within the solution in two ways. Whole volumes of solution may be transported from place to place, taking solute molecules with them. This is bulk flow or mass flow and results from such things as convection flows and other large scale disturbances within the solution. As far as living organisms are concerned, bulk flow may be achieved through cytoplasmic streaming, transpiration streams and similar processes. It is likely to be associated more with distribution of materials (whether in solution or not) over large cellular or intercellular dimensions than with transfer across the lesser dimension of a lipid bilayer membrane.

The second mode of solute movement is diffusion, where random thermal motion at the molecular level causes all solute molecules to move continuously. If the solution is completely homogeneous then any molecules which move out of a particular unit volume will be replaced by an identical number moving into that unit volume and, under all but the most stringent experimental conditions, the exchange of solute molecules will not be detectable. On the other hand, if there is a concentration gradient within the solution there will be a net flow of solute molecules from the high concentration end of the gradient, towards the low concentration end. Note that this gradient can be a chemical gradient of uncharged molecules (e.g. a sugar), an electrical gradient of a charged ion (e.g. K^+) or a combination of the two. This diffusion process is extremely relevant to the behaviour of every cell, since there is likely to be a concentration gradient across the plasma membrane for just about every solute of importance to the cell.

3.6.2 Transport systems

To traverse the biological membranes a solute must leave the aqueous phase for the lipoidal environment of the membrane, traverse that, and then re-enter the aqueous phase on the other side of the membrane. Unaided simple diffusion of molecules across biological membranes depends considerably on their solubility in lipids.

There are exceptions to this generalisation, though, as some small polar molecules (such as water) enter cells more readily than would be expected from their solubility in lipid. They behave as though they are traversing the membrane by simple diffusion through gaps or pores which are transiently generated by random movements of the acyl chains of the membrane phospholipids. Since transfer of these materials (like that of molecules which are soluble in the hydrophobic lipid bilayer of the membrane, such as O_2 and CO_2) depends on diffusion, their rate of movement is proportional to the concentration differential on the two sides of the membrane, and the direction of movement is from the high to the low concentration side. No metabolic energy is expended and no specific membrane structures are involved in this mode of transfer, but net transfer ceases when the transmembrane concentrations are equalised.

In some, though probably rare, cases these concentration gradients across membranes will be oriented so that solute flows in the direction required by the cell and at a rate suitable for its metabolism. However, in most cases the gradient will be adverse, or the concentration differential so small that the rate of diffusion is inadequate. Furthermore, only a minority of compounds pass through biological membranes *in vivo* by simple diffusion, and the vast majority of metabolites that the cell needs to absorb or excrete are too polar to dissolve readily in lipid and too large in molecular size to make use of transient pores. To cope with these circumstances the membrane is equipped with solute transport systems. This argument applies to intracellular membranes bounding compartments within the cell as well as to the plasma membrane (see Garrill, 1995 for discussion of tonoplast and mitochondrial transport).

The essential component of any transport system is a transporter molecule, a protein which spans the membrane and assists transfer of the metabolite across the lipid environment of the membrane. With both passive and active transporters, substrate translocation depends on a conformational change in the transporter such that the substrate binding site is alternately presented to the two faces of the membrane. These transporters are glycoproteins of around 500 amino acids arranged into three major domains: 12 α-helices spanning the membrane, a highly charged cytoplasmic domain between helices 6 and 7, and a smaller external domain, between helices 1 and 2, which bears the carbohydrate moiety. Sequence homology between the N- and C-terminal halves of the protein suggest that the 12 α-helix structure has arisen by the duplication of a gene encoding a 6-helix structure. Ion channels are different as their polypeptide subunits form a β-barrel containing a pore. A loop of the polypeptide is folded into the barrel and amino acids of this loop determine the size and ion selectivity of the channel. This transporter alternates between open and closed conformations (Jennings, 1995).

If the transfer is passive, that is without a requirement for metabolic energy, then the transport process is described as facilitated diffusion. Such a process still depends upon a concentration differential existing between the two sides of the membrane, transfer occurring ‘down the gradient’ (towards the compartment which has the lower concentration). However, transfer is much faster than would be predicted from the solubility of the metabolite in lipid, the high rate of transfer depending on the fact that the transporter and the transporter - metabolite complex are highly mobile in the lipid environment of the membrane. The major differences from simple diffusion is that facilitated diffusion exhibits saturation kinetics and (usually) high substrate specificity.

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

Showing saturation kinetics means that as the concentration of the metabolite being transported is increased, the rate of transport increases asymptotically towards a theoretical maximum value at which all the transporters are complexed with the metabolite being transported (i.e. transporters are saturated).

Facilitated diffusion can transport a specific substrate very rapidly; but can only equalise the concentrations of the transported metabolite on the two sides of the membrane. Yet in many cases the cell needs to transfer a metabolite against its concentration gradient. The prime example will be where the cell is absorbing a nutrient available at only a low concentration; if growth of the cell is not to be limited by the external concentration of the nutrient, the cell must be able to accumulate the nutrient to concentrations greater than those existing outside. In which case an adverse gradient of concentration will have to be established and maintained. Neither simple diffusion nor facilitated diffusion can do this; to achieve it the cell must expend energy to drive the transport mechanism. Such a process is called active transport.

Active transport is a transporter-mediated process in which movement of the transporter-substrate complex across the membrane is energy dependent. The transporter exhibits the same properties as a facilitated transport transporter (saturation kinetics, substrate specificity, sensitivity to metabolic inhibitors). In addition to these properties, active transport processes characteristically transfer substrate across the membrane against a chemical and/or electrochemical gradient, and are subject to inhibition by conditions or chemicals which inhibit metabolic energy generation.

The mechanism is often a co-transport in which the movement of an ion down its electrochemical gradient is coupled to transport of another molecule against its concentration gradient. When the ion and the transported substrate move in the same direction the co-transporter is called a symport, whereas transporters which transport the two in opposite directions are termed antiporters. The electrochemical gradients, most usually of protons or K^+ in fungi, are created by ion pumps in which hydrolysis of ATP phosphorylates a cytoplasmic domain of the ion channel. Consequential conformational rearrangement of the protein then translocates the ion across the membrane and reduces the affinity of the binding site to release the ion at the opposite membrane face. Dephosphorylation restores the pump to its active conformation (and may translocate another ion or molecule in the opposite direction).

3.7 Transport strategy

Complex interactions occur in transport of anions, cations and non-electrolytes; interactions which may depend on metabolic, chemical, biophysical and/or electrochemical relationships between a number of different molecular species and with the rest of metabolism. There are indications of what might be called transport strategy in operation in most cells. Single uptake systems are rarely encountered; dual or multiple systems are the norm, the different components being suited to different environmental conditions the organism may encounter. Multiple uptake systems for a single substance are found widely in plants as well as fungi. They inevitably result in complex uptake kinetics which might be indicative of physically separate transport transporters, each showing Michaelis-Menten kinetics (like the glucose transporters in *Neurospora*), or of single molecules exhibiting kinetics modulated by their environment (like the glucose transporter in *Coprinus;* Fig. 3.5). Whatever the physical basis, the regulatory properties of the components of such 'families' of transport processes appear to be interlinked to ensure that nutrient uptake is maintained at a reliable level whatever the variation in substrate availability in the environment.

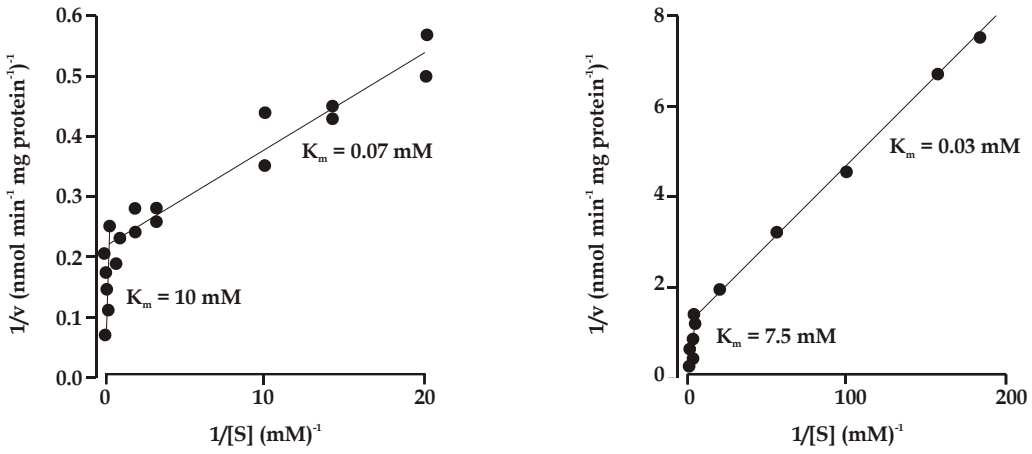


Fig. 3.5. Initial rates of uptake of two glucose analogues as a function of concentration in *Neurospora* (left) and *Coprinus* (right). Note the biphasic nature of the plots, indicating that there are at least two kinetic entities in each case, one with a much higher substrate affinity than the other. Data taken from Neville *et al*. (1971), and Moore and Devadatham (1979).

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

Probably the most important generalisation that can be made about transport processes, though, is that for almost all of them the active extrusion of protons from the fungal cell seems to be of significance. The proton gradient thereby established provides for uptake of sugars, amino acids and other nutrients by proton co-transport down the gradient, and is directly involved in cation transport (like the K^+/H^+ exchange or antiport).

Thus, each fungus possesses multiple uptake systems for most nutrients but the same basic process (active H^+ extrusion) provides the energisation for most if not all. An aqueous suspension of yeasts may be a fairly homogeneous population, expressing relatively uniform transport properties, but a yeast colony on a solid medium, and most definitely a mycelial fungus on any solid substratum will inevitably be expressing different transport processes in different regions of the mycelium. This conclusion stems from consideration of such data as those dealing with the amino acid composition of suspensions of yeast cells. Recall that the composition of the amino acid pool varies with age, with the growth rate, and with the composition of the medium. Many of these observed differences in amino acid pool reflect differences in transport processes. Applying these observations to mycelia of filamentous fungi, different hyphal cells in a filamentous mycelium will be expressing different transport capabilities because of inevitable differences in age, growth rate and composition of the substratum on which the hypha is growing.

What is true for amino acid uptake is probably also true for other nutrients and it is quite evident that uptake systems must be regulated not only by reference to their own substrates but in relation to other substrates and other metabolic conditions as well. The majority of the transport proteins which have been characterised can be grouped into families which appear to be conserved between different organisms. As more genomes are sequenced, computer analysis of many transport proteins has become feasible, allowing deduced protein sequences of unknown function to be characterised by sequence similarity with known membrane transport proteins (Andre, 1995).

A crucial point, which has not yet been taken into account, is that the sorts of transport systems so far considered will inevitably alter the solute concentrations of the cell and thereby influence the movement of that all-pervading nutrient, water.

3.8 Water relationships

Water, of course, is the solvent within which the majority of life processes are played out, but it is also a significant (even if often overlooked) component of innumerable biochemical processes (Ayres and Boddy, 1986). For example, every hydrolytic enzyme reaction uses a molecule of water, every condensation reaction produces a molecule of water and respiration of 1 g of glucose produces 0.6 g of water. The water relationships of the fungal cell are an important aspect of its overall economy.

Water availability is determined by its potential energy - referred to as the water potential, symbolised by the Greek letter psi (Ψ). Zero water potential is the potential energy of a reference volume of free, pure water. The water in and around living fungal cells will have positive or negative potential energy relative to that reference state, depending on the effect(s) of osmotic, turgor, matrix and gravitational forces. Water will flow spontaneously along a water potential gradient, from high to low potentials, though in the normal state for most fungi this will mean from a negative to a more negative potential. The lower the water potential the less available is the water for physiological purposes and the greater is the amount of energy that must be expended to make the water available.

On the face of it, two things need to be considered. One is some sort of compensation for change in the solute relationships of the cell resulting from uptake of some substrate - such a process would further reduce the potential of the cell water and increase the tendency of external water to influx. The other is to provide the cell with a means to regulate its water uptake even though the external water potential is uncontrollable. In fact, of course, these are just two facets of the same problem. In either case the fungus must cope with water potential stress and the evidence indicates that solute transport systems provide the mechanism which permits this. The internal maintenance of turgor pressure by movement of water across the membrane is related to transport of ions across the membrane and to the breakdown of macromolecules and biosynthesis of solutes. Inorganic ions usually make the greatest percentage contribution to the osmotic potential of the protoplasm. The main ions involved are K^+ and Na^+, with Cl^- being moved to balance the cation content. Some organic solutes also make major contributions, including glycerol, mannitol, inositol, sucrose and proline (Eamus and Jennings, 1986).

The most immediate response to water potential stress is change in cell volume by the rapid flow of water into or out of the cell. The consequent change in turgor affects the cell membrane permeability and electrical properties so that the cell can restore the volume by transporting ions or other solutes across the membrane and/or by synthesising solutes or by obtaining them by degrading macromolecules. Response to water potential stress can be extremely rapid. Experimentally this is particularly evident in fungal protoplasts, the size of which alters soon after change in the solute concentration of the suspending medium. Such behaviour attests to the ready permeability of the cell membrane to water even though it is quite obvious that water molecules are unable to penetrate the lipid (hydrophobic) environment of the membrane. The route taken by water molecules has not been established but they are thought to penetrate by migration through pores or channels in proteins which span the membrane. These proteins may have other functions; although no such research has been done with fungi, there is evidence that in the rabbit corneal endothelium water molecules flow

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

preferentially through a glucose transporter protein. The managed flow of water, coordinated with control of the wall synthetic apparatus, must be a prime factor in controlling the inflation of fungal cells which is responsible for so many of the changes in cell shape which characterise fungal cell differentiation. Furthermore, since most of the increase in size during maturation of fruit bodies and related structures is itself an expansion due to inflation of the constituent cells (rather than an increase in cell number), management of water movement is an equally important aspect of gross morphogenesis.

Turgor also contributes to flow along the fungal hypha. As this is a filamentous structure, flow of water and solutes within the hypha (i.e. translocation) is of enormous importance. Although our current view of apical growth requires that fungi can organise rapid translocation and specific delivery of microvesicles containing enzymes and substrates for wall synthesis, the evidence indicates that more general water flow along the hypha is driven by a turgor gradient and that solutes are translocated by this turgor-driven bulk flow. Translocation of nutrients of all sorts in this manner is of crucial importance to morphogenesis because it must be the main way in which developing multicellular structures, such as a fruit body developing on a vegetative colony, are supplied with nutrients and water. Translocation is ably discussed by Jennings (1995; see his Chapter 14) and the mechanism can best be illustrated by quoting his description of the way in which *Serpula lacrymans* (the major timber decay organism in buildings in northern Europe) translocates carbohydrate:

> "Mycelium attacks the cellulose in the wood, producing glucose, which is taken into the hyphae by active transport. Inside the hypha, glucose is converted to trehalose, which is the major carbohydrate translocated. The accumulation of trehalose leads to the hypha having a water potential lower than outside. There is a flux of water into the hyphae and the hydrostatic pressure so generated drives the solution through the mycelium. The sink for translocated material is the new protoplasm and wall material produced at the extending mycelial front. The mechanism of translocation in *S. lacrymans* is thus the same as that now accepted for translocation in the phloem of higher plants, namely osmotically driven mass flow.(Jennings, 1995; p. 459)."

This description could be paraphrased to apply it to other circumstances by, for example, featuring nutrients other than carbohydrates (but note that the molecule which is translocated may be a different species from those which are initially absorbed or finally used) and/or alternative sinks, such as fruit bodies or particular tissues in fruit bodies. Importantly, this bulk flow does not have to be unidirectional *within a tissue*. Because the tissue is comprised of a community of hyphae, different hyphae in that community may be translocating in different directions. Carbon is translocated simultaneously in both directions along rhizomorphs of *Armillaria mellea* (Granlund *et al*., 1985). In mycorrhizas, carbon sources from the host plant and phosphorus absorbed by the hyphae from the soil must move simultaneously in opposite directions. Indeed, the flow of carbon in mycorrhizas must be fairly complex as carbon can be transferred between two different plants which are connected to the same mycorrhizal system (Watkins *et al*., 1996). Cairney (1992) has proposed that contrary flows in individual hyphae may be achieved if there is a spatial separation between nutrient flow through the cytoplasm and a bulk flow of water in the reverse direction through the hyphal walls. Although much remains to be learned, there is clear evidence that nutrients (including water in that category) can be delivered over long distances through hyphal systems, and that the flows can be managed and targeted to specific destinations.

3.9 Intermediary metabolism

Metabolism is the sum total of the chemical changes which occur in a fungus; changes which are both destructive (catabolism) and constructive (anabolism). The different metabolic processes are interlinked in a very complex manner in a living cell, but for study and description metabolism must be subdivided and categorised. It is important to recognise that these subdivisions are not absolute in terms of cellular biochemistry, but are extremely useful in terms of its interpretation. It is also important to note that much of the biochemistry described in this chapter is common to all free-living organisms. In the sections which follow, the general metabolism is described first and then any particular mycological points are emphasised.

A fungus grows at its maximum rate (μ_{max}) when all the nutrients it requires are freely available. If a nutrient is in short supply, growth rate will be reduced to that which can be supported by the amount of the limiting nutrient which is available. Metabolic pathways which characteristically operate when a fungus is growing at or near its maximum rate are described as primary pathways; secondary pathways become operational (or amplified) when growth rate is limited in some way to a level below the maximum (Bu'Lock, 1967).

The fundamental function of metabolism is the utilization of nutrients to form ATP, reduced nucleotide coenzymes (NADH and NADPH) and the compounds which serve as precursors of cellular components, especially macromolecular constituents. This section will be concerned with aspects of metabolism responsible for formation of substrates which serve biosynthetic pathways and compounds providing energy and reducing power - the so-called primary or intermediary metabolism.

3.10 Carbon metabolism

The major source of energy and reducing power is the catabolism of carbohydrate. Although other carbon-

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

containing compounds can be utilised for these purposes by most cells, the full sequence of enzymic processes are conventionally represented as involving the controlled release of energy by the use of atmospheric oxygen to convert glucose to CO_2 and water. This overall process is described as respiration and its chemically balanced (or stoichiometric) summary equation is:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + \text{energy}$$

Note that this equation does not even begin to describe the biochemical mechanisms which achieve the indicated chemical transformation, but it does emphasise that for the conversion of each mole of glucose, six moles of oxygen must be absorbed from the atmosphere, and six moles of CO_2 and six moles of water appear within the cell - and 2900 kJ of free energy (i.e. energy capable of doing some work) are released. To put this into more readily grasped units, respiration of 1 g of glucose uses 1.07 g (747 cm^3) oxygen and produces 1.47 g (747 cm^3) CO_2 and 0.6 g water, releasing 16.1 kJ of energy.

This basic chemistry can be demonstrated by simply combusting glucose under suitably controlled conditions. Obviously, the living cell cannot do this, but instead uses a sequence of enzymically controlled reactions. These are conveniently divided into three phases or subpathways: glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. The indications are that these pathways are much the same in fungi as they are in other organisms (Blumenthal, 1965, 1968; Lindenmeyer, 1965; Niederpruem, 1965; Cochrane, 1976; Watson, 1976; Fothergill-Gilmore, 1986; Jennings, 1995; Van Laere, 1995).

3.10.1 Glycolysis: conversion of glucose to pyruvate

The word glycolysis describes the conversion of glucose to pyruvate without implying a particular pathway. In fact, there are three enzymic pathways which might be used, though one does tend to predominate.

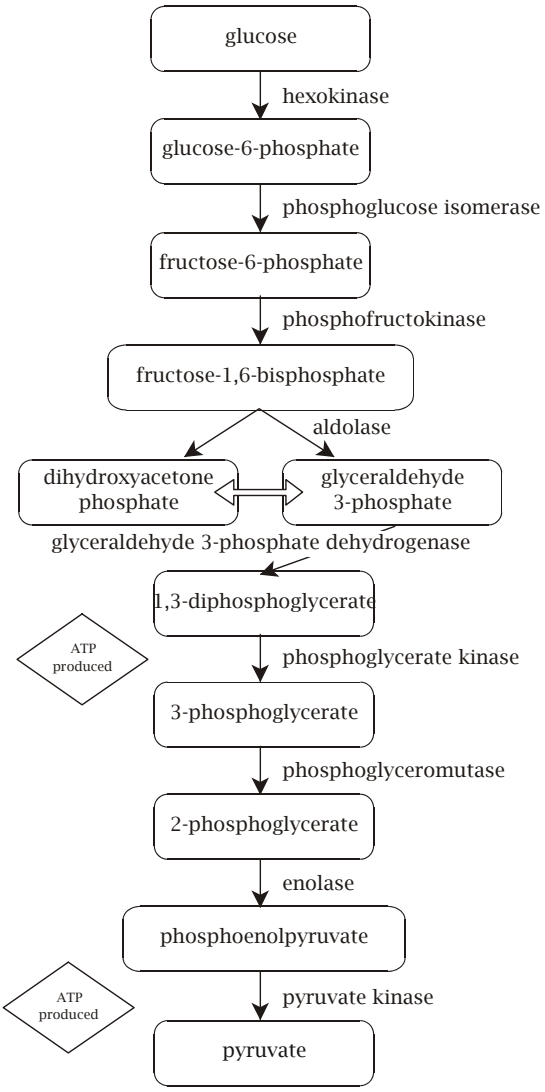


Fig. 3.6. The Embden-Meyerhof-Parnass (EMP) glycolytic pathway.

The *Embden-Meyerhof-Parnass (EMP) pathway* is the major pathway in most species; it comprises nine enzymic steps, all of which occur in the cytoplasm (Fig. 3.6). The net outcome of the reactions summarised in Fig. 3.6 is that one molecule of glucose is converted to two molecules of pyruvic acid plus two molecules of ATP and two molecules of $NADH_2$. Thus, the energy yield ($2ATP + 2NADH_2$, since the latter do represent potential chemical work), is rather small; the main function of the EMP pathway being conversion of glucose to pyruvate for processing in the TCA cycle.

A commonly encountered alternative glycolytic pathway is the *pentose phosphate pathway (PPP)*, also called the hexose monophosphate pathway (HMP) (Fig. 3.7). In strictly chemical terms such a description is true; glucose 6-phosphate is diverted out, undergoes a range of chemical conversions, and then fructose 6-phosphate and glyceraldehyde 3-phosphate feed back into the EMP pathway. The EMP provides the cell with its major intermediate for energy generation (pyruvate), the PPP provides pentose sugars for nucleotide synthesis (which includes coenzymes and energy carriers as well as RNA and DNA), erythrose phosphate for the synthesis of aromatic amino acids through the shikimic acid pathway, and $NADPH_2$ - the coenzyme which is most often used in biosynthetic reactions that require reducing power, especially fat and oil synthesis. So although the PPP can theoretically achieve complete glycolysis (six cycles through the reaction sequence would completely oxidise a molecule of glucose to CO_2), it is more likely to be involved in furnishing biosynthetic intermediates. The PPP does, of course, also provide a route for utilization of pentose sugars which become available as carbon sources, and for interconverting hexose and pentose phosphates.

The *Entner-Doudoroff (ED)* pathway proceeds via 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate, which gives rise to pyruvate and glyceraldehyde 3-phosphate (Fig. 3.8). It is a common

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

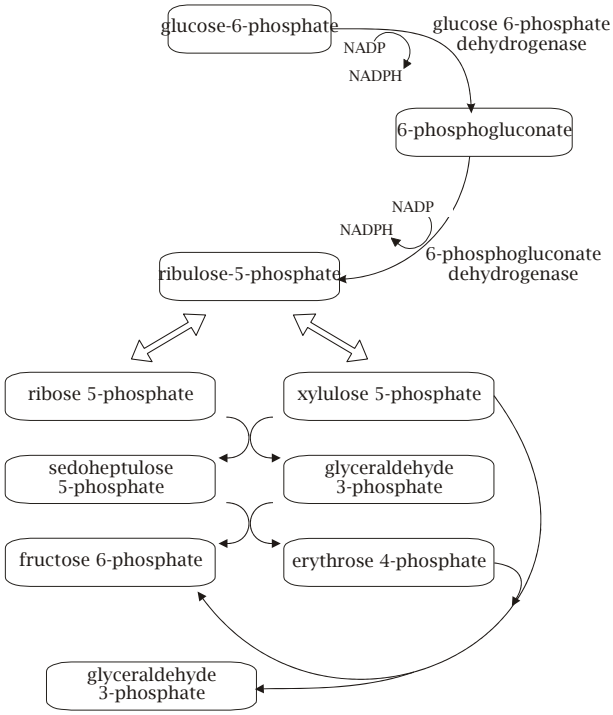


Fig. 3.7. The pentose phosphate pathway (PPP), also called the hexose monophosphate pathway (HMP).

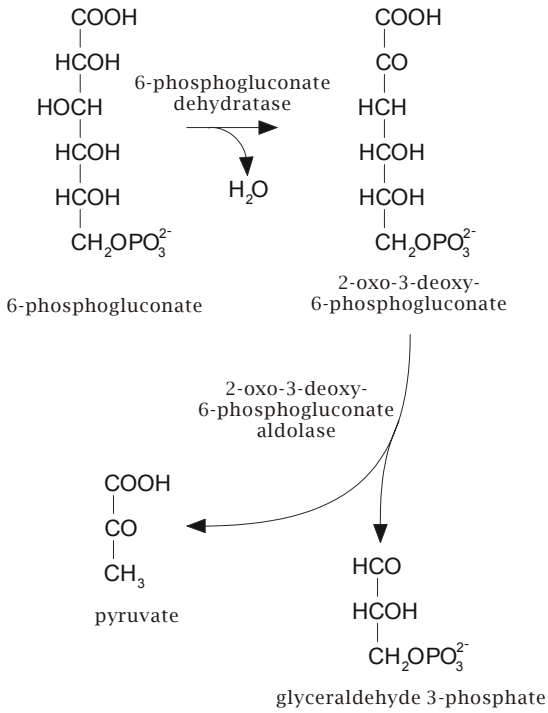


Fig. 3.8. The Entner-Doudoroff (ED) pathway.

glycolytic pathway in bacteria, but has been demonstrated in only a few fungi though where it does occur it seems to be the major glycolytic route.

The use of different glycolytic pathways in any cell will reflect the relative contribution their intermediates are required to make to the functions of the cell; they will change with age, activity and nutrition. In general, since the PPP provides intermediates for biosynthesis, use of this pathway increases in rapidly growing and in differentiating cells, and is minimised in those which are resting or quiescent. Stimulation of PPP activity can often be demonstrated by imposing nutritional conditions requiring expenditure of $NADPH_2$, as by the provision of ammonium or nitrate as sole nitrogen sources.

3.10.2 The tricarboxylic acid (TCA) cycle: oxidation of pyruvate

Whichever glycolytic pathway produces pyruvate, this latter molecule is formed in the cytoplasm and must then be transported into the mitochondrion where it is converted to acetyl coenzyme A (acetyl-CoA). This step is achieved by the pyruvate dehydrogenase complex - a combination of enzymes which first decarboxylate pyruvate and then transfer the resulting acetyl group to coenzyme A.

The TCA cycle is cyclic because its 'end-product', oxaloacetate, reacts with acetyl-CoA to introduce what remain of the pyruvate carbon atoms into a reaction sequence (Fig. 3.9) the primary function of which is to convert pyruvate formed in glycolysis entirely to CO_2, the released energy being captured primarily in $NADH_2$. The overall stoichiometry is that one molecule of pyruvate with three molecules of water forms three molecules of CO_2 and releases 10 protons, the latter appearing in the form of three molecules of $NADH_2$, and one each of $FADH_2$ and the 'high energy' compound GTP. The succinate dehydrogenase enzyme is bound to the inner mitochondrial membrane (and, because of this, is often used as a marker for the presence of mitochondria in fractionated cell extracts), the other enzymes of the TCA cycle occur in the mitochondrial matrix.

A common variant of the TCA cycle is the *glutamate decarboxylation loop* in which 2-oxoglutarate is aminated to glutamate rather than being oxidatively decarboxylated to succinate. The glutamate is decarboxylated to 4-aminobutyrate; transamination between the latter and 2-oxoglutarate yielding succinate semialdehyde which, on oxidation, feeds back into the TCA cycle as succinate (Fig. 3.9). The enzymes of this loop have been found to be at high activity in fruit bodies, especially caps, of the basidiomycete *Coprinus cinereus* (Moore and Ewaze, 1976; Moore, 1984a). The glutamate decarboxylation loop is the normal route of TCA metabolism in this organism and also operates in *Agaricus bisporus* (see section 4.2.2; for review see Kumar and Punekar, 1997).

3.10.3 Oxidative phosphorylation

Through glycolysis and the TCA cycle, all of the carbon contained in the substrate glucose is released as CO_2. However, very little energy is released, most of it being trapped in $NADH_2$ (with a small amount in GTP and

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

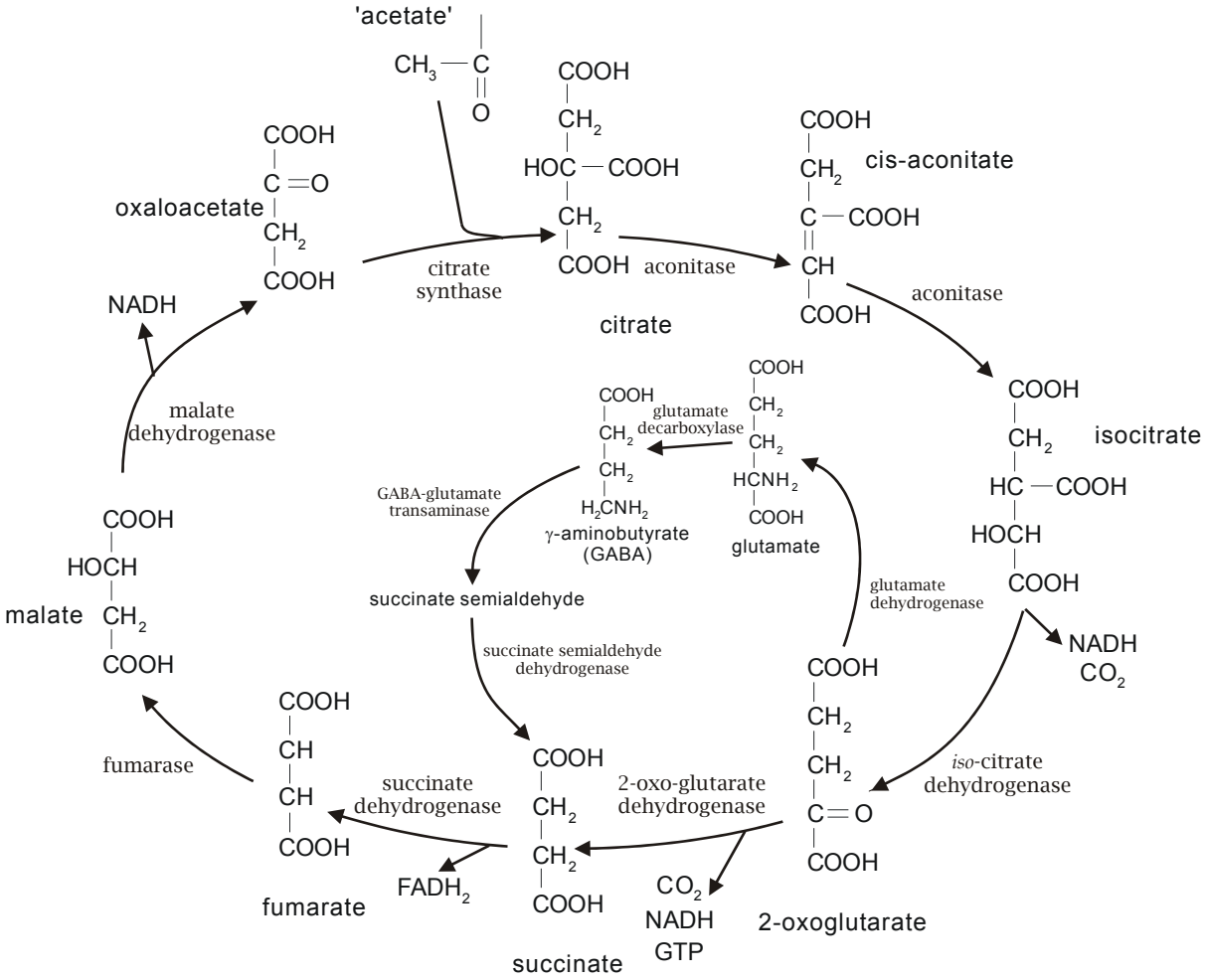


Fig. 3.9. Oxidation of pyruvate: the tricarboxylic acid (TCA) cycle. The glutamate decarboxylation loop is shown in the centre of the main cycle.

$FADH_2$). The energy represented in the form of the reduced coenzymes is recovered as ATP through the electron transport chain, located on the inner mitochondrial membrane, in the process known as *oxidative phosphorylation*.

The electron transport chain transfers electrons from the reduced coenzymes through a series of reactions until the electrons are finally passed to oxygen, reducing it to water. Stepwise transfer of electrons between components of the electron transport chain leads to the pumping of protons from the mitochondrial matrix into the intermembrane space (Bowman and Bowman, 1996). The resulting proton gradient (the pH in the intermembrane space is about 1.4 units lower than that of the matrix) is used to generate ATP.

Transfer of a pair of electrons from one molecule of $NADH_2$ to oxygen leads to proton pumping at three sites in the chain, at each of which the consequent proton gradient can be used to synthesise one molecule of ATP. The ATP is synthesised by an enzyme complex located on the matrix side of the inner mitochondrial membrane. As protons move down a channel in this complex (the channel, known as the F_0 sector, is composed of at least four hydrophobic subunits forming the proton channel located in the membrane) the associated F_1 sector (containing five different subunits) projects into the matrix and is responsible for ATP synthesis (Bowman and Bowman, 1996).

3.10.4 Gluconeogenesis and the synthesis of carbohydrates

Although I am primarily concerned here with catabolism, the pathways described permit, with a few modifications, sugar synthesis. It has been stressed above that glycolysis and the TCA cycle provide opportunities for the fungus to make use of a very wide range of potential carbon and energy sources; but one which is successfully growing on acetate, for example, is clearly required to synthesise all of those compounds which have more than two carbon atoms chained together. In such circumstances glycolysis cannot simply be reversed because the steps governed by kinases (hexokinase, phosphofructokinase and pyruvate kinase) are irreversible, so for these steps in particular additional enzymes are required for gluconeogenesis (Fig. 3.10). The first steps in conversion of pyruvate to carbohydrate are carried out by pyruvate carboxylase, which synthesises oxaloacetate which is then decarboxylated and phosphorylated to phosphoenolpyruvate by phosphoenolpyruvate

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

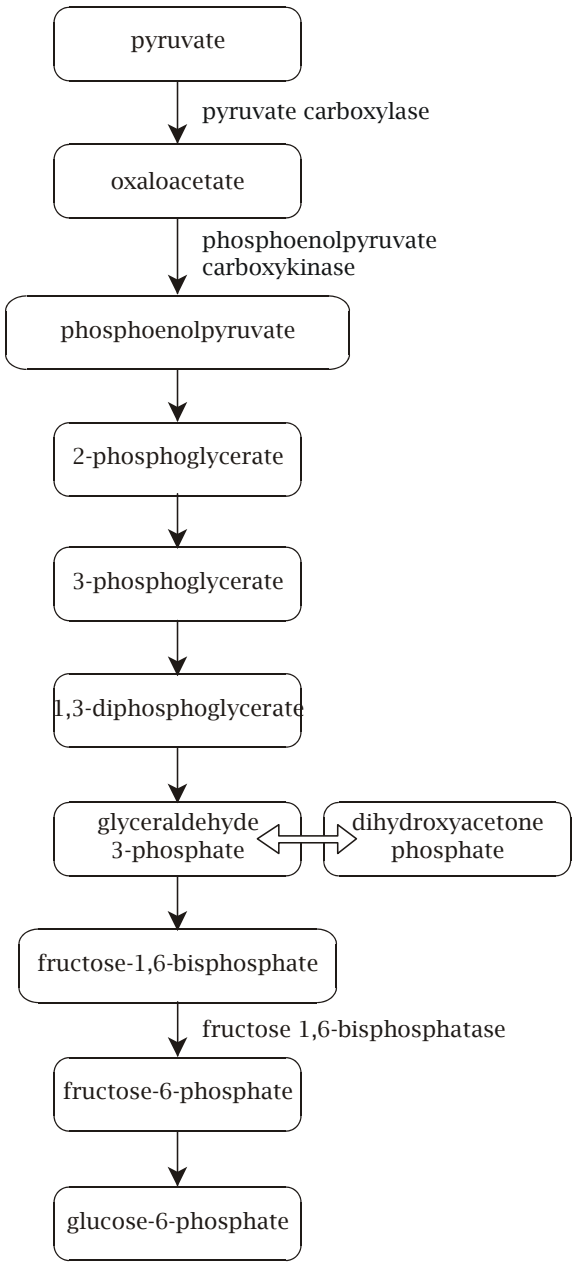


Fig. 3.10. Gluconeogenesis: the making of carbohydrates.

carboxykinase. The phosphoenolpyruvate can then be converted to fructose 1,6-bisphosphate by reversal of the EMP pathway, but an additional enzyme, fructose bisphosphatase, is required to generate fructose 6-phosphate. As the sugar phosphates are readily interconvertible, once this compound is formed oligosaccharide and polysaccharide synthesis can proceed. The structures of many of the polysaccharides formed have been shown earlier in this chapter.

Glycolysis and gluconeogenesis are obviously alternatives which demand close control to assure metabolic balance. I have already noted the sorts of controls which are exercised over phosphofructokinase the key glycolytic control point, it is interesting to note that fructose bisphosphatase responds inversely to the same molecules, being allosterically activated by citrate but inhibited by AMP.

Before leaving carbohydrate metabolism, it is worth mentioning here that the sugar alcohol mannitol and the disaccharide trehalose are almost always found among the water-soluble cytoplasmic carbohydrates in fungi; trehalose is the most widely-distributed sugar in fungi. Mannitol and trehalose seem to serve as transient storage compounds (i.e. molecules capable of immediate mobilisation when required). For example, both have been identified as substrates used for the metabolism associated with spore germination (discussion in Blumenthal, 1976). However, mannitol can certainly serve an osmoregulatory function in the marine fungus *Dendryphiella* (Jennings and Austin, 1973) and may serve the same purpose in fruit bodies of the cultivated mushroom, *Agaricus bisporus*, in which it can be accumulated to concentrations of up to 50% of the dry weight (Hammond and Nichols, 1976); similar concentrations have been encountered in fruit bodies of *Lentinula edodes* (Tan and Moore, 1994; see section 4.2.5). In *A. bisporus*, mannitol is synthesised by reduction of fructose by an NADP-linked mannitol dehydrogenase.

Trehalose synthesis/accumulation/degradation cycles occur at a number of stages in development of a fungus so it is quite clear that this sugar is the 'common currency' of the fungal carbohydrate economy. It is synthesised, as trehalose 6-phosphate, by the enzyme trehalose phosphate synthase from glucose 6-phosphate and the sugar nucleotide UDP-glucose.

3.11 Fat catabolism

Fats are molecules of glycerol in which the three hydroxyl groups are replaced with three fatty acid molecules. In degradation the first step is carried out by lipase which removes the fatty acids from the glycerol (see Fig. 3.8). The latter can be converted to glyceraldehyde 3-phosphate and thereby enter glycolysis, but it represents only about 10% by weight of a fat molecule, the bulk being represented by the fatty acids which consist of long carbon chains (e.g. palmitic acid, C_{16}; stearic acid, C_{18}).

These chains are degraded by sequential removal of the terminal two carbon atoms in the form of an acetyl group attached to CoA (Fig. 3.11). Because the cleavage occurs at the second (β) carbon atom, this process is called β-oxidation and it takes place in the mitochondrial matrix. Each such cleavage is oxidative, enzymes passing the H atoms to the coenzymes NAD and FAD. Thus, degradation of palmitic acid requires seven cleavages and yields eight molecules of acetyl-CoA (which enter the TCA cycle), seven $NADH_2$ and seven $FADH_2$ (both of which enter the electron transport chain for oxidative phosphorylation). Oxidation of fatty acids releases considerable amounts of energy; for example, one molecule of palmitic acid will give rise to about 100 molecules of ATP. This is why fats are such effective energy storage compounds.

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

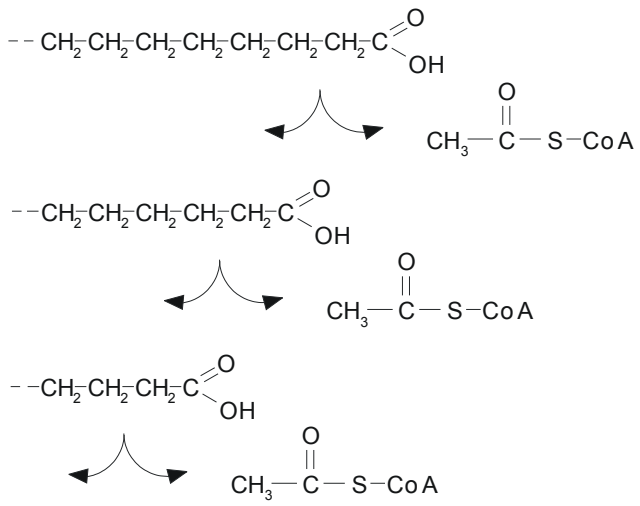


Fig. 3.11. β-oxidation of fatty acids.

3.12 Nitrogen metabolism

Ultimately, all nitrogen in living organisms is derived from the native element in the atmosphere. Each year an amount between 100 and 200 million tonnes of atmospheric nitrogen is reduced to ammonium by the nitrogenase enzyme system of *nitrogen-fixing* bacteria and blue-green algae.

3.12.1 Formation of amino groups

If amino groups are not available directly by absorption of amino acids, they have to be formed and the most immediate source is by the assimilation of ammonium. The only route of ammonium assimilation which can be considered pretty well universal in fungi is the synthesis of glutamate from ammonium and 2-oxoglutarate by the enzyme glutamate dehydrogenase. Many filamentous fungi and yeasts have been shown to produce two glutamate dehydrogenase enzymes; one linked to the coenzyme NAD and the other linked to NADP.

As can be appreciated from Figure 3.12, the interconversion of 2-oxoglutarate and glutamate is a reaction which occupies a central position in metabolism and is one at which important pathways in both carbon metabolism and nitrogen metabolism come together. The reaction is readily reversible and it is often considered that NAD-linked glutamate dehydrogenase (NAD-GDH) has a deaminating or catabolic role (glutamate → 2-oxoglutarate + ammonium), while the NADP-linked enzyme provides the aminating or anabolic function (2-oxoglutarate + ammonium → glutamate). Stewart and Moore (1974) showed that the NAD-GDH predominated in mycelia of *Coprinus cinereus* whether they were grown with glutamate as sole nitrogen source (which would demand deamination) or with ammonium (which requires amination). The key seems to be that this reaction is so important in intermediary metabolism that different organisms have evolved different patterns of *endogenous* regulation, especially in relation to morphogenetic processes. Certainly, in *Coprinus* the NADP-GDH normally appears at high activity only in the cap tissue of the mushroom fruit body where it is located in the basidia apparently protecting meiosis and sporulation from inhibition by ammonium, i.e. acting as an ammonium detoxifier rather than ammonium assimilator (see sections 4.2.3 and 6.5.3).

This example aside, NADP-GDH is generally the most important enzyme involved in ammonium assimilation in mycelia and its activity is often increased when ammonium is provided as a growth-limiting, sole nitrogen source. However, in some fungi an alternative enzyme system appears to scavenge for ammonium when it becomes limiting, this is the glutamine synthetase/glutamate synthase system. Glutamine synthetase is widely, perhaps universally, distributed and is responsible for synthesis of glutamine. However, glutamine synthetase can have a high affinity for ammonium and, in combination with glutamate synthase (which converts glutamine + 2-oxoglutarate to two molecules of glutamate), forms an ammonium assimilation system which can recover ammonium even when this molecule is present at low concentrations. The net result (2-oxoglutarate + NH_4^+ → glutamate) is the same as the reaction promoted by NADP-GDH, but the cost is higher as glutamine synthetase uses ATP to make glutamine. The glutamate synthase mechanism is common in bacteria, but is encountered in fewer fungi, but has been demonstrated in *Neurospora crassa*, *Aspergillus nidulans* and several yeasts and mycorrhizas.

Some yeasts and a larger number of filamentous fungi can utilize nitrate as sole source of nitrogen. Chemically, nitrate is first converted to nitrite which is then converted to ammonium, but the enzymic steps are quite complicated and not yet fully understood. The complexity of the reaction is reflected in the large number of mutant genes, in both *Neurospora* and *Aspergillus*, which have been found to affect nitrate assimilation (Marzluf, 1996). The first stage is performed by nitrate reductase which, generally in fungi, has a cofactor

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

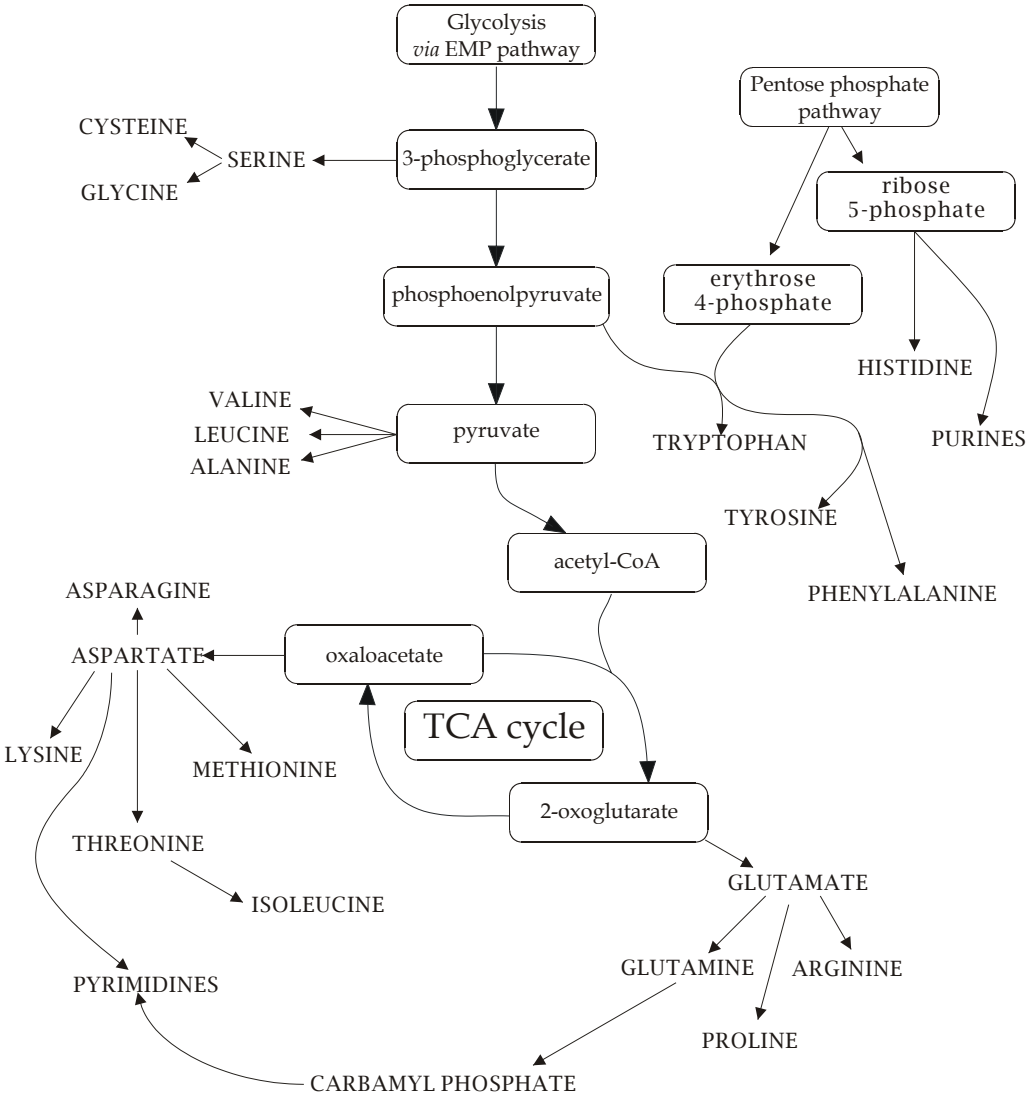


Fig. 3.12. A flow chart illustrating pathways of nitrogen redistribution by showing the metabolic origins of amino acids, purines and pyrimidines.

containing molybdenum and requires NADPH (NADH in at least some yeasts). Nitrate is thought to bind to the molybdo-cofactor of the enzyme prior to being reduced by removal of an oxygen atom. Removal of this allows the nitrate formed to be bound to nitrite reductase, through its nitrogen atom, for the reduction to ammonium. The ammonium formed from nitrate is immediately used for the reductive amination of 2-oxoglutarate to glutamate.

Conversion of NO_3^- to NH_3 is a chemical reduction requiring considerable energy expenditure. In fact the equivalent of four $NADPH_2$ molecules (880 kJ of energy) are used to reduce one NO_3^- ion to NH_3, which is additional to the energy demand for assimilation of the ammonium (one $NADPH_2$ is used for assimilation via NADP-GDH, 1 $NADPH_2$ + 1 ATP for assimilation through glutamine synthetase and glutamate synthase). Given these additional energy demands, it is not surprising that the nitrate reduction machinery is produced only when nitrate is the sole available source of nitrogen, being induced by nitrate and rapidly repressed by the presence in the medium of ammonium or alternative sources of reduced nitrogen.

3.12.2 Disposal of excess nitrogen

The constituents of living cells are in a continual state of flux; all components being subjected to turnover as old materials are catabolized and new ones synthesised. When proteins and other nitrogen-containing compounds are broken down, either as part of this turnover process or as externally supplied nutrients, the carbon can be disposed of as CO_2, hydrogen as water and nitrogen either as ammonium or as urea. The use of protein as a *carbon* source has been discussed above. In these circumstances the organism (animal, plant or fungus) suffers an excess of nitrogen and must excrete it. Experiments with the basidiomycetes *Agaricus bisporus*, *Coprinus*

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

cinereus and *Volvariella volvacea* have shown that one third to one half of the nitrogen contained in the protein given as substrate is excreted as ammonium into the medium (Kalisz *et al*., 1986). Obviously this resulted in very considerable increases in the ammonium concentration in the medium, about 40 mM for each organism in the 25 ml culture volumes used in these *in vitro* experiments. Since ammonium is toxic this type of nutritional strategy must rely on rapid dissipation/dilution of the excreted ammonium in nature. In terrestrial mammals metabolising protein the toxicity of ammonium is avoided by excretion of urea formed through the urea cycle (Fig. 3.13). However, the enzyme urease seems generally to be constitutive in fungal mycelia so any urea formed is likely to be dissimilated to NH_3 and CO_2. Nevertheless there are circumstances in which fungi accumulate urea. Especially large accumulations have been found in basidiomycete fruit bodies (Reinbothe and Tschiersch, 1962; Reinbothe *et al*., 1967), where it seems likely that it acts as an 'osmotic metabolite' controlling water entry into cells during expansion. Urea also accumulates in the cap of the agaric *Coprinus cinereus*, probably for the same purpose, and in this case urease activity has been shown to be lacking in cap cells, though present in other fruit body tissues and constitutive in mycelia (Ewaze *et al*., 1978). Thus, the capacity to synthesise, and even accumulate, urea is well developed in fungi but it seems that it is ammonia which is excreted to dispose of excess nitrogen.

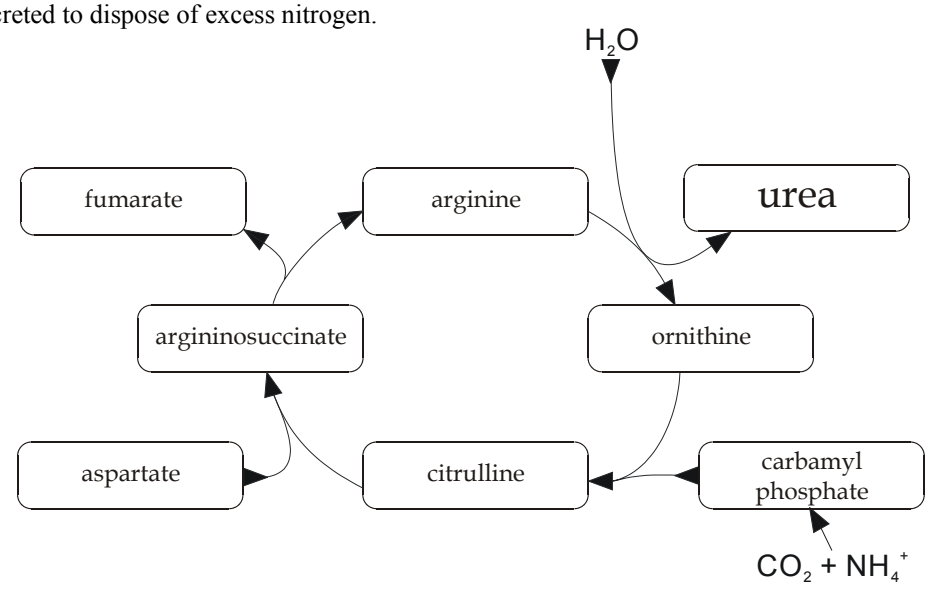


Fig. 3.13. The urea cycle for disposal of excess nitrogen. The ammonium molecule which contributes to carbamyl phosphate and the amino group of aspartate are both 'discarded' into the urea molecule.

3.13 Secondary metabolism

When all nutrients are available to a fungus its rate of nutrient utilization is maximised and the growth rate is exponential. As a nutrient becomes depleted the rate of growth slows and eventually stops. The progress of metabolism is correspondingly altered, a number of special biochemical mechanisms appear (or become amplified) and a range of novel secondary metabolites are produced. The subject is huge, and this section is intended only to give an indication of the chemical versatility of fungi; it is not intended to be a comprehensive treatment.

3.13.1 Definition

The term secondary metabolite seems to have been coined by J. D. Bu'Lock (1961) in preference to continued use of the phrase 'natural products', on the grounds that some of the materials so described were a good deal less natural than others. He contrasted the metabolism leading to secondary metabolites with general metabolism (which we might more tidily call primary metabolism), observing that primary metabolism exhibits basic biochemical patterns which are common to widely differing organisms, and he defined secondary metabolites as being of restricted distribution (exhibiting even species-specific distribution) and having no obvious function in general metabolism.

This final phrase should be read: 'no *obvious* function'. Formation of secondary metabolites is common in plants and microorganisms (conventionally, animals are not widely considered to produce secondary metabolites) and many organisms expend considerable effort and energy in secondary metabolism so some selective advantage must accrue. Possible roles and functions will be discussed in section 3.13.4, but it is important here to appreciate that secondary metabolism *is* functional, though (i) we may be too ignorant of the biochemistry and physiology involved to appreciate the function and/or (ii) most of our interest (and many an example among the fungi) is likely to be concentrated on abnormal cultures grown in abnormal conditions to yield a product of commercial value. There are many potential differences between primary and secondary metabolites and there are examples of both which have commercial value. Consequently, definitions tend to

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

become blurred. The most reliable one, though, remains that secondary metabolites are '…restricted in their distribution, being found in less than every species in a single family.' (Campbell, 1984). Thus, secondary metabolism is a common feature. It consists of a relatively small number of enzymological processes (often of relatively low substrate specificity) which convert a few important intermediates of primary metabolism into a wide range of products (Bu'Lock, 1967). These later stages of secondary metabolism are so varied that individual secondary metabolites tend to have the narrow species-distribution which is their essential character.

3.13.2 Main fungal pathways and products

Primary and secondary metabolism are coextensive - they can occur at the same time in the same cell and draw carbon-containing intermediates from the same sources. In many respects, however, secondary metabolism has a greater biochemical complexity than is evident in primary metabolism.

A few secondary metabolites are derived directly from glucose without cleavage of the glucose carbon chain, such as the kojic acid produced by *Aspergillus* species (Turner, 1971; Turner and Aldridge, 1983), but the majority have their origins in a small number of the intermediates in pathways dealt with above. Acetyl CoA is, perhaps, the most important, being a precursor for terpenes, steroids, fatty acids and polyketides; phosphoenolpyruvate and erythrose 4-phosphate initiate synthesis of aromatic secondary metabolites through the shikimic acid pathway by which aromatic amino acids are synthesised, whilst other secondary metabolites are derived from other (non-aromatic) amino acids (Fig. 3.14).

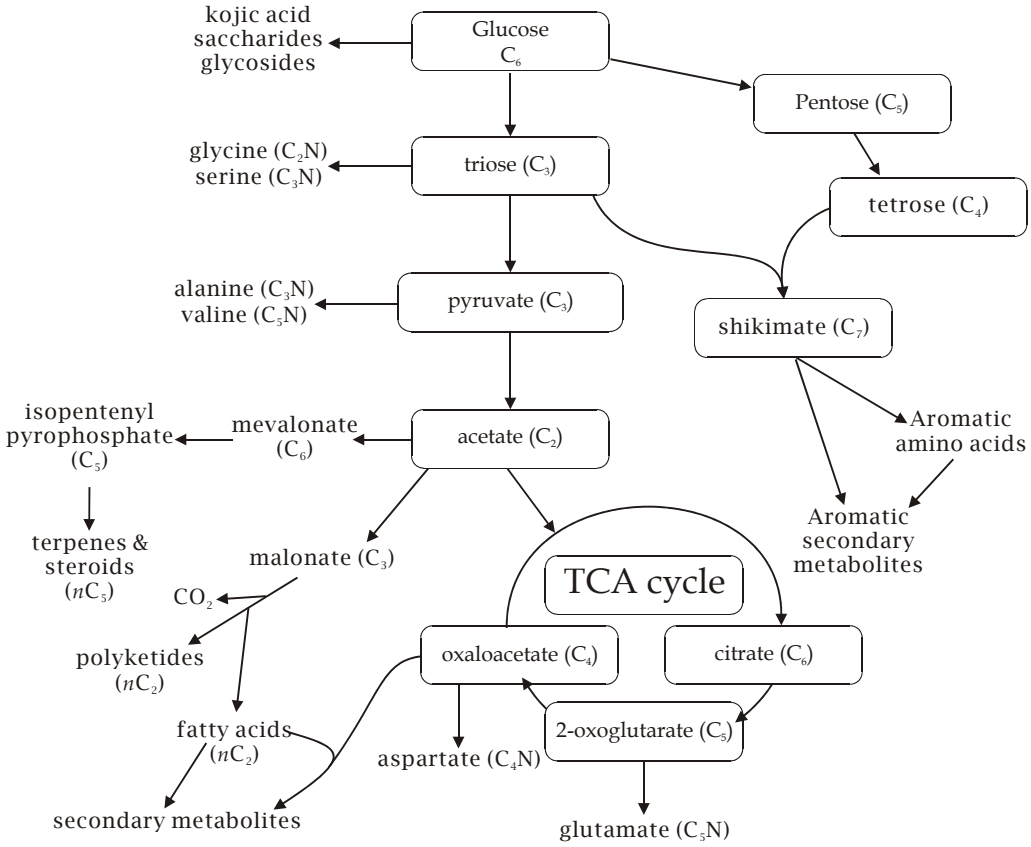


Fig. 3.14. Metabolic route map summarising the relationships between primary metabolism and the major pathways for synthesis of secondary metabolites. Compare with Fig. 3.12.

3.13.2.1 The mevalonic acid pathway: terpenes, carotenoids and sterols

Terpenes, carotenoids and steroids are widely distributed in nature and though the fungal products are not unusual in that sense, many of the end-products have chemical structures which are unique to fungal metabolism. All of these compounds are related by the occurrence within their structures of five carbon atoms arranged as in the hydrocarbon isoprene. The terpenes are the simplest of the naturally occurring isoprenoid compounds and are derived by condensation of a precursor isoprene unit to form an isoprenoid chain (Fig. 3.15).

Strictly, terpene is the name of the hydrocarbon with the formula C_9H_{16}, but the term is used generally to refer to the ten-carbon isoprenoids which may be open-chain (i.e. acyclic) or cyclic compounds. Synthesis of acyclic terpenes starts when dimethylallylpyrophosphate condenses with a molecule of isopentenyl-pyrophosphate to form the 9-carbon monoterpene geranylpyrophosphate (Fig. 3.16); addition of another isopentenylpyrophosphate gives the 15-carbon sesqui- (one-and-a-half) terpene farnesylpyrophosphate; and of another, the 20-carbon diterpene geranylgeranylpyrophosphate, and so on (Turner, 1971; McCorkindale, 1976).

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

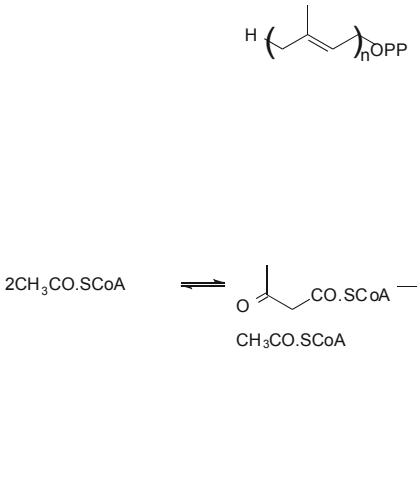


Fig. 3.15. Derivation of terpenes from isoprene units . The basic isoprenoid repeat structure is shown at the top; the lower panel shows the synthesis of mevalonate from two molecules of acetyl-coenzyme A.

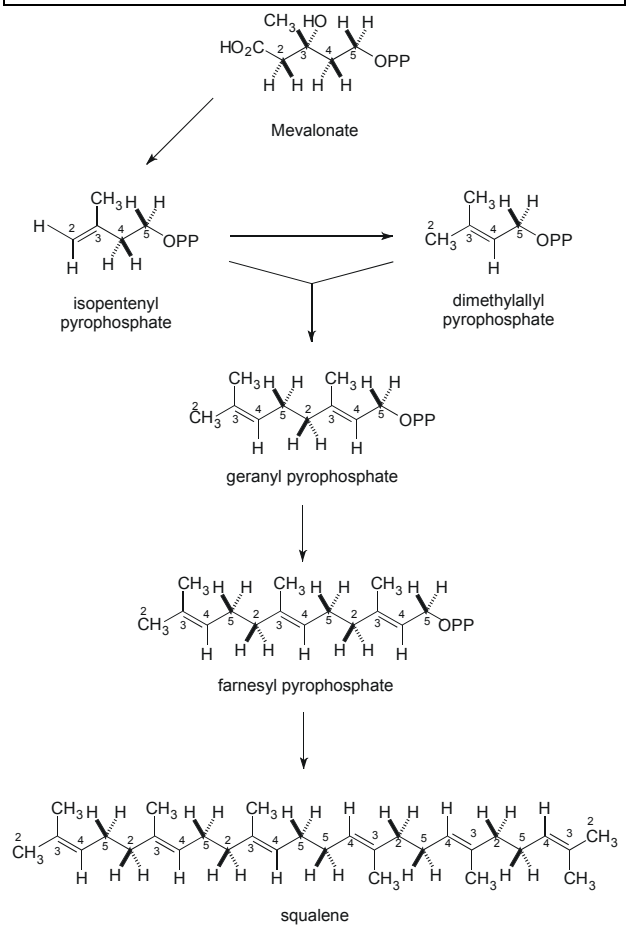


Fig. 3.16. Synthesis of acyclic terpenes by successive condensations. Dimethylallyl-pyrophosphate condenses with isopentenyl-pyrophosphate to form geranylpyrophosphate. Redrawn after Turner (1971).

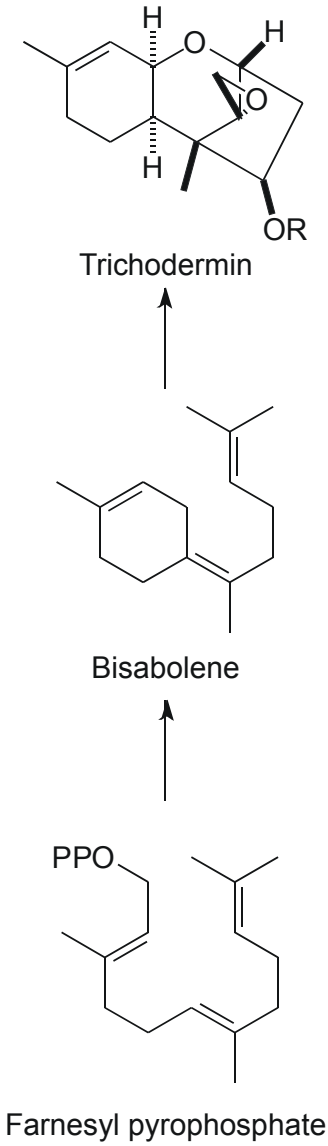


Fig. 3.17. Sesquiterpenes: possible cyclisations of farnesylpyrophosphate to produce the trichothecane nucleus.

As many as 24 isoprene units may condense in this way, though secondary metabolites usually have between two and five. Not all such molecules are secondary metabolites; the ubiquinones of the respiratory chain are polyprenoid quinones (Stone and Hemming, 1967). Sesquiterpenes are the largest group of terpenes isolated

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

from fungi. Most of the fungal sesquiterpenes are based on carbon skeletons which can be derived by cyclisation of farnesylpyrophosphate as suggested in the schemes shown in Fig. 3.17 (most of which are plausible possibilities rather than defined metabolic pathways).

Diterpenes are derived by cyclization of geranylgeranyl pyrophosphate (Fig. 3.18) and include the gibberellins, a mixture of plant growth promoters which were first isolated from culture filtrates of the plant pathogen *Gibberella fujikuroi* (the sexual form of *Fusarium moniliforme*) and only later shown to be endogenous plant hormones.

The major significance of the triterpenes (C_{30}) is that the acyclic triterpene squalene is the precursor of sterols. Squalene is formed by a head-to-tail condensation of two sesquiterpene (farnesylpyrophosphate) units; it is so called because it is found in high concentration in shark liver oil, but in fungi as well as in other organisms sterols (and cyclic triterpenes) are derived by cyclization of squalene oxide (Fig. 3.19). Cholesterol is the quantitatively predominant sterol in animals where it serves to control membrane fluidity, and ergosterol, so named because it was first isolated from the ergot fungus (*Claviceps purpurea*), probably fulfils a similar role in fungi, influencing permeability characteristics of the membrane (Hemmi *et al*., 1995; Parks and Casey, 1995) and as it is unique to fungi, ergosterol synthesis is a potential target for antifungal agents (Barrettbee and Dixon, 1995). However, other sterols, even cholesterol, occur commonly and a very wide range of sterols has been detected in fungi (Goodwin, 1973; Weete and Gandhi, 1996) which have scope for characterizing strains (Muller *et al*., 1994). Lipid, sterol and phospholipid contents differed between yeast and mycelial forms of *Candida albicans* and *Mucor lusitanicus*, so there is a morphogenetic connection, too (Goyal and Khuller, 1994; Funtikova *et al*., 1995). The sequential order of steps in sterol synthesis is well established as mutations in nearly every step of the yeast sterol biosynthetic pathway have been induced and selected (Parks *et al*., 1995).

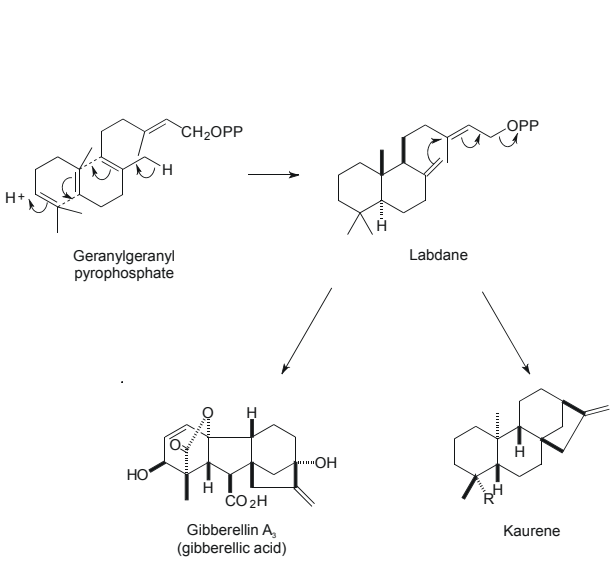


Fig. 3.18. Cyclisation of the diterpene geranyl-geranyl pyrophosphate producing gibbane and kaurane structures. Gibberellic acid is a plant growth hormone.

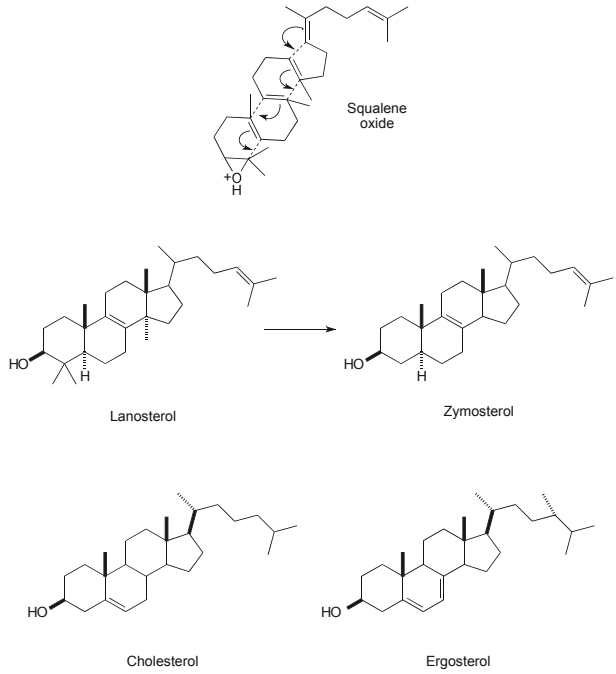


Fig. 3.19. Sterols (and cyclic triterpenes) are derived by cyclisation of squalene oxide.

In much the same manner as squalene is formed by head-to-tail condensation of sesquiterpenes, head-to-tail condensation of two diterpenes generates the C_{40} carotenoid pigments. These include acyclic compounds like lycopene, the pigment responsible for the red colour of tomatoes, and monocyclic and dicyclic compounds like α-, β- and γ-carotene (Fig. 3.20). The carotenes and some keto-derivatives are widely synthesised (Goodwin, 1976; Armstrong and Hearst, 1996) and carotenoids are good taxonomic markers for some fungi (Valadon, 1976), though not all fungi produce them. Mutants of *Mucor circinelloides* with defects in all enzymatic steps from farnesyl pyrophosphate to β-carotene, together with some regulatory mutants, have been described (Navarro *et al*., 1995) so the pathway is well established and open to detailed analysis.

Continued condensation of isoprene units can produce terpenes with very long carbon chains. Natural rubber is a highly polymerised isoprene compound, containing of the order of 5000 isoprene units. The latex of the major commercial source, the rubber palm *Hevea brasiliensis*, is an aqueous colloidal suspension of particles of the hydrocarbon. The milk-like juice which is exuded from broken fruit bodies of *Lactarius*, stems of *Mycena*

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

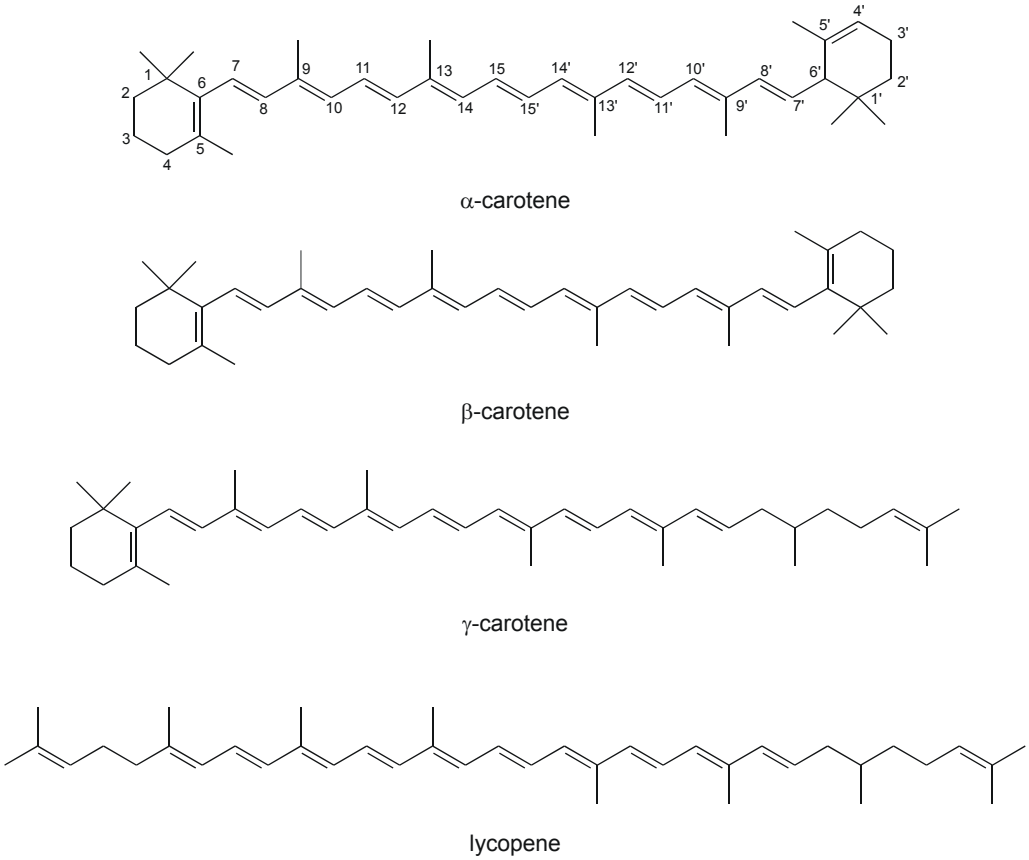


Fig. 3.20. Fungal carotenoid pigments.

and gill edges of *Lacrymaria* is sometimes also called 'latex' but is chemically very different from rubber palm latex, being only superficially similar in appearance. The fungal product probably differs in structure between genera, but the 'latex' or 'milk' of *Lactarius rufus* has been shown to contain mannitol, glucose and lactarinic acid ($CH_3[CH_2]_{11}CO[CH_2]_4COOH$; structural formula shown in Fig. 3.25). The latter is a modified fatty acid (6-oxo-octadecanoic acid, also known as 6-ketostearic acid).

3.13.2.2 The malonate pathway: polyketides

More secondary metabolites are synthesised through the polyketide pathway in fungi than by any other pathway (Turner, 1976). Polyketides are characteristically found as secondary metabolites among ascomycetes, and especially the imperfect deuteromycetes; they are rarely encountered in basidiomycetes and are produced by only a few organisms other than fungi. Polyketides are more correctly described as poly-β-ketomethylenes, the fundamental acyclic chain from which they are derived being comprised of $-CH_2CO-$ units. Just as linear poly-isoprenoid chains can 'fold' and cyclise, so too the polyketides can cyclise to produce a wide range of different molecules (Fig. 3.21). As might be expected from the chemical nature of the repeating unit, synthesis of polyketides involves transfer of acetyl groups which are ultimately derived from acetyl-CoA. In fact the synthesis of polyketides has a lot in common with fatty acid biosynthesis. Fatty acids are catabolized by stepwise removal of 'acetyl-units' (β-oxidation, see Fig. 3.11) but their synthesis requires malonyl-CoA which is formed by carboxylation of acetyl-CoA by the enzyme acetyl-CoA carboxylase: $CH_3COSCoA + HCO_3^- + ATP \rightarrow COOH.CH_2COSCoA + ADP + Pi$.

Synthesis of fatty acids is carried out by a complex of enzymes called the fatty acid synthetase system (Walker and Woodbine, 1976; Kohlwein *et al*., 1996). The reactions occur between substrate molecules which are chemically bound to the enzyme proteins; precursors are not found free in the cytoplasm. In the first reaction 'acetate' from acetyl-CoA is transferred to a peripheral sulfydryl group on the enzyme complex (releasing CoA), then a malonyl group (from malonyl-CoA) is transferred to an adjacent (central) sulfydryl group on the protein and a condensation reaction occurs between the enzyme-bound substrates to form acetoacetate which is still bound to the enzyme at the central sulfydryl group [CH_3COCH_2CO-S-enzyme]. This is then reduced, dehydrated and reduced again to form a butyryl-enzyme complex [$CH_3CH_2CH_2CO$-S-enzyme]. For chain lengthening, the butyryl residue is transferred to the peripheral sulfydryl so that a malonyl group can be brought in to the central one, permitting repetition of the condensation, reduction, dehydration, reduction cycle. Thus, malonyl-CoA provides all the carbon of long chain fatty acids except for the two terminal atoms, which derive from the 'acetate' initially introduced to the peripheral sulfydryl binding site.

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

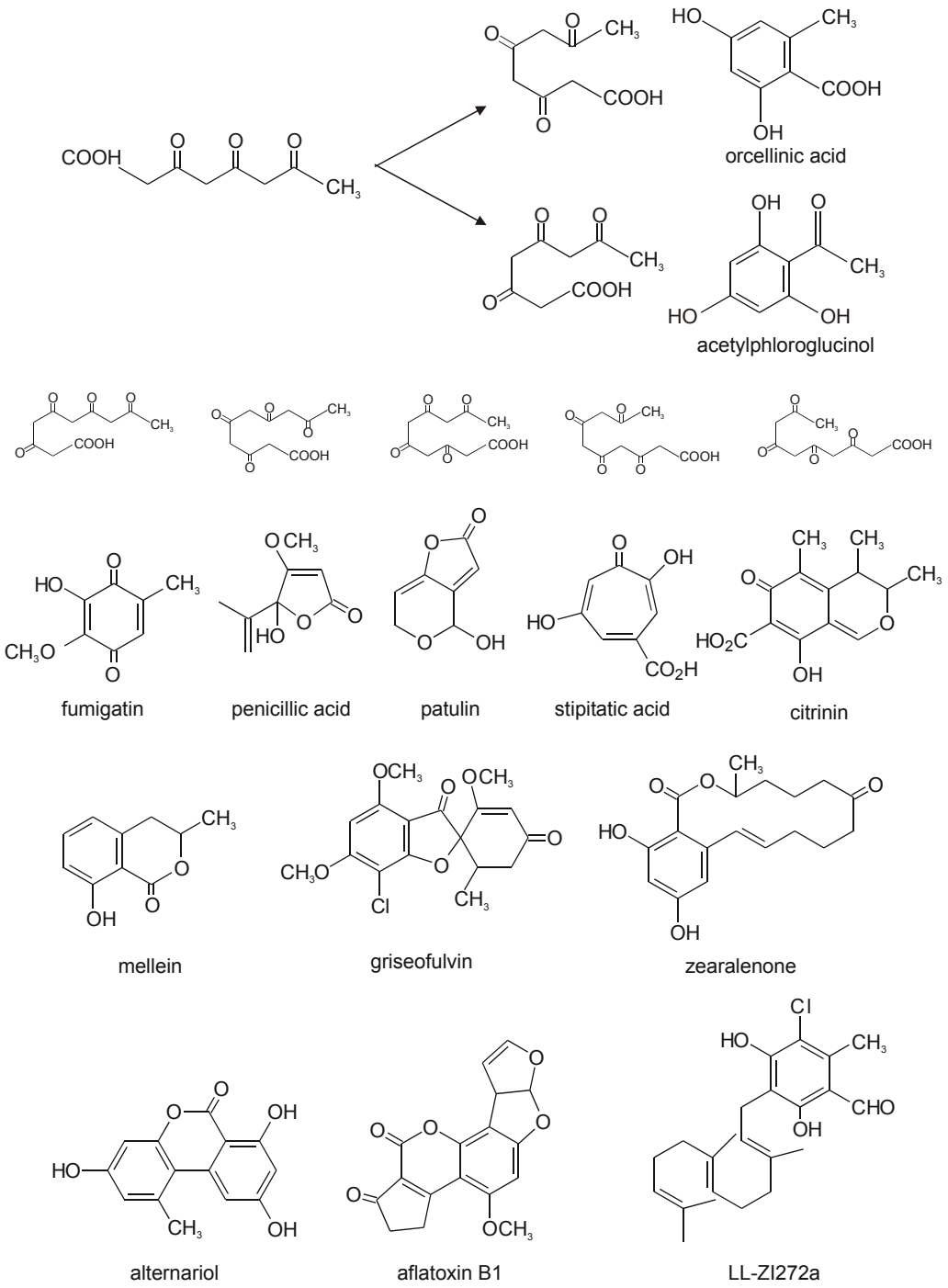


Fig. 3.21. Polyketide chains can fold in a variety of ways and internal aldol condensations form closed aromatic rings. The alternative cyclisations of a tetraketide are shown at the top, together with the potential products orsellinic acid and acetylphloroglucinol. A pentaketide can cyclise in five ways, and these are shown schematically across the centre of the figure. The bottom section shows the structues of a small selection of polyketides discussed in the text in section 3.15.2.2. LL-Z1272α is an antibiotic isolated from *Fusarium* species in which the polyketide-derived aromatic rings have a sesquiterpene substituent.

This brief description of fatty acid synthesis can be echoed by a description of polyketide synthesis which is initiated by condensation of an acetyl unit with malonyl units, requires the respective CoA derivatives, and seems to occur on the enzymes involved. Again, free precursors are not found. Precise details of the mechanism of polyketide synthesis are still uncertain, however, but the similarities observed imply that the chain-building mechanism must be much the same between fatty acids and polyketides. However, for polyketides the reduction process does not occur and successive rounds of condensation generate polymers with the -CH_2CO- ('ketide') repeating unit, the number of which can be used to designate the chain formed as a triketide, pentaketide,

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

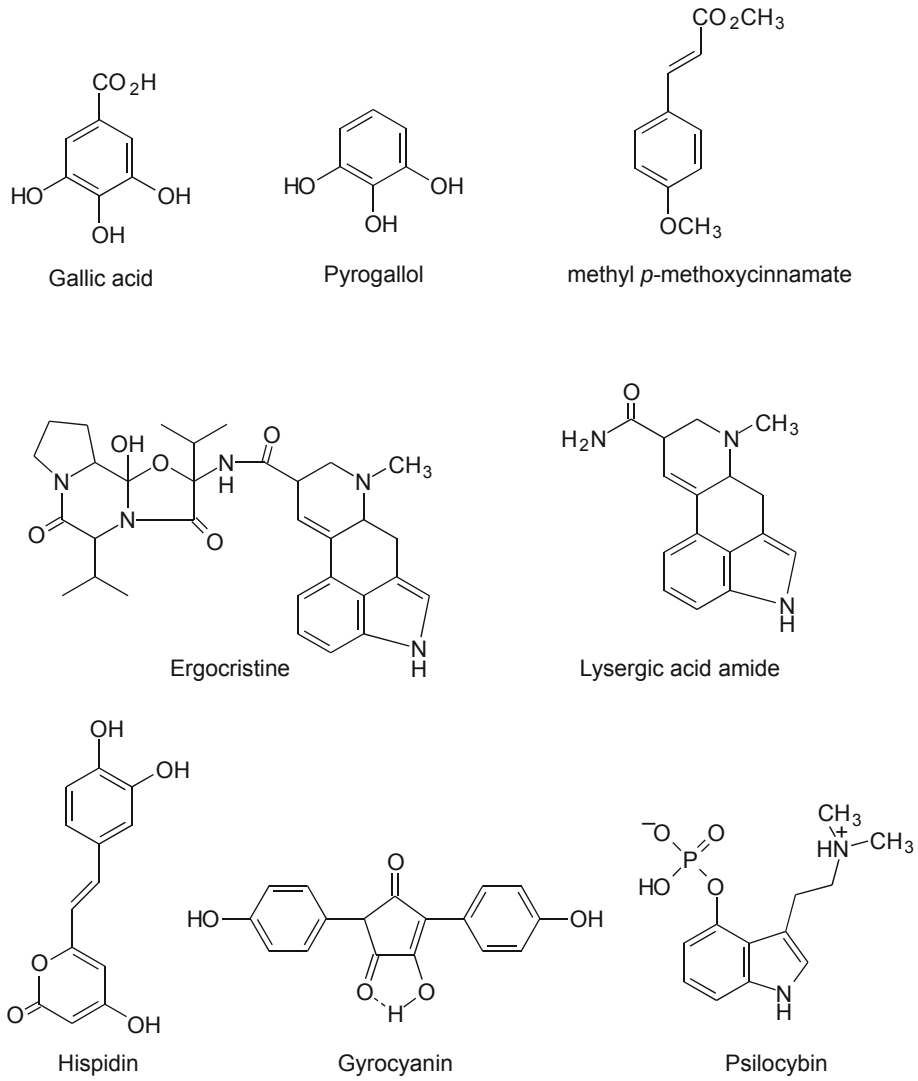


Fig. 3.22. The shikimate-chorismate pathway produces a variety of aromatic compounds as well as the amino acids phenylalanine, tyrosine and tryptophan. Gallic acid, pyrogallol, and methyl *p*-methoxycinnamate are relatively simple compounds produced by many plants as well as fungi. Ergocristine, which is one of the ergot alkaloids (from *Claviceps purpurea*), lysergic acid amide and psilocybin, the halucinogenic principle of the original 'magic' mushroom (*Psilocybe*), are all essentially tryptophan derivatives. Gyrocyanin is a product of *Gyroporus cyanescens* which oxidises to a blue pigment in injured fruit bodies, and hispidin is the precursor of a polymer which seems to be responsible for toughening the fruit bodies of *Polyporus hispidus.*

octaketide, etc. The methylene (-CH_2-) and carbonyl (=C:O) groups of these chains can interact in internal aldol condensations to form closed aromatic rings (Fig. 3.21). A great variety of cyclisations thus become possible and this, in part, accounts for the wide range of polyketides which are encountered. There are limits, however; it seems that compounds in which the uncyclized residue of the CH_3-end of the chain is shorter than that from the -COOH end are not found (Turner, 1976).

The variety of cyclizations only partly accounts for the range of observed polyketides because other chemical modifications, especially dehydration, reduction, oxygenation by hydroxylation, decarboxylation, substitution and oxidation can generate further derivatives (Turner, 1976), and differences in the order in which such reactions occur can result in an enormous variety of actual and potential biosynthetic pathways.

Polyketide secondary metabolites are too numerous to be documented here (see Turner, 1971; Turner and Aldridge, 1983) but they include (Fig. 3.21) the tetraketides orsellinic acid, fumigatin (from *Aspergillus fumigatus*), penicillic acid (from *Penicillium* species, and *not* to be confused with penicillins which are modified dipeptides, patulin and tropolones like stipitatic acid (from *Penicillium stipitatum*). Pentaketides include the antibiotic citrinin (originally isolated from *P. citrinum*, but commonly found in ascomycetous fungi) and mellein, derivatives of which can be useful taxonomic and phylogenetic characters in Xylariaceae (Whalley and

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

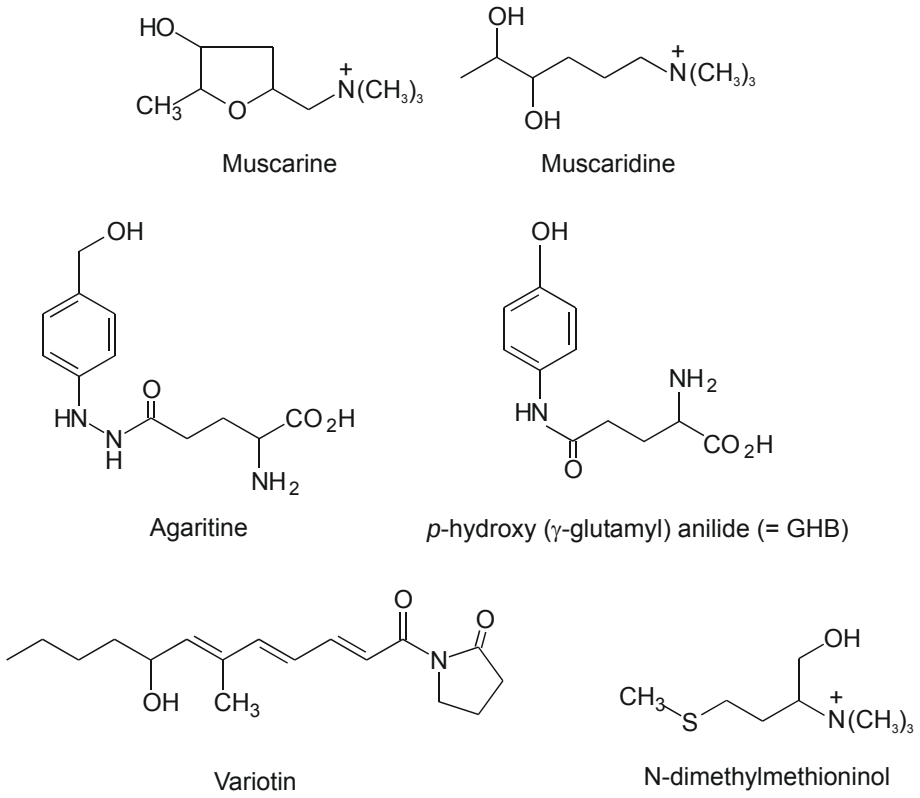


Fig. 3.23. Non-aromatic amino acids may also be modified and accumulated as secondary metabolites. Muscarine and muscaridine, the main toxins of *Amanita muscaria*, are synthesised from glutamate, and agaritine and *p*-hydroxy (γ-glutamyl) anilide (also known as glutaminyl hydroxybenzene, or GHB) from *Agaricus* spp., are *N*-acylated glutamate molecules. Agaritine may account for up to 0.3% of the dry weight in *A. bisporus* fruit bodies and GHB may be involved in controlling basidiospore dormancy and is the most likely precursor for *Agaricus* melanin in the spore walls (Hammond and Wood, 1985). Variotin is an antifungal agent isolated from *Paecilomyces varioti* and is γ-aminobutyric acid *N*-acylated with a hexaketide. *N*-dimethylmethioninol is a volatile amine formed by decarboxylation of methionine by *Penicillium camemberti*. It is responsible for the aroma of Camembert cheese.

Edwards, 1987). Heptaketides include the antifungal agent griseofulvin (originally isolated from *Penicillium griseofulvum*) and alternariol (from *Alternaria* species). Nonaketides include zearalenone, a toxin produced in stored grain contaminated by *Fusarium*, the aflatoxins which are produced in mouldy groundnut meal by *Aspergillus* species (Moss, 1996), and the tetracycline antibiotics produced by actinomycetes. Other, more complex compounds also arise and there are some compounds in which polyketide-derived aromatic rings are attached to sesquiterpenes, such as the antibiotic LL-Z1272α isolated from *Fusarium* species.

Sterigmatocystin and the aflatoxins are among the most toxic, mutagenic, and carcinogenic natural products known. The sterigmatocystin biosynthetic pathway in *Aspergillus nidulans* is estimated to involve at least 15 enzymatic activities, while certain *A. parasiticus*, *A. flavus*, and *A. nomius* strains contain additional activities that convert sterigmatocystin to aflatoxin. Brown *et al*. (1996) characterized a 60 kb region of the *A. nidulans* genome and found it contained 25 co-ordinately regulated transcripts representing most, if not all, of the functions needed for sterigmatocystin biosynthesis.

3.13.2.3 Other secondary metabolic pathways

The previous two sections deal with special aspects of metabolism which give rise to particular classes of secondary metabolite, but there are many opportunities in primary metabolism for secondary metabolites to arise by greater or lesser modification of primary intermediates. Thus, important secondary metabolites can be formed as derivatives of amino acids, and fatty acids and are briefly discussed below.

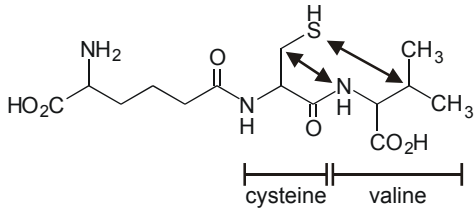
The primary role of the shikimate-chorismate pathway is the synthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan (Fig. 3.14), but the pathway provides intermediates for the synthesis of other aromatic compounds as secondary metabolites (Turner, 1971; Towers, 1976). Plants synthesise a particularly large variety of compounds by this route, though it is not so widely used in fungi, where the polyketide pathway is used more to make aromatic ring compounds. Nevertheless, numerous shikimate-

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

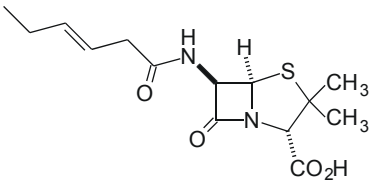
chorismate derivatives of quite common occurrence in plants have been isolated from fungi, such as gallic acid, pyrogallol, and methyl *p*-methoxycinnamate (Fig. 3.22). Among compounds more particularly associated with fungi are the ergot alkaloids (from *Claviceps purpurea*) which include ergocristine and lysergic acid amide and the hallucinogenic principle of the original 'magic' mushroom (*Psilocybe*), psilocybin which are all essentially tryptophan derivatives. Also, basidiomycete pigments like gyrocyanin, which oxidises to a blue pigment in injured fruit bodies of *Gyroporus cyanescens*, and hispidin, a polymer of which may be responsible for toughening of the fruit bodies of *Polyporus hispidus* (Bu'Lock, 1967) are derivatives of the shikimate-chorismate pathway.

Non-aromatic amino acids may also be modified (Wright and Vining, 1976). The muscarines and muscaridines (Fig. 3.23), which are the main toxic constituents of *Amanita muscaria* (but are also found in *Inocybe* and *Clitocybe* species), are synthesised from glutamate (Turner and Aldridge, 1983). Agaritine and related molecules like glutaminyl hydroxybenzene (or GHB) from *Agaricus* spp. (originally *A. bisporus*, the cultivated mushroom) are substituted (strictly, *N*-acylated) glutamate molecules, while the antifungal agent variotin (from *Paecilomyces varioti*) is γ-aminobutyric acid (GABA, shown in Fig. 3.9) *N*-acylated with a hexaketide (Fig. 3.23). Volatile amines formed by decarboxylation of neutral amino acids form part of the distinctive odours of some fungi; the fungus causing stinking smut of wheat (*Tilletia tritici*) produces large quantities of trimethylamine, and the aroma of Camembert cheese depends on the formation of *N*-dimethyl methioninol by *Penicillium camemberti*.

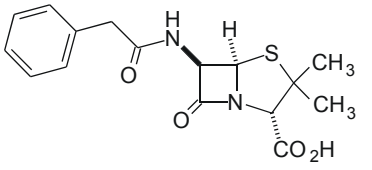
A variety of fungal secondary metabolites are derived from peptides; two or more amino acids linked through a peptide bond. Among those derived from dipeptides are the penicillins and cephalosporins (Weil *et al*., 1995a, 1995b). These could rate as the most important compounds ever to be isolated from fungi as they gave rise to a new era in medicine and a new branch of biotechnology. The basic structure of both antibiotics is derived from cysteine and valine (Fig. 3.24) though the variable acyl group may come from another amino acid as in penicillin G where the acyl group is phenylalanine (illustrated in Fig. 3.24). Biosynthesis involves the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, which is also illustrated in Fig. 3.24, but does not involve ribosomes. Instead, amino-acid activating domains of peptide synthetases determine the number and order of the amino acid constituents of the peptide secondary metabolites. *In vitro* reconstruction of the gene sequences of these multifunctional enzymes produces hybrid genes that encode peptide synthetases with altered amino acid specificities able to synthesise peptides with modified amino acid sequences (Stachelhaus *et al*., 1995). Also derived from peptides are the *Amanita* toxins of which there are many, but which can be represented by α-amanitin and phalloidin. Because of the resemblance between *Amanita phalloides* and edible field mushrooms, these toxins are involved in the majority of cases of mushroom poisoning (e.g. O'Brien and Khuu, 1996). Many fungi produce siderophores for acquiring iron which, though an essential nutrient, is not readily available in aquatic or terrestrial environments or in animal hosts. These iron-binding compounds also originate as peptides formed from modified amino acids (Turner, 1971; Turner and Aldridge, 1983; Wright and Vining, 1976; Winkelmann, 1986; Guerinot, 1994).



δ-(α-amino-adipoyl)cysteinylvaline



Penicillin F



Penicillin G

Fig. 3.24. The penicillin antibiotics are representative of secondary metabolites which are derivatives of peptides. This figure shows the structural formulae of two penicillins. At the top is the precursor δ-(α-amino-adipoyl)cysteinylvaline. The cysteine and valine residues of this compound are identified and the double-headed arrows show the bonds which have to be made to create the penicillin

Chemical modification of fatty acids produces a variety of secondary metabolites, most particularly the polyacetylenes, many of which have been obtained from basidiomycetes. These compounds have straight carbon chains varying between C_6 and C_{18}, though C_9 and C_{10} are most common in fungi. They are conjugated acetylenes (i.e. with triple bonds between adjacent carbons, e.g. hexatriyene from *Fomes annosus*, Fig. 3.25), or systems containing both ethylenic (i.e. with double bonds between carbon atoms) and acetylenic structures (e.g. nemotinic acid, Fig. 3.25). Polyacetylenes are derived from fatty acids by a series of dehydrogenation reactions (Turner, 1971; Turner and Aldridge, 1983). Also formed from fatty acids are cyclopentanes like brefeldin, which has been extracted from *Penicillium*, *Nectria* and *Curvularia* species (Fig. 3.25). Lactarinic acid was referred to above (section 3.13.2.1).

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

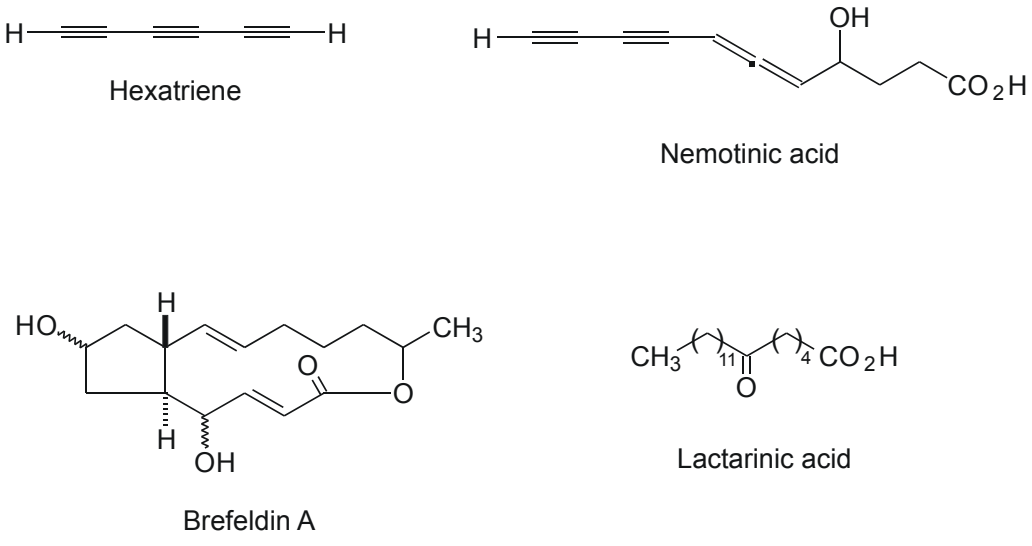


Fig. 3.25. Chemical modification of fatty acids produces a variety of secondary metabolites including polyacetylenes and cyclopentanes. Lactarinic acid is a major component of the 'milk' or 'latex' which exudes from injured fruit bodies of *Lactarius* spp. Lactarinic acid is 6-ketostearic acid and is very different from true latex which is an isoprene compound.

3.13.3 Regulation

Secondary metabolism characterises the periods occurring after one or more nutrients become growth-limiting. Indeed, Bu'Lock (1961) describes cell growth and secondary biosynthesis as incompatible, competing processes; the synthesis of secondary metabolites being suppressed while the cells in a culture are multiplying. A further implication is that a fungal culture passes through a sequence of distinct phases: initially it is not nutrient-limited and primary metabolism and cell growth and proliferation predominate, but those activities use nutrients and eventually one becomes limiting. When that happens, secondary metabolic activities are triggered.

It is important to appreciate that this fungal culture will be, for the most part, a diverse population of cells of different age and metabolic state so the behaviour observed for the culture as a whole is the average of all the members of this population. Surface cultures, whether the substratum is solid or liquid, are particularly diverse and inhomogeneous; submerged cultures a little less so as, at least, they lack surface and aerial components. Synchronised cultures, if they can be obtained, provide by definition a population of cells which are progressing through their cell cycles in unison, so the observed behaviour of the culture most accurately reflects individual cell behaviour. Synchronised cultures are, of course, very artificial; the natural state of fungal cultures being the unsynchronised, inhomogeneous one. This means that the descriptions of the phases in the growth of a culture which follow can be interpreted at a number of levels: they can be seen as averaged descriptions of the 'life experiences' of individual cells, or as averaged accounts of happenings in the whole culture. The latter is the usual interpretation, but then the ability to observe the expected phases will depend on the degree of homogeneity in the population. Surface cultures are usually too inhomogeneous to permit identification of the phases; submerged batch cultures (i.e. one batch of liquid medium inoculated with a single starter culture and cultivated to the desired end-point) are usually sufficiently uniform to demonstrate the major characteristics.

The phases through which such a batch culture progresses have been defined and named as the balanced phase, storage phase and maintenance phase by Borrow *et al*. (1961); or the trophophase (balanced phase) and idiophase (storage phase + maintenance phase) by Bu'Lock (1965). Descriptions like this are convenient for describing the course of a fermentation but further than this, they do describe coordinated changes in physiology which are quite readily measurable. The balanced phase is the period of rapid growth extending from the onset of growth to the time of exhaustion of the first nutrient. This is the period of rapid proliferation and both the chemical composition and the microscopic appearance of the mycelium remain constant throughout the balanced phase of growth. Both features are remarkably independent of the initial nutrient concentrations of the medium. The storage phase is one in which reserves of carbon and other elements are accumulated in mycelium by redistribution of the components of cells which have ceased rapid proliferation. The dry weight and fat and carbohydrate content of the mycelium increased in nitrogen-limited cultures given sufficient glucose though at about the time that the dry weight reaches its maximum, the uptake of nutrients other than glucose ceases. Timing of individual events within this phase differs, implying that nutrient exhaustion initiates a number of independent metabolic changes which proceed during the storage phase. Among these changes are those processes which are called secondary metabolism. There is, of course, a transition between the balanced and storage phases; the events occurring during the transition depend on the

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

nature of the limiting nutrient. The onset of storage phase is signalled by cessation of cell proliferation; but during storage phase the biomass continues to increase as reserves are redistributed and accumulated. Eventually, however, the mycelial dry weight (biomass) reaches a maximum and this defines the start of maintenance phase. During this phase gross features like dry weight, nucleic acid content and total carbohydrate remain approximately constant but there is considerable turnover as carbon sources, either from the medium or mobilised from internal reserves, are metabolized. During the course of this metabolism secondary metabolites can be accumulated to their maximum levels.

Thus, the fundamental control factor initiating secondary metabolism seems to be nutrient limitation, but any of a wide range of nutrients can be effective in this and, as we have seen, there is a wide range of secondary metabolic pathways which might be initiated.

3.13.4 Role

Secondary metabolites may be obtained by chemical extraction of material collected in the field, the traditional way in which organic chemists obtained and catalogued 'natural products', but the special attraction of fungi is that so many of them will produce secondary metabolites when grown in the laboratory in liquid media. This ability has proved a great convenience for academic study of secondary metabolism and has been the essential prerequisite of its exploitation for commercial purposes. It must be stressed, though, that except for yeasts and the water moulds growth in liquids and, especially, growth in submerged culture, is physiologically abnormal for fungi. Furthermore, many of the fungal strains most favoured for commercial production of secondary metabolites are biochemically defective in some way; a strain which 'wastes' nutritional resources in order to overproduce a commercially useful product being the organism of choice (Aharonowitz, 1980). In attempting to assign a role (or roles) in nature to secondary metabolism and secondary metabolites it must be recognised that our basic information is fragmentary, is concentrated in areas which have relevance to commercial exploitation and is, therefore, inevitably at its weakest in areas relating to the natural environment and natural behaviour of the fungus.

Nevertheless, a consistent argument can be developed along the lines that, rather than being some sort of luxurious biochemical extravagance, secondary metabolism does serve important physiological roles. Bu'Lock (1961) clearly stated that we must suppose that formation of secondary metabolites confers selective advantage on the producing organism. He further observed that secondary metabolites are known in such variety that no single intrinsic property accounting for production of secondary metabolites in general can be found that is common to all of them. Bu'Lock suggested that the selective advantage of secondary metabolism might be that the synthetic activities that characterise it may serve to '.maintain mechanisms essential to cell multiplication in operative order when that cell multiplication is no longer possible.'

In other words it is cell multiplication which drives balanced growth by providing a continuously expanding sink for the products of primary metabolism. When that growth process stops, secondary metabolism, as a differentiation process, provides an alternative sink permitting a number of general synthetic and nutrient uptake mechanisms to continue operating, especially during the maintenance phase, but without requiring close integration of processes because the end-product (the secondary metabolite) is of no special significance. This fundamentally economic explanation could well provide a general, very unspecific, role to secondary metabolite production and thereby account for its common occurrence. However, an unsatisfactory aspect of this notion is that the other characteristic of secondary metabolism, namely that secondary metabolites are individually of such restricted distribution as often to represent species-specific markers, must then be left to chance. That is, it may be that all fungi need to embark upon secondary metabolism as their growth becomes nutrient-limited but the particular metabolite that an individual species synthesises is an arbitrary 'choice'. Bu'Lock (1961) argued that advantages other than the economic one (such as ecological advantages conferred by antibiotics; structural and physiological advantages due to synthesis of pigments and polyphenols in cell walls, etc.) should be viewed as incidental to the main economic role.

An alternative (and not necessarily exclusive) argument has been developed. Campbell (1984) points out that in several ways secondary metabolism is more sophisticated in its biochemistry, especially in the stereospecificity of terpene and polyketide biosyntheses. In this respect secondary metabolism represents a considerable evolutionary advance over primary metabolism and must therefore be of very considerable selective advantage. He also emphasises the distributional definition of secondary metabolites - that they are metabolites which are (individually) of limited or restricted distribution and points out the Darwinian prediction that competition would be most severe between varieties of the same species or species of the same genus. This being the case, it might be expected that variety- or species-specific processes providing competitive advantage would arise as a consequence, and some would result in variety- or species-specific metabolites.

Campbell (1984) lists a number of physiological processes in which limited distribution of the metabolites is a necessary prerequisite of the function. These include sex hormones, pheromones and tropic agents; hormones and growth factors involved in morphogenesis; agents affecting spore germination and outgrowth; chelating agents; structural and extracellular protective agents; and host-specific toxins. The majority of the processes in this list contribute in some way to fungal morphogenesis. In addition, protection or defence is especially important and is viewed in its widest interpretation as taking in processes as diverse as cell wall

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

reinforcement, toxin production (e.g. to deter grazing animals) and cell-cell communication (Gloer, 1995; Shearer, 1995). Thus, there is no shortage of physiological functions which depend upon uniqueness of the effective molecule. As our knowledge of the biological nature of these processes increases, so our understanding of the part played by secondary metabolites will increase. Indeed, many of the generalised interpretations of the function of 'secondary metabolism' may well be overtaken by events as knowledge accumulates of the molecular architecture of the cell. Several polypeptides, including the nuclear lamins, several vesicular transport proteins, the oncogene product Ras and fungal peptide pheromones require the post-translational attachment of a farnesyl group, an isoprenoid lipid moiety derived from mevalonate, to the carboxyl-terminus of the protein (Caldwell *et al*., 1995; Dimsterdenk *et al*., 1995). Should this protein prenylation be labelled as an aspect of secondary metabolism, or is the isoprenoid pathway just another way of making function-specific post-translational modifications to peptides rather than a secondary metabolic route? Which is more significant, the biochemistry or the language used to describe it?

The study of secondary metabolism was dominated in its early years by the desire of organic chemists to document the occurrence of natural products. It is an area of biological chemistry which has a particular history and we are still left with conventions and distinctions which are purely matters of definition or semantics. For example, it was stated above that, conventionally, animals are not considered to produce many secondary metabolites. This is a matter of the convention in the use of the term, rather than an indication of a profound biochemical difference between the different groups of organisms. For the sorts of reasons discussed immediately above, chemicals employed in defence, offence, communication and control in fungi, bacteria and plants are expected to have limited distribution and therefore to be classified as secondary metabolites. Animals produce toxins, antitoxins, hormones and pheromones too. They are not called secondary metabolites because their function has often been discovered before isolation of the chemical itself, so there is no history of long lists of natural animal products which lack obvious function, 'secondary metabolites'. It's a matter of semantics, but it seems that such instances as the spider which excretes a moth pheromone (Stowe *et al*., 1987) to attract prey to a last supper are genuine examples of what in a plant or fungus would be called secondary metabolism rather than chemical mimicry - the phrase used in zoological vocabulary. A more extreme example is the presence of the hallucinogen bufotenin in the toad *Bufo* and in a species of the toadstool *Amanita* (Metzenberg, 1991). This has been mentioned before as another possible example of horizontal transmission at some stage in evolution (section 1.7), but why should it be described by different terms in the two organisms?

References

Abuzinadah, R. A., Finlay, R. D. & Read, D. J. (1986). The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. II. Utilization of protein by mycorrhizal plants of *Pinus contorta*. *New Phytologist*, **103**: 495-506.

Abuzinadah, R. A. & Read, D. J. (1986a). The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. I. Utilization of peptides and proteins by ectomycorrhizal fungi. *New Phytologist*, **103**: 481-493.

Abuzinadah, R. A. & Read, D. J. (1986b). The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. III. Protein utilization by *Betula*, *Picea* and *Pinus* mycorrhizal association with *Hebeloma crustuliniforme*. *New Phytologist*, **103**: 507-514.

Aharonowitz, Y. (1980). Nitrogen metabolite regulation of antibiotic biosynthesis. *Annual Review of Microbiology*, **34**: 209-233.

Ander, P. (1994). The cellobiose-oxidizing enzymes CBQ and CbO as related to lignin and cellulose degradation - a review. *FEMS Microbiology Reviews*, **13**: 297-312.

Andre, B. (1995). An overview of membrane transport proteins in *Saccharomyces cerevisiae*. *Yeast*, **11**: 1575-1611.

Armstrong, G. A. & Hearst, J. E. (1996). Carotenoids. 2. Genetics and molecular biology of carotenoid pigment biosynthesis. *FASEB Journal*, **10**: 228-237.

Ayres, P. G. & Boddy, L. (1986). *Water, Fungi and Plants*. Cambridge University Press, Cambridge, U.K.

Bajwa, R. & Read, D. J. (1985). The biology of mycorrhiza in the Ericaceae. IX. Peptides as nitrogen sources for the ericoid endophyte and for mycorrhizal and non-mycorrhizal plants. *New Phytologist*, **101**: 459-467.

Barrettbee, K. & Dixon, G. (1995). Ergosterol biosynthesis inhibition - a target for antifungal agents. *Acta Biochimica Polonica*, **42**: 465-479.

Beguin, P. & Aubert, J. P. (1994). The biological degradation of cellulose. *FEMS Microbiology Reviews*, **13**: 25-58.

Blumenthal, H. J. (1965). Glycolysis. In *The Fungi* (G. C. Ainsworth & A. S. Sussman, eds): 229-268. Academic Press, New York & London.

Blumenthal, H. J. (1968). Glucose catabolism in fungi. *Wallerstein Laboratory Communications*, **31**: 171-191.

Blumenthal, H. J. (1976). Reserve carbohydrates in fungi. In *The Filamentous Fungi, Biosynthesis and Metabolism* (J. E. Smith & D. R. Berry, eds): 292-307. Edward Arnold Ltd, London.

Bonnen, A. M., Anton, L. H. & Orth, A. B. (1994). Lignin-degrading enzymes of the commercial button mushroom, *Agaricus bisporus*. *Applied and Environmental Microbiology*, **60**: 960-965.

Bowman, B. J. & Bowman, E. J. (1996). Mitochondrial and vacuolar ATPases. In *The Mycota, Biochemistry and Molecular Biology* (R. Brambl & G. A. Marzluf, eds): 57-83. Springer-Verlag, Berlin, Heidelberg, New York.

Bremner, J. M. (1967). Nitrogenous compounds. In *Soil Biochemistry* (A. D. McLaren & G. H. Peterson, eds): 19-66. Marcel Dekker, New York.

Broda, P., Birch, P. R. J., Brooks, P. R. & Sims, P. F. G. (1996). Lignocellulose degradation by *Phanerochaete chrysosporium* - gene families and gene expression for a complex process. *Molecular Microbiology*, **19**: 923-932.

Brown, D. W., Yu, J. H., Kelkar, H. S., Fernandes, M., Nesbitt, T. C., Keller, N. P., Adams, T. H. & Leonard, T. J. (1996).

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences of the U S A*, **93**: 1418-1422.
Bu'Lock, J. D. (1961). Intermediary metabolism and antibiotic synthesis. *Advances in Applied Microbiology*, **3**: 293-342.
Bu'Lock, J. D. (1967). *Essays in Biosynthesis and Microbial Development*. John Wiley & Sons, New York.
Byrde, R. J. W. (1982). Fungal pectinases, from ribosome to plant cell wall. *Transactions of the British Mycological Society*, **79**: 1-14.
Cairney, J. W. G. (1992). Translocation of solutes in ectomycorrhizal and saprotrophic rhizomorphs. *Mycological Research*, **96**: 135-141.
Caldwell, G. A., Naider, F. & Becker, J. M. (1995). Fungal lipopeptide mating pheromones: a model system for the study of protein prenylation. *Microbiological Reviews*, **59**: 406-422.
Campbell, I. M. (1984). Secondary metabolism and microbial physiology. *Advances in Microbial Physiology*, **25**: 1-60.
Chang, Y. (1967). The fungi of wheat straw compost. II. Biochemical and physiological studies. *Transactions of the British Mycological Society*, **50**: 667-677.
Cochrane, V. W. (1976). Glycolysis. In *The Filamentous Fungi, Biosynthesis and Metabolism* (J. E. Smith & D. R. Berry, eds): 65-91. Edward Arnold Ltd, London.
Cooke, R. C. & Rayner, A. D. M. (1984). *Ecology of Saprotrophic Fungi*. Longman, London.
Cullen, D. & Kersten, P. J. (1996). Enzymology and molecular biology of lignin degradation. In *The Mycota, Biochemistry and Molecular Biology* (R. Brambl & G. A. Marzluf, eds): 295-312. Springer-Verlag, Berlin, Heidelberg, New York.
Dimsterdenk, D., Schafer, W. R. & Rine, J. (1995). Control of Ras mRNA level by the mevalonate pathway. *Molecular Biology of the Cell*, **6**: 59-70.
Eamus, D. & Jennings, D. H. (1986). Water, turgor and osmotic potentials of fungi. In *Water, Fungi and Plants* (P. G. Ayres & L. Boddy, eds): 27-48. Cambridge University Press, Cambridge, U.K.
Edwardson, J. M. & Marciniak, S. J. (1995). Molecular mechanisms in exocytosis. *Journal of Membrane Biology*, **146**: 113-122.
Elisashvili, V. I. (1993). Physiological regulation of ligninolytic activity in higher basidium fungi. *Microbiology*, **62**: 480-487.
Eriksson, K. E. L., Habu, N. & Samejima, M. (1993). Recent advances in fungal cellobiose oxidoreductases - review. *Enzyme and Microbial Technology*, **15**: 1002-1008.
Ewaze, J. O., Moore, D. & Stewart, G. R. (1978). Co-ordinate regulation of enzymes involved in ornithine metabolism and its relation to sporophore morphogenesis in *Coprinus cinereus*. *Journal of General Microbiology*, **107**: 343-357.
Fermor, T. R. & Wood, D. A. (1981). Degradation of bacteria by *Agaricus bisporus* and other fungi. *Journal of General Microbiology*, **126**: 377-387.
Fothergill-Gilmore, L. A. (1986). The evolution of the glycolytic pathway. *Trends in Biochemical Sciences*, **11**: 47-51.
Funtikova, N. S., Katomina, A. A. & Mysyakina, I. S. (1995). Dimorphism and lipid composition of the fungus *Mucor lusitanicus*. *Microbiology*, **64**: 238-239.
Garrill, A. (1995). Transport. In *The Growing Fungus* (N. A. R. Gow & G. M. Gadd, eds): 163-181. Chapman & Hall, London.
Gloer, J. B. (1995). The chemistry of fungal antagonism and defense. *Canadian Journal of Botany*, **73**: S1265-S1274.
Goodwin, T. W. (1973). Comparative biochemistry of sterols in eukaryotic micro-organisms. In *Lipids and Biomembranes of Eukaryotic Micro-organisms* (J. A. Erwin, eds): 1-40. Academic Press, New York.
Goodwin, T. W. (1976). Carotenoids. In *The Filamentous Fungi, {Bvol II}, Biosynthesis and Metabolism* (J. E. Smith & D. R. Berry, eds): 423-444. Edward Arnold Ltd, London.
Goyal, S. & Khuller, G. K. (1994). Structural and functional role of lipids in yeast and mycelial forms of *Candida albicans*. *Lipids*, **29**: 793-797.
Granlund, H. I., Jennings, D. H. & Thompson, W. (1985). Translocation of solutes along rhizomorphs of *Armillaria mellea*. *Transactions of the British Mycological Society*, **84**: 111-119.
Grant, W. D., Rhodes, L. L., Prosser, B. A. & Asher, R. A. (1986). Production of bacteriolytic enzymes and degradation of bacteria by filamentous fungi. *Journal of General Microbiology*, **132**: 2353-2358.
Guerinot, M. L. (1994). Microbial iron transport. *Annual Review of Microbiology*, **48**: 743-772.
Guiseppin, M. L. F. (1984). Effects of dissolved oxygen concentration on lipase production by *Rhizopus delemar*. *Applied Microbiology and Biotechnology*, **20**: 161-173.
Hammond, J. B. W. & Nichols, R. (1976). Carbohydrate metabolism in *Agaricus bisporus* (Lange) Sing.:Changes in soluble carbohydrates during growth of mycelium and sporophore. *Journal of General Microbiology*, **93**: 309-320.
Hatakka, A. (1994). Lignin-modifying enzymes from selected white-rot fungi - production and role in lignin degradation. *FEMS Microbiology Reviews*, **13**: 125-135.
Hemmi, K., Julmanop, C., Hirata, D., Tsuchiya, E., Takemoto, J. Y. & Miyakawa, T. (1995). The physiological roles of membrane ergosterol as revealed by the phenotypes of *syr1/erg3* null mutant of *Saccharomyces cerervisiae*. *Bioscience Biotechnology and Biochemistry*, **59**: 482-486.
Hilt, W. & Wolf, D. H. (1995). Proteasomes of the yeast *S. cerevisiae*: genes, structure and functions. *Molecular Biology Reports*, **21**: 3-10.
Jennings, D. H. & Austin, S. (1973). The stimulatory effect of the non-metabolized sugar 3-O-methyl glucose on the conversion of mannitol and arabitol to polysaccharide and other insoluble compounds in the fungus *Dendryphiella salina*. *Journal of General Microbiology*, **75**: 287-294.
Jennings, D. H. (1995). *The Physiology of Fungal Nutrition*. Cambridge University Press, Cambridge, U.K.
Kalisz, H. M., Moore, D. & Wood, D. A. (1986). Protein utilization by basidiomycete fungi. *Transactions of the British Mycological Society*, **86**: 519-525.
Kalisz, H. M., Wood, D. A. & Moore, D. (1987). Production, regulation and release of extracellular proteinase activity in basidiomycete fungi. *Transactions of the British Mycological Society*, **88**: 221-227.

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8

Kalisz, H. M. (1988). Microbial proteinases. *Advances in Biochemical Engineering/Biotechnology*, **36**: 1-65.
Kalisz, H. M., Wood, D. A. & Moore, D. (1989). Some characteristics of extracellular proteinases from *Coprinus cinereus*. *Mycological Research*, **92**: 278-285.
Kirk, T. K., Tien, M., Kersten, P. J., Kalyanaraman, B., Hamel, K. E. & Farrell, R. L. (1990). Lignin peroxidase from fungi: *Phanerochaete chrysosporium*. *Methods in Enzymology*, **188**: 159-171.
Kohlwein, S. D., Daum, G., Schneiter, R. & Paltauf, F. (1996). Phospholipids - synthesis, sorting subcellular traffic - the yeast approach. *Trends in Cell Biology*, **6**: 260-266.
Kumar, S. & Punekar, N. S. (1997). The metabolism of 4-aminobutyrate (GABA) in fungi. *Mycological Research*, **101**: 403-409.
Leake, J. R. & Read, D. J. (1990). Chitin as a nitrogen source for mycorrhizal fungi. *Mycological Research*, **94**: 993-995.
Lemaire, M. (1996). The cellulosome - an exocellular multiprotein complex specialised in cellulose degradation. *Critical Reviews in Biochemistry & Molecular Biology*, **31**: 201-236.
Lindenmeyer, A. (1965). Terminal oxidation and electron transport. In *The Fungi* (G. C. Ainsworth & A. S. Sussman, eds): 301-348. Academic Press, New York & London.
Lodha, B. C. (1974). Decomposition of digested litter. In *Biology of Plant Litter Decomposition* (C. H. Dickinson & J. F. Pugh, eds): 213-241. Academic Press, London.
Marzluf, G. A. (1996). Regulation of nitrogen metabolism in mycelial fungi. In *The Mycota, Biochemistry and Molecular Biology* (R. Brambl & G. A. Marzluf, eds): 357-368. Springer-Verlag, Berlin, Heidelberg, New York.
McCorkindale, N. J. (1976). The biosynthesis of terpenes and steroids. In *The Filamentous Fungi, Biosynthesis and Metabolism* (J. E. Smith & D. R. Berry, eds): 369-422. Edward Arnold Ltd, London.
Metzenberg, R. L. (1991). The impact of molecular biology on mycology. *Mycological Research*, **95**: 9-13.
Moore, D. & Ewaze, J. O. (1976). Activities of some enzymes involved in metabolism of carbohydrate during sporophore development in *Coprinus cinereus*. *Journal of General Microbiology*, **97**: 313-322.
Moore, D. & Devadatham, M. S. (1979). Sugar transport in *Coprinus cinereus*. *Biochimica et Biophysica Acta*, **550**: 515-526.
Moore, D. (1984). Developmental biology of the *Coprinus cinereus* carpophore: metabolic regulation in relation to cap morphogenesis. *Experimental Mycology*, **8**: 283-297.
Moss, M. O. (1996). Mycotoxins. *Mycological Research*, **100**: 513-523.
Mukhamedzhanova, T. G. & Bezborodov, A. M. (1982). Effect of nitrogen and carbon sources on lipase accumulation by the fungus *Rhizopus oryzae* 1414. *Mikrobiologia*, **18**: 16-22.
Muller, M. M., Kantola, R. & Kitunen, V. (1994). Combining sterol and fatty acid profiles for the characterization of fungi. *Mycological Research*, **98**: 593-603.
Navarro, E., Sandmann, G. & Torresmartinez, S. (1995). Mutants of the carotenoid biosynthetic pathway of *Mucor circinelloides*. *Experimental Mycology*, **19**: 186-190.
Neville, N. M., Suskind, S. R. & Roseman, S. (1971). A derepressible active transport system for glucose in *Neurospora crassa*. *Journal of Biological Chemistry*, **246**: 1294-1301.
Niederpruem, D. J. (1965). Tricarboxylic acid cycle. In *The Fungi* (G. C. Ainsworth & A. S. Sussman, eds): 269-300. Academic Press, New York & London.
O'Brien, B. L. & Khuu, L. (1996). A fatal Sunday brunch - *Amanita* mushroom poisoning in a gulf coast family. *American Journal of Gastroenterology*, **91**: 581-583.
Parks, L. W. & Casey, W. M. (1995). Physiological implications of sterol biosynthesis in yeast. *Annual Review of Microbiology*, **49**: 95-116.
Parks, L. W., Smith, S. J. & Crowley, J. H. (1995). Biochemical and physiological effects of sterol alterations in yeast - a review. *Lipids*, **30**: 227-230.
Peberdy, J. F. & Ferenczy, L. (1985). *Fungal Protoplasts: Applications in Biochemistry and Genetics*. Marcel Dekker, New York.
Radford, A., Stone, P. J. & Taleb, F. (1996). Cellulase and amylase complexes. In *The Mycota, Biochemistry and Molecular Biology* (R. Brambl & G. A. Marzluf, eds): 269-294. Springer-Verlag, Berlin, Heidelberg, New York.
Read, D. J., Leake, J. R. & Langdale, A. R. (1989). The nitrogen nutrition of mycorrhizal fungi and their host plants. In *Nitrogen, Phosphorus and Sulphur Utilization by Fungi* (L. Boddy, R. Marchant & D. J. Read, eds): 181-204. Cambridge University Press, Cambridge, U.K.
Read, D. J. (1991). Mycorrhizas in ecosystems - Nature's response to the "Law of the Minimum". In *Frontiers of Mycology* (D. L. Hawksworth, eds): 101-130. CAB International, Wallingford, U.K.
Reddy, C. A. & Dsouza, T. M. (1994). Physiology and molecular biology of the lignin peroxidases of *Phanerochaete chrysosporium*. *FEMS Microbiology Reviews*, **13**: 137-152.
Reinbothe, H. & Tschiersch, B. (1962). Harnstoff-metabolismus bei basidiomyceten. I. Zur harnstoffbiosynthese in *Agaricus bisporus* Lange und *Lycoperdon perlatum* Pers. *Flora*, **152**: 423-446.
Reinbothe, H., Wasternack, C. & Miersch, J. (1967). Harnstoff-metabolismus bei basidiomyceten. IV. Untersuchungen zur physiologie des harnstoffs. *Flora*, **158**: 27-57.
Samuels, R. I. & Paterson, I. C. (1995). Cuticle degrading proteases from insect moulting fluid and culture filtrates of entomopathogenic fungi. *Comparative Biochemistry and Physiology B - Biochemistry & Molecular Biology*, **110**: 661-669.
Schatz, G. & Dobberstein, B. (1996). Common principles of protein translocation across membranes. *Science*, **271**: 1519-1526.
Shearer, C. A. (1995). Fungal competition. *Canadian Journal of Botany*, **73**: S1259-S1264.
Spinner, S. & Haselwandter, K. (1985). Proteins as nitrogen sources for *Hymenoscyphus* (= *Pezizella*) *Iericae*. In *Proceedings of the 6th North American Conference on Mycorrhizae* (R. Molina, eds): 422. Forest Research Laboratory, Oregon State University, Corvallis, Oregon.
St Leger, R. J. (1995). The role of cuticle-degrading proteases in fungal pathogenesis of insects. *Canadian Journal of*

Botany, **73**: S1119-S1125.
Stachelhaus, T., Schneider, A. & Marahiel, M. A. (1995). Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. *Science*, **269**: 69-72.
Stewart, G. R. & Moore, D. (1974). The activities of glutamate dehydrogenases during mycelial growth and sporophore development in *Coprinus lagopus* (*sensu* Lewis). *Journal of General Microbiology*, **83**: 73-81.
Stone, K. J. & Hemming, F. W. (1967). The stereochemistry of hexahydroprenol, ubiquinone and ergosterol biosynthesis in the mycelium of *Aspergillus fumigatus*. *Biochemical Journal*, **104**: 43-56.
Stowe, M. K., Tumlinson, J. H. & Heath, J. H. (1987). Chemical mimicry: bolas spiders emit components of moth prey species sex pheromones. *Science*, **236**: 964-967.
Swift, M. J. (1977). The ecology of wood decomposition. *Science Progress (Oxford)*, **64**: 175-199.
Tan, Y. H. & Moore, D. (1994). High concentrations of mannitol in the shiitake mushroom *Lentinula edodes*. *Microbios*, **79**: 31-35.
Towers, G. H. N. (1976). Secondary metabolites derived through the shikimate-chorismate pathway. In *The Filamentous Fungi, Biosynthesis and Metabolism* (J. E. Smith & D. R. Berry, eds): 460-474. Edward Arnold Ltd, London.
Turner, W. B. (1971). *Fungal Metabolites*. Academic Press, London & New York.
Turner, W. B. (1976). Polyketides and related metabolites. In *The Filamentous Fungi, Biosynthesis and Metabolism* (J. E. Smith & D. R. Berry, eds): 445-459. Edward Arnold Ltd, London.
Turner, W. B. & Aldridge, D. C. (1983). *Fungal Metabolites II*. Academic Press, London & New York.
Valadon, L. R. G. (1976). Carotenoids as additional taxonomic characters in fungi. *Transactions of the British Mycological Society*, **67**: 1-15.
van Laere, A. (1995). Intermediary metabolism. In *The Growing Fungus* (N. A. R. Gow & G. M. Gadd, eds): 211-238. Chapman & Hall, London.
Walker, P. & Woodbine, M. (1976). The biosynthesis of fatty acids. In *The Filamentous Fungi, Biosynthesis and Metabolism* (J. E. Smith & D. R. Berry, eds): 137-158. Edward Arnold Ltd, London.
Wardle, K. C. & Schisler, L. C. (1969). The effects of various lipids on growth of mycelium of *Agaricus bisporus*. *Mycologia*, **61**: 305-314.
Watkins, N. K., Fitter, A. H., Graves, J. D. & Robinson, D. (1996). Carbon transfer between C-3 and C-4 plants linked by a common mycorrhizal network, quantified using stable carbon isotopes. *Soil Biology & Biochemistry*, **28**: 471-477.
Watson, K. (1976). The biochemistry and biogenesis of mitochondria. In *The Filamentous Fungi, Biosynthesis and Metabolism* (J. E. Smith & D. R. Berry, eds): 92-120. Edward Arnold Ltd, London.
Weete, J. D. & Gandhi, S. R. (1996). Biochemistry and molecular biology of fungal sterols. In *The Mycota, Biochemistry and Molecular Biology* (R. Brambl & G. A. Marzluf, eds): 421-438. Springer-Verlag, Berlin, Heidelberg, New York.
Weil, J., Miramonti, J. & Ladisch, M. R. (1995). Cephalosporin C: mode of action and biosynthetic pathway. *Enzyme and Microbial Technology*, **17**: 85-87.
Weil, J., Miramonti, J. & Ladisch, M. R. (1995). Biosynthesis of cephalosporin C: regulation and recombinant technology. *Enzyme and Microbial Technology*, **17**: 88-90.
Whalley, A. J. S. & Edwards, R. L. (1987). Xylariaceous fungi: use of secondary metabolites. In *Evolutionary Biology of the Fungi* (A. D. M. Rayner, C. M. Brasier & D. Moore, eds): 423-434. Cambridge University Press, Cambridge, U.K.
Winkelmann, G. (1986). Iron complex products (siderophores). In *Biotechnology 4* (H. J. Rehn & G. Reed, eds): 215-243. VCH Verlagsgesellschaft, Weinheim.
Wood, D. A. (1980a). Inactivation of extracellular laccase of *Agaricus bisporus*. *Journal of General Microbiology*, **117**: 339-345.
Wood, D. A. (1980b). Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *Journal of General Microbiology*, **177**: 327-338.
Wright, J. L. C. & Vining, L. C. (1976). Secondary metabolites derived from non-aromatic amino acids. In *The Filamentous Fungi, Biosynthesis and Metabolism* (J. E. Smith & D. R. Berry, eds): 475-502. Edward Arnold Ltd, London.

Extracted from: Moore, D. (1998). *Fungal Morphogenesis*. Cambridge University Press: New York. 469 pp. ISBN 0 521 55295 8