*An introduction to*

# Biochemistry of Fungal Development

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TABLE 6.2. Effect of spore-population density at different temperatures on the formation of SG spores, the degree of spherical growth, and the degree of germ-tube formation from spores of *Aspergillus niger* after 15 h cultivation. From Anderson and Smith (1972).

| Spores per milliliter medium, $\times 10^6$ | No. of SG spores, % | | | Size of SG spores $\mu$ | | | No. of SG spores with germ tubes, % | | |
|---|---|---|---|---|---|---|---|---|---|
| | Temperature | | | Temperature | | | Temperature | | |
| | 30° | 41° | 44° | 30° | 41° | 44° | 30° | 41° | 44° |
| 4 | 51 | 32 | 30 | 10·4 | 14·0 | 9·2 | 97 | 37 | 0 |
| 3 | 73 | 76 | 64 | 9·5 | 15·4 | 9·8 | 96 | 30 | 0 |
| 2 | 98 | 90 | 75 | 9·5 | 15·0 | 10·2 | 99 | 52 | 0 |
| 1 | 97 | 95 | 93 | 9·0 | 13·8 | 9·7 | 96 | 75 | 0 |
| 0·5 | 99 | 98 | 95 | 7·8 | 13·0 | 9·3 | 100 | 91 | 0 |
| 0·1 | 100 | 97 | 94 | 7·1 | 12·1 | 7·3 | 97 | 99 | 0 |

enlargement at 44°, whilst retaining the ability to form a complex reproductive structure and viable conidia, is extremely puzzling. However, studies on this phenomenon should provide valuable information relating to the control of both the vegetative and asexual reproductive phases of this fungus. Also, as a culture technique for studies on conidiation it should simplify the studies of regulatory mechanisms preceding conidiation and of the processes accompanying conidiophore development and conidia production.

## BIOCHEMISTRY OF CONIDIATION

Filamentous, multicellular fungi are inherently difficult systems from which to obtain meaningful results on specific biochemical changes associated with the process of differentiation. As with other microbial systems, most physiological and biochemical studies have been concerned with the analysis of crude homogenates of the entire culture. Such methods may closely indicate the true changes occurring at specific phases of development in synchronously growing unicellular cultures. However, with mycelial cultures, and in particular because of the phenomenon of apical growth, there exists within each mycelium and indeed each hypha a spatial distribution of differing biochemical activities. As a result, particular aspects of fungal differentiation, e.g. conidiation, may not necessarily involve the entire thallus, and since normally the vegetative cells far outnumber the cells actively involved in conidiation important, specific and highly characteristic biochemical changes may be masked by the vegetative physiology. The problems

inherent in the analytical methods are further multiplied in static mycelial cultures where variation of the physiological and biochemical status of the mycelium is increased by the additional variants of oxygen tension and nutrient concentration. Submerged batch or continuous cultivation reduces these complications only if the mycelium grows in a filamentous form rather than in pellet form, the more usual condition of growth in submerged cultures. With pellet growth nutrients and oxygen are only readily available to the peripheral hyphae and autolysis rapidly

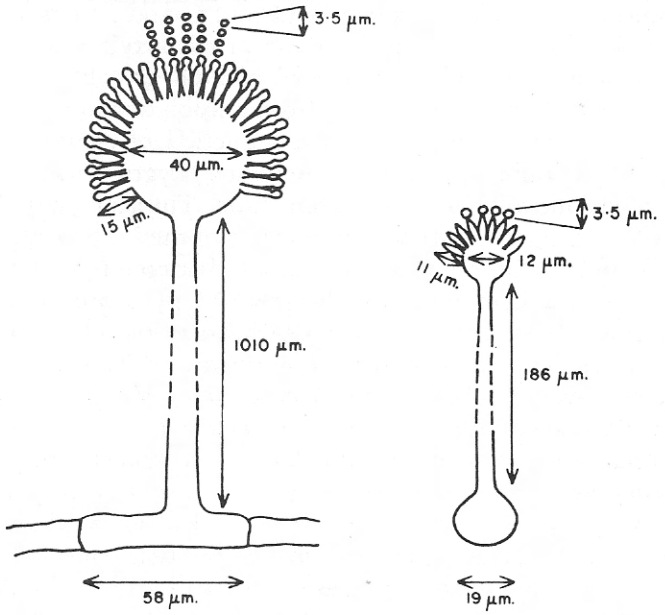


Fig. 6.21. Comparison of the typical subaerial conidiophore of *Aspergillus niger* produced from conidiating mycelium and from microcycle conidiation. From Anderson and Smith (1971).

occurs at the centre while dense growth continues at the edge. Although submerged growth is more desirable for biochemical studies of conidiation it is often beset with the problem that conidiation is generally suppressed in submerged culture even in those species which conidiate freely in static surface culture. However, it has clearly been shown that this difficulty can be overcome by careful manipulation of the cultural conditions.

Numerous studies have dealt specifically with quantitative and qualitative changes in enzyme levels during differentiation of several fungi and correlations between increasing levels of specific enzymes and

the changing requirements of differentiation have also been described.

*Carbon metabolism.* Oxidative metabolism appears to be essential to conidiogenesis in *Neurospora* and environmental conditions which favour glycolysis inhibit conidial development (Turian, 1969). Thus a four-day vegetative mycelium produces 80% more ethanol than a corresponding conidial mycelium; the addition of *p*-chloromercuribenzoate almost eliminates alcohol production and causes the M culture to conidiate. Inhibition of the tricarboxylic acid cycle with fluoroacetate favours glycolysis and C mycelium reverts to M mycelium.

Differences in relative enzyme activities in C and M cultures are as expected; thus alcohol dehydrogenase and carboxylase activities are greater in M mycelia. The occurrence of high glycolytic activity in filtrates M mycelium is confirmed by manipulation of the environment in various ways, such as by the induction of conidiation in an amino acid/ammonium medium by increased oxygen tension or by the addition of glycine to an ammonium medium. Therefore, conidiation is considered to be a morphogenetic expression of the Pasteur effect. Thus conidiation must be regulated by the balance between the oxidative and glycolytic pathways, probably at the point of pyruvate. The relative concentrations of reduced and oxidized NAD may also play a regulatory role. An active hexosemonophosphate pathway which appears during conidiogenesis would be expected to decrease the activity of the Embden-Meyerhof-Parnas pathway through competition for glucose-6-phosphate. In M hyphae, alcohol dehydrogenase has been cytochemically detected by oxidative assay and demonstrates a dense, uniform distribution of activity except at hyphal tips. In the conidiating hyphae, alcohol dehydrogenase becomes less dense in distribution especially in the budding apices. Cytochrome oxidase activity, localized in the mitochondria, is confined to the subapical zone of vegetative hyphae while at the initiation of conidiation it becomes dispersed throughout the proconidial buds.

During induction of conidiogenesis in *Neurospora crassa* by acetate, enzymic analyses demonstrate a much more active isocitrate lyase than in mycelium conidiating in sucrose medium. The increased conidiogenesis that occurs at 37°C compared with 25°C is also mirrored by a higher isocitrate lyase activity. Bicarbonate addition to the 25° culture also increases isocitrate lyase levels. Thus three different environmental conditions that induce increased conidiation also result in higher isocitrate lyase activity. The glyoxylate formed by isocitrate lyase is transaminated with alanine to form glycine.

However, it may well be that a source of glyoxylate rather than an active glyoxylate cycle is vital for conidial development since conidia

are formed at 25° under conditions of relatively low isocitrate lyase activity. In this case, the glyoxylate could be formed by the splitting of pentose produced by the pentose phosphate pathway. The nitrogen source in these experiments is nitrate and its assimilation through nitrate reductase could provide a mechanism of NADP regeneration essential for the continued function of the pentose phosphate pathway. Addition of ammonium salts to the medium inhibits conidiation. $NH_3$ is preferentially utilized and probably uncouples nitrate reductase from the pentose phosphate pathway and prevents conidiation.

Induction of oxidative metabolism is essential for the expression of the conidiation potential; for example it can be induced by nitrate which has the effect of re-oxidizing $NADPH_2$ which can be coupled with glucose-6-phosphate dehydrogenase. A flavin type of metabolism coupled through $NADPH_2$-NADP regeneration to the direct oxidation of sugars via the hexose monophosphate pathway predominates during conidial differentiation. Several enzymes, including succinic dehydrogenase, NAD nucleotidase and NAD-dependent glutamate dehydrogenase, also show increased activites during conidiogenesis. Table 6.3 and Fig. 6.22 summarize the differences between purely vegetative mycelium and conidial cultures investigated for a number of biochemical characters (Turian, 1969).

Somewhat similar observations have been made with *Aspergillus* spp. $NADPH_2$-dependent isocitrate dehydrogenase and isocitrate lyase show much higher specific activities at the period preceding conidiophore development in *A. niger* than during vegetative growth of mycelium of the same physiological age. These enzymes are also active during conidiophore maturation in replacement culture (Fig. 6.23) and in continuous culture. Malate dehydrogenase, aconitase, NADP-dependent isocitrate dehydrogenase and malate synthetase are relatively similar in pre-conidiating and vegetative mycelium and do not appear to be highly activated during conidiation in replacement culture and continuous culture. In flask culture studies glycine-alanine transferase is detectable quantitatively only in pre-conidiating mycelium. It can be somewhat tentatively concluded that in *A. niger* the tricarboxylic acid cycle is only ticking over slowly during the rapid vegetative phase of growth due to catabolite repression, and since α-oxoglutarate dehydrogenase can never be detected it is possible that the tricarboxylic acid cycle serves purely a synthetic function. A major distinction between conidiating and non-conidiating mycelia in the systems so far examined is the synthesis of glyoxylate and possibly glycine through NADP-dependent isocitrate dehydrogenase and isocitrate lyase. The importance of glycine in RNA synthesis cannot be underestimated and the in-

TABLE 6.3. Biochemical analysis and physiological differentiation of mycelial and conidial cultures of *Neurospora crassa*. From Turian (1969)*.

| Criterion | Mycelial cultures | Conidial cultures |
|---|---|---|
| | Specific activity | |
| Enzymes: | | |
| Glucose 6-phosphate dehydrogenase | 760 | 605† |
| NADP nucleotidase | 309 | 12,618 |
| $NADPH_2$-cytochrome c reductase | 205 | 743 |
| Succinate-cytochrome c reductase‡ | 648 | 771 |
| Succinate dehydrogenase‡ | 92 | 109 |
| Cytochrome oxidase | 8,720 | 8,070 |
| Isocitrate lyase | 45 | 95 |
| Pyruvate carboxylase | 14 | 7 |
| Ethanol dehydrogenase | 1,659 | 125 |
| | Specific production | |
| Chemical Composition: | | |
| Ethanol§ (mg./g. dry wt.) | 896 | 118 |
| Acetaldehyde (mg./g. dry wt.) | 2.1 | 0·3 |
| Carotenoids ($\mu$g/% dry wt.) | 290 | 12,000 |
| Physiological activity: | | |
| $Q_{O_2}$ | 12·8 | 18·9 |
| $CO_2/O_2$ | 4·2 | 1·2 |

Age of cultures 3–4 days; Enzymes—specific activity; chemical composition = specific production.
*3–4 day-old cultures were examined.
† Value was underestimated by rapid destruction of NADP (see value for NADP nucleotidase).
‡ In isolated mitochondria instead of cell-free extracts.
§ Cultures were 4 days old.

creased activities of the enzymes which ultimately lead to increased glycine production may be intricately involved in regulating the RNA synthesis concerned with differentiation.

Recently there has been an extensive examination of carbon catabolism during differentiation of *Aspergillus* fungi. In these experiments, *in vitro* enzyme determinations were coupled with the radiorespirometric analysis of glucose metabolism *in vivo* in order to give a more reliable estimation of the *in vivo* changes occurring in glucose catabolism. It was found using the replacement fermenter technique that during conidiophore development the pentose phosphate pathway enzymes were higher in activity than the Embden-Meyerhof-Parnas pathway enzymes and this when taken together with the radiorespirometric analysis strongly implies that the direct oxidation of glucose

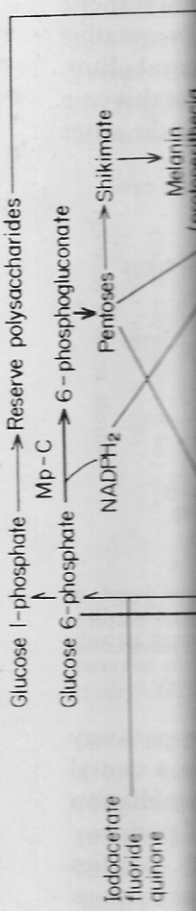

Fig. 6.22. Diagram of the principal metabolic pathways of morphogenesis of *Neurospora crassa*: carotenoid pigmented conidia, differentiation stimulated by extracellular acetate or glycine, increase in temperature (37°), or suppression of glycolysis. FA = fluoroacetate, an inhibitor of conidiation. M, Mp and C indicate predominant pathways associated with undifferentiated mycelium (M), with fertile mycelium producing protoperithecial asci (Mp) and microconidia, and with the differentiation of macroconidia (C). Note the double role in conidiation (C) of gluconeogenesis from acetate, and the reducing power of $NADPH_2$ (favourable to lipid synthesis). From Turian (1969).

through the pentose phosphate pathway may be of importance during conidiophore development. One of the main functions of the pentose phosphate pathway in cellular metabolism is to produce $NADPH_2$ essential for reductive biosynthesis.

Undoubtedly one of the main enzymic changes associated with conidiogenesis in filamentous fungi is the stimulation of the pentose phosphate pathway for carbon catabolism. The high biosynthetic demands of the process obviously cannot be met from normal vegetative metabolism, and this necessitates a major change in carbon catabolism. How are such changes in enzyme pathways brought about? In this case the enzymic pathways are already present and functional, and the onset

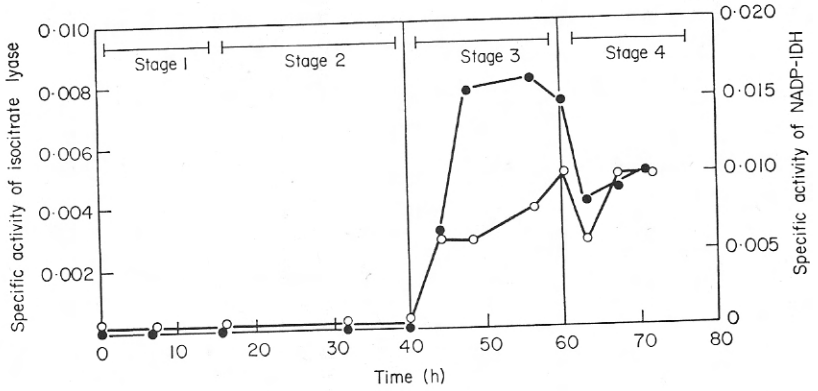


Fig. 6.23. Activities of NADP-isocitrate dehydrogenase and isocitrate lyase of *Aspergillus niger* during growth and morphogenesis in replacement fermenter culture. ●——●, Isocitrate lyase activity; ○——○, NADP-isocitrate dehydrogenase activity.

of conidiation leads to concomitant partial repression of one pathway and stimulation of the other. If it is considered that there is a causal relationship between the activity of these pathways and conidiation then how are these pathways regulated? Perhaps control of pathway activity is by way of critical levels of essential intermediates. Studies with *Aspergillus niger* during differentiation in several cultural conditions have shown that major changes can occur in the levels of certain intermediates of glycolysis. The fact that there was little apparent correlation between intermediate levels in the two systems would substantiate the results of Wright with *Dictyostelium discoideum* (Chapter 2) that the intracellular concentrations of certain metabolites essential to differentiation can vary from one study to another and yet normal morphogenesis can occur. This would imply that critical changes in the concentration of

metabolites essential to differentiation can be met and balanced by compensatory mechanisms.

Further studies on a wider range of cellular intermediates may well be of value in determining the mechanism of initiation of conidiogenesis.

*Esterase activity.* Studies with lipolytic esterases have established a causal relationship between a biochemical event and a morphological character in a fungus (Lloyd *et al.*, 1972). In flask cultures of *Aspergillus niger* esterases are always present in mycelial extracts during conidiation irrespective of the mode of induction but cannot be detected by electrophoresis in the vegetative mycelium of cultures which would ultimately conidiate or in mycelium of sterile cultures. In replacement

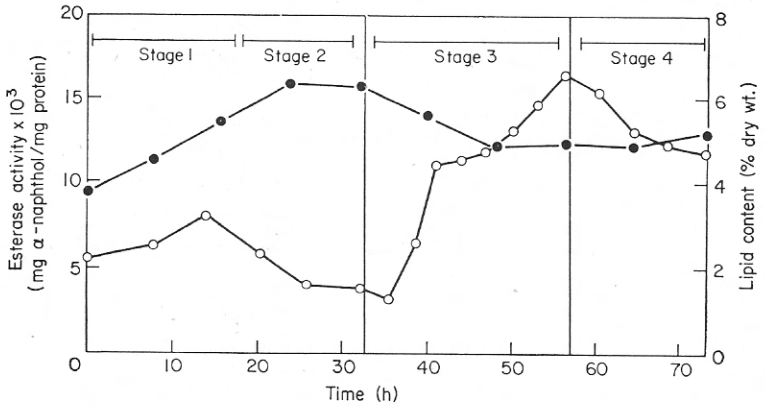


Fig. 6.24. Comparison of esterase activity and lipid content of Aspergillus niger during growth and morphogenesis in replacement fermenter culture. ○———○, esterase activity of the organism; ●———●, lipid content of the organism. Lloyd *et al.* (1972).

fermenter techniques quantitative esterase determinations demonstrate a low basal level of esterase activity during vegetative development (Fig. 6.24). Esterase activity increases greatly immediately prior to vesicle and phialide formation and persists in these structures once formed. The increase in esterase activity during conidiation has also been shown cytochemically to occur in the conidiophore tip prior to the formation of the vesicle and phialides and in the latter structures after their formation. Such studies must imply that lipids function as a source of carbon and energy during conidiation. Esterase production is also associated with conidiation induced in continuous culture and in microcycle conidiation. That esterase activity plays an important role in conidiophore differentiation must now be accepted since the esterases are always present at conidiation irrespective of the method of induction.

*Nucleic acid studies*. Very few studies have been made to relate nuclear expression with conidiogenesis. This is undoubtedly due in part to the poverty of investigations on basic DNA/RNA studies in filamentous fungi.

Recently there have been several interesting studies with *Trichoderma viride*, a fungus which requires a light stimulation for conidiation. Because of this light dependency it is possible to precisely time the onset of conidiation. When 8-azaguanine or 5-fluorouracil are applied within a certain time range relative to light induction and removed thereafter, there is complete suppression of growth. The inhibition by 5-fluorouracil can be overcome by the addition of uracil. Chromatographic studies of RNA species suggest that the continuous synthesis of RNA during critical periods is a prerequisite for photo-induced conidiation. This may mean that new species of RNA are being transcribed at these periods.

The technique of DNA/RNA hybridization has been used extensively in bacterial studies where it has been possible to demonstrate that transcription of different RNA species occurs during specific growth phases. When this technique was used with differentiating *Trichoderma viride* it did reveal differences in RNA species during early vegetative growth but did not detect changes in RNA transcription directly following photo-induction (Stavy, Galum and Gressel, 1972). This lack of detection of photo-induced transcription may be explained by one or more of the following reasons: the transition is not regulated by transcriptional control but perhaps by translational control; new transcription does take place but only a very small fraction of the genome is transcribed and is not detected by the hybridization technique; the change in RNA transcription is substantial but restricted to the relatively few aerial hyphae which become conidiophores and is masked by the massive vegetative contribution.

Undoubtedly, the photo-induced conidiation demonstrated in *Trichoderma viride* makes this a valuable model for differentiation in the fungi. The rapid and exact timing of the light-mediated induction has immense experimental potential and it is surprising that this system has not yet been widely appreciated. In particular, studies with this fungus may help to resolve the argument as to whether control of differentiation is primarily at the level of transcription or translation.

Studies on the biochemistry of conidiogenesis in filamentous fungi are only now beginning to produce meaningful results. The results of many older studies must be considered with much reservation because of the complex nature of the experimental systems. Conidiogenesis is a very complex type of development and for this reason great care must

be taken to appreciate the role of the environment in controlling this process.

The discovery of microcycle conidiation in *Aspergillus niger* and in other fungi could well be of considerable importance since (*a*) it allows conidiation to occur with a minimum of vegetative growth and consequently less masking of important biochemical events; (*b*) a high degree of synchrony can be induced in large populations of cells; and (*c*) the entire cycle can be carried out in a fermenter where many environmental parameters can be suitably monitored.

### Ultrastructural Aspects of Conidiation

Although there have been many studies on the metabolic aspects of conidiation, there have been few attempts to thoroughly record the ultrastructural changes associated with this developmental process. In particular, it is of major importance to attempt to integrate studies at a biochemical level with structural and ultrastructural observations. Only by considering both aspects together can a composite understanding of conidiation be forthcoming.

Weiss and Turian (1966) have observed several ultrastructural differences between vegetative and conidiating cultures of *Neurospora*. Mitochondria from conidiating mycelium are much more swollen than mitochondria from vegetative cultures, and ribosomes are freely dispersed in the conidia but grouped in zones in vegetative hyphae. Some differences in wall structure have also been noted.

In the growing conidiophore stalk of *Aspergillus niger* (Oliver, 1972) the organelles of the cell are arranged in a similar manner to those in mycelial hyphae. The apex is singularly free of organelles other than vesicles and further behind is the zone of ribosomes and mitochondria and then nuclei. Vacuolation develops in the older parts of the stalk in association with bodies morphologically similar to autophagic vacuoles.

As the conidiophore tip begins to swell to form the vesicle, the apical vesicles (or microvesicles to distinguish them from the conidiophore vesicle) can be seen randomly dispersed throughout the entire volume of the swollen head. As the vesicle dome matures the microvesicles become concentrated in clusters at the sites of development of the metulae. As the metulae develop apical microvesicles and plasmalemmasomes can be seen associated with the wall surface. Further development of metulae, phialides and conidia is effected by a budding cycle in which a uninucleate growing cell becomes cut off from the adjacent cell and then matures autonomously. Throughout this process microvesicles are always present at the growing apex and are normally