Changes in Carbon Catabolic Pathways during Synchronous Development of Conidiophores of Aspergillus niger

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SUMMARY

Carbon catabolism was studied during the synchronous development of conidiophores of Aspergillus niger. The activities of key enzymes of the Embden–Meyerhof–Parnas (EMP) and pentose phosphate (PP) pathways were measured and studies of $^{14}$CO$_2$ production from [1-$^{14}$C]- and [6-$^{14}$C]glucose were made. The enzyme activities together with the radiorespirometric studies showed that both the EMP and PP pathways were present and operative at all stages of conidiophore development but that the relative activities of the pathways differed. The highest activity of the EMP pathway occurred during the initial period of vegetative growth before any signs of morphological change. High EMP activity and high 6-C/1-C ratios were obtained in a medium which did not support conidiophore initiation but allowed vegetative growth to occur. In contrast, high activity of the PP pathway and very low 6-C/1-C ratios accompanied each stage of conidiophore development. The results suggest that the PP pathway predominates during conidiophore development of this fungus.

INTRODUCTION

Radiorespirometric studies with Endothia parasitica (McDowell & DeHertogh, 1968) and Aspergillus nidulans (Carter & Bull, 1969) indicated an enhanced contribution of the PP pathway prior to and during conidiation. Similarly, an active PP pathway was found to operate throughout conidiogenesis in Neurospora crassa (Turian, 1962), while Daly, Sayre & Pazur (1957) have demonstrated that the PP pathway was the major respiratory pathway during conidiation of the obligate rust fungus Puccinia carthami growing on safflower.

Recent studies on carbon catabolism during the growth cycle of Aspergillus niger (Smith, Valenzuela-Perez & Ng, 1971) growing in flask culture indicated that the contribution of the EMP pathway was highest in conidiating mycelium, whereas the contribution of the PP pathway was highest in young vegetative mycelium growing under conditions that would not induce conidiation (Valenzuela-Perez & Smith, 1971). It was considered that the use of glutamate in these experiments to induce conidiation masked the high reductive biosynthetic needs of conidiation and it is probable that if a tricarboxylic acid cycle intermediate had been used to induce conidiation (Galbraith & Smith, 1969) there would have been a higher involvement of the PP pathway during conidiation of this fungus.

This report describes the changes that occurred in certain enzymes of the EMP and PP pathways during the initiation and synchronous development of conidiophores of Aspergillus niger. Enzyme determinations were also carried out on extracts of mycelium grown in a medium which did not support conidiophore initiation but did allow normal vegetative growth to occur. In view of the difficulties involved in assessing the significance of enzyme studies in vitro the measurements of enzyme activities were supplemented with measurements of $^{14}$CO$_2$ produced from [1-$^{14}$C]- and [6-$^{14}$C]glucose.
METHODS

Organism and cultural conditions. The fungus used was *Aspergillus niger* van Tieghem (IMI 41873) and the maintenance of stock cultures and the production of conidia were as previously described (Galbraith & Smith, 1969). Growth and synchronous development of conidiophores and conidia were obtained by means of a replacement fermentor culture technique (Anderson & Smith, 1971). Several media were used in this technique and all contained the following minerals in 1 l distilled water: KH$_2$PO$_4$, 1.0 g; MgSO$_4$.7H$_2$O, 0.25 g; CuSO$_4$.5H$_2$O, 0.234 mg; FeSO$_4$.7H$_2$O, 6.32 mg; ZnSO$_4$.7H$_2$O, 1.1 mg; MnCl$_2$.4H$_2$O, 3.5 mg; CaCl$_2$, 46.7 mg. The medium for growth of the fermentor inoculum contained in 1 l (mineral solution): (NH$_4$)$_2$SO$_4$, 3.96 g; and glucose, 20 g. The pH was adjusted to 2.3 for loose colonial growth. The medium for the initial phase of growth in the fermentor (LN medium) contained in 1 l: (NH$_4$)$_2$SO$_4$, 0.66 g; and glucose, 10.0 g. The initial pH of this medium was 4.5 and was not further adjusted. The first replacement medium (MN+ citrate medium) contained in 1 l: (NH$_4$)$_2$SO$_4$, 1.98 g; and citric acid, 12.6 g. The pH was adjusted to 3.0 with NaOH. The second replacement medium (glucose nitrate medium) contained in 1 l distilled water: NaNO$_3$, 2.55 g; glucose, 20 g; and mineral salts as for M except that KH$_2$PO$_4$ was increased to 12.96 g and K$_2$HPO$_4$, 1.13 g was included.

By means of the medium replacement technique the differentiation of the conidiophore can be divided into four distinct morphogenetic stages: (i) conidiophore initiation; (ii) conidiophore growth; (iii) vesicle and phialide formation; and (iv) conidiospore production. Initiation and growth of the conidiophores occurs in the original LN medium. The first medium replacement at 36 h induces vesicle and phialide formation and the second medium replacement at 56 h leads to heavy conidia production. Without medium replacement the conidiophores will not develop beyond the immature stalk stage. Conidiophore initiation did not occur in LN medium under continuous oxygen limited conditions (LNO medium), although vegetative growth was not restricted. Enzyme determinations were carried out on extracts from all stages of the synchronous development of the conidiophores and also from the completely vegetative mycelium from LNO medium.

Preparation of extracts. The mycelium was harvested by filtration, washed free of medium, pressed between filter papers to remove excess water, and disrupted by means of a glass homogenizer attached to an M. S. E. Waring blender rotor shaft (Watson & Smith, 1967). A ratio of 1 g wet weight of mycelium to 10 ml extraction medium containing 0.05 M-tris-HCl buffer, pH 7.0, was used and all operations were carried out between 0 and 4 °C. The homogenate was centrifuged at 26 000g for 15 min and the supernatant used immediately for enzymatic determinations. For comparative enzyme studies equal weights of fresh mycelium were used, the conditions of extraction rigorously standardized, and the final cytoplasmic fractions made to equal volumes.

Enzyme assays. A Unicam SP 800 spectrophotometer with scale expansion, constant-temperature cell housing, automatic cell changer, and constant-wavelength accessory was used to measure the enzymatic activities of the extracts. All assays were carried out at 25 °C by observing either the oxidation or the reduction of nicotinamide adenine dinucleotides (NAD and NADP). Enzyme activities were measured by noting the change in absorbance at 340 nm which was recorded continuously for 3 to 5 min and the results calculated from the linear portion of the curve of absorbance against time. Endogenous activity, which was determined using an assay system minus substrate, was always subtracted. All activities were assayed at optimum pH and at substrate concentrations which ensured enzyme saturation and extracts were added at concentrations which limited the rate of the reaction. The en-
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10 20 30 40 50

**Fig. 1.** Changes in activities of EMP and PP pathway enzymes from mycelium from LN and replacement media. All concentrations of enzymes are expressed as units of enzyme activity/g fresh weight of mycelium. (a) EMP pathway enzymes: ●, hexokinase; ○, phosphofructokinase; △, pyruvate kinase; and ▲, fructose diphosphatase. (b) PP pathway enzymes: □, glucose-6-phosphate dehydrogenase; ■, phosphogluconate dehydrogenase; ●, transketolase.

zymes were stable for several hours if kept at 0 to 4 °C. Enzyme activities were measured in at least three, usually six, separate experiments.

Hexokinase was assayed as described by Gibbs & Turner (1964), phosphofructokinase by Slater (1953), fructose diphosphatase by Rosen (1966), pyruvate kinase by Bucher & Pfleiderer (1955), glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase by Kornberg & Horecker (1955) as modified by Waygood & Rohringer (1964) and transketolase by Casselton (1966).

Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951).

**Presentation of enzyme results.** One unit of enzyme activity is defined as the amount of enzyme which converts 1 μmol of substrate/min at 25 °C. An extinction coefficient of $6.22 \times 10^6$ cm$^2$/mol of nucleotide at 340 nm (Horecker & Kornberg, 1948; Wold & Ballou, 1957) was used in these calculations. Activity was related either to fresh weight of mycelium and
expressed as units of activity/g mycelium or as specific activity and expressed as units of activity/mg protein. In Fig. 1 and 2 vertical lines divide the diagrams at points of medium replacement.

Radiorespirometry. Radiorespirometric studies were carried out as previously described (Smith et al. 1971). The interpretation of the data is based on the assumption that the first carbon atom of glucose is oxidized preferentially to CO₂ when still a constituent atom of the C₆ compound (6-phospho-β-glucanate) and not a further breakdown product of glucose. Therefore, it is essential to limit the time of the experiment and for this reason the cumulative radiochemical recoveries were taken at the point of substrate exhaustion. At this time it is considered that the different catabolic sequences are minimized thus making the ¹⁴CO₂ data reliable for pathway estimation. Preliminary experiments showed that the 1 μmol of glucose used in these experiments was completely utilized within 1 h of incubation. Radiorespirometric analyses were carried out at 0.5 and 1 h and in general the ¹⁴CO₂ readings were proportional.

RESULTS

Activities of EMP pathway enzymes during growth in LN and replacement media

The activities of the enzymes hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) together with the gluconeogenic enzyme fructose diphosphatase (FDP) in extracts of mycelium during synchronous development of conidiophores are shown in Fig. 1 and 2.
Fig. 3. Changes in activities of EMP and PP pathway enzymes from mycelium from LNO medium. All concentrations of enzymes are expressed as units of enzyme activity/g fresh weight of mycelium. (a) EMP pathway enzymes; (b) PP pathway enzymes. Symbols as in Fig. 1.

The pattern of activity of HK and PFK was similar in LN medium both initially increased in activity to 7 h and then decreased. PK activity remained low during the early stages of growth in this medium and then increased slightly. The activity of FDP remained low throughout.

In the first replacement medium HK and PK remained consistently low, whereas the activity of PFK increased. FDP was present only in trace levels.

In the second replacement medium the specific activity of HK, PFK and PK increased initially and then rapidly decreased. FDP showed a pattern of constant low activity. On a fresh weight basis there was a general pattern of decrease in activity.

**Activities of PP pathway enzymes during growth in LN and replacement media**

The activities of the enzymes glucose 6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (PGADH) and transketolase (TK) in extracts of mycelium during synchronous development of conidiophores are shown in Fig. 1 and 2.

In LN medium the activities of each enzyme increased up to 16 h and then decreased.
Table 1. Changes in $6\text{-C}/1\text{-C}$ ratios during growth in LN, replacement, and LNO media

<table>
<thead>
<tr>
<th>Age of culture (h)</th>
<th>Isotope</th>
<th>C.p.m./2.5 mg dry wt/h</th>
<th>Recovery (%)</th>
<th>$6\text{-C}/1\text{-C}$</th>
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<tr>
<td></td>
<td></td>
<td><strong>Replacement culture</strong></td>
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<tr>
<td>Inoculum</td>
<td>1-C</td>
<td>52 057</td>
<td>4.1</td>
<td>0.73</td>
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<td></td>
<td>6-C</td>
<td>38 373</td>
<td>2.8</td>
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</tr>
<tr>
<td>LN medium</td>
<td>7</td>
<td>237 227</td>
<td>18.8</td>
<td>0.31</td>
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<tr>
<td></td>
<td>6-C</td>
<td>74 183</td>
<td>5.5</td>
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<td></td>
<td>16</td>
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<tr>
<td></td>
<td>6-C</td>
<td>11 849</td>
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<td>26</td>
<td>181 473</td>
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<tr>
<td></td>
<td>6-C</td>
<td>26 152</td>
<td>1.9</td>
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<td>32</td>
<td>137 270</td>
<td>10.9</td>
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<tr>
<td></td>
<td>6-C</td>
<td>71 757</td>
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<td>134 344</td>
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<tr>
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<td><strong>2nd replacement medium</strong></td>
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<td>29 972</td>
<td>2.2</td>
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<td><strong>LNO medium</strong></td>
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<tr>
<td></td>
<td>8</td>
<td>42 394</td>
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<tr>
<td></td>
<td>6-C</td>
<td>34 209</td>
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</table>

Total activity in each flask: $[\text{1-}^{14}\text{C}]$glucose, 1 259 607 c.p.m. (0.4 μCi); $[\text{6-}^{14}\text{C}]$glucose, 1 344 645 c.p.m. (0.4 μCi)

After each medium replacement there was an initial increase in enzyme activity followed by a steady decrease.

At all phases of development the activities of the PP pathway enzymes were much in excess of the EMP pathway enzymes.

Activities of EMP and PP pathway enzymes during growth in LNO medium

The activities of the EMP pathway enzymes were all considerably higher in this medium than in LN medium (Fig. 3). The activities of HK and PK increased rapidly in the early stages of growth; whereas HK decreased after 16 h PK continued to increase. PFK was initially low in activity but steadily increased throughout the experimental period. FDP remained at trace levels throughout.

G6PDH and TK both increased until 16 h and then decreased while the activity of PGADH steadily increased throughout the experimental period.
When glucose is metabolized through the EMP pathway carbon atoms 1 and 6 are equivalent and the 6-C/1-C ratio of isotopic carbon produced should be equal to unity. However, if the PP pathway is also functioning this ratio should give values below one (Beevers & Gibbs, 1954). The value of the ratio will depend on the relative contribution of each pathway.

The 6-C/1-C ratio altered significantly during synchronous development of the conidiophores. The ratio was highest in the initial inoculum decreasing to a low level in LN medium at 26 h and then increasing considerably at 32 h (Table I).

In the first replacement medium the ratio again dropped and remained constant over 12 h. A further decrease occurred in the ratio in the second replacement medium followed by a slight increase after 7 h.

In LNO medium the ratios remained consistently high throughout the experimental period.

DISCUSSION

Numerous studies have dealt specifically with quantitative changes in enzyme levels during differentiation and suggestive correlations have been described between the increasing levels of specific enzymes and the changing requirements of differentiation (see Smith & Galbraith, 1971). However, it is still uncertain whether enzyme activity in vitro always truly reflects enzyme activity in vivo. In the present experiments enzyme determinations in vitro were coupled with the radiorespirometric analysis of glucose dissimilation in vivo and when taken together give a more reliable estimation of the changes in vivo in glucose catabolism during differentiation.

The enzymes of the PP and EMP pathways were active throughout the entire experimental period. In general, during conidiophore differentiation the PP pathway enzymes were higher in activity than the EMP pathway enzymes and when considered with the radiorespirometric results strongly imply that the direct oxidation of glucose through the PP pathway may be of considerable importance during conidiophore development in Aspergillus niger. These observations are in substantial agreement with other studies on glucose catabolism during fungal differentiation (Daly et al. 1957; Turian, 1962; McDowell & DeHertogh, 1968; Carter & Bull, 1969), and would indicate a major role for the PP pathway and its intermediary products (pentoses, NADPH₂) during differentiation.

The observation that at any one time G6PDH was the most active PP pathway enzyme is of interest. Osmond & Appreess (1969) have pointed out that this enzyme may be involved in the control of the pathway. Similarly, a number of other reports have shown that an increase of the PP pathway was accompanied by an increase of this enzyme and that treatments that lead to the oxidation of NADPH₂ caused an immediate stimulation of G6DPH (Holzer & Witt, 1960; Novello & McLean, 1968).

In LN medium TK was the second most active enzyme, and Venkataraman & Racker (1961) have indicated that an increase in activity of TK would prevent the accumulation of erythrose-4-phosphate which is a known inhibitor of glucose-6-phosphate isomerase, thus allowing the increase in PP pathway activity without due inhibition of the EMP pathway. This may be the situation in LN and LNO media where the activity of both pathways was high.

The high activity of the PP pathway after replacement into the nitrate medium (second
replacement) may be due to the presence of the nitrate since nitrate assimilation involves the
utilization of NADPH by nitrate reductase and the availability of this nucleotide is con-
sidered to limit the pathway (Osmond & Apprees, 1969).

No conclusions can be made regarding the role of the EMP pathway during conidiophore
development. The activity of the EMP enzymes paralleled the total glucose concentration
in the medium. In the early stages in LN medium and in the second replacement medium,
where glucose was also the carbon source, enzyme activity was highest decreasing as the
glucose became exhausted (Anderson & Smith, 1971). In the first medium replacement where
citrate was the carbon source and also at the time when active differentiation was occurring
the EMP pathway enzymes were low in activity.

The partial anaerobic conditions that prevail in LN medium for the initial 14 h (Anderson
& Smith, 1971) may also influence the activity of the EMP enzymes. In LNO medium the
activity of EMP pathway enzymes was initially high and increased as the growing conditions
became more anaerobic. Similar observations on the influence of oxygen tension on glyco-
lysis have been made for Aspergillus niger (Franke, Eichhorn & Jilge, 1963) and for Fusarium
Zini (Heath, Nasser & Koffler, 1956) and continued high EMP activity may suppress mor-
phological differentiation (Schwalb & Miles, 1967; Crocken & Tatum, 1968; Warburg,

The activity of the PP pathway, as measured by the 6-C/1-C ratio, was lower in LNO
medium than in LN medium, although the specific activities of the respective enzymes were
not markedly affected. Fluorometric analysis of the glycolytic intermediates of this system
has shown that the concentration of glucose-6-phosphate (G-6-P) is, in general, much higher
in mycelium from LNO medium than that from LN medium (Smith & Ng, 1972), e.g. in
LN medium at 7 and 16 h the concentrations of G-6-P were 0.003 and 0.007 µmol/g dry
weight respectively, whereas in LNO medium the corresponding values were 0.145 and 0.045
µmol/g dry weight. Thus in LN medium the low steady state level of G-6-P coupled with a
possible high affinity for G-6-P of G6PDH may regulate the pathway of glucose catabolism.
In LNO medium the higher steady state levels of G-6-P may allow a greater proportion to be
metabolized by the EMP pathway. Thus the partial anaerobic conditions in LNO medium
may act by stimulating G-6-P formation and not by directly inhibiting PP enzyme activity.

However, it is probable that conidiation requires the establishment of a balance between
oxidative and glycolytic pathways and that conidiation can be considered to be a morpho-
getic expression of the Pasteur effect (Turian, 1969).

The present results differ in some detail from those obtained in the previous study on
carbon catabolism during differentiation of Aspergillus niger (Smith et al. 1971). It is difficult
to make a true comparison between the systems, each of which ultimately leads to conidia-
tion, because of the changing environmental conditions imposed in the fermentor studies.
However, unpublished results have suggested that transcription for conidiation takes place
early in each growth cycle, viz. between 32 and 36 h in the glutamate medium (Smith et al.
1971) and approximately in the middle phase in LN medium. Thus, if a comparison is made
between the first 48 h of growth in glutamate medium and the growth phase in LN medium
some interesting observations can be made. In each case the patterns of activity of HK and
PK are similar, whereas PFK shows a steady increase in the glutamate medium and a
decrease in LN medium. How much the changes in PFK are related to glucose depletion or
changing aeration rates is not yet clearly understood.

The presence of glutamate must undoubtedly influence the contribution of the PP path-
way since one of the main functions of this pathway is to produce the NADPH$_2$ essential for
reductive biosynthesis, in particular, amino acid synthesis from inorganic sources. The
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ability of the mycelium to incorporate glutamic acid from the medium directly into the amino acid pool could greatly reduce the involvement of the PP pathway for this particular aspect of cellular biosynthesis. Furthermore, the presence of glutamate in the medium could particularly mask the high biosynthetic needs of the differentiating system. Although the contribution of the PP pathway is always less in the glutamate medium than in LN medium there is a marked increase in the PP pathway in the former as measured by 6-C/1-C ratios as differentiation progresses (Smith et al. 1971).

REFERENCES


